

**Biochemical Basis of the Temperature-Inducible
Constitutive Protease Activity of the
RecA441 Protein of *Escherichia coli***

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We compared the biochemical properties of the RecA441 protein to those of the wild-type RecA protein in an effort to account for the constitutive protease activity observed in *recA441* strains. The two RecA proteins have similar properties in the absence of single-stranded DNA binding protein (SSB protein), and the differences that do exist shed little light on the temperature-inducible phenotype observed in *recA441* strains. In contrast, several biochemical differences are apparent when the two proteins are compared in the presence of SSB protein, and these are conducive to a hypothesis that explains the temperature-sensitive behavior observed in these strains. We find that both the single-stranded DNA (ssDNA)-dependent ATPase and LexA-protease activities of RecA441 protein are more resistant to inhibition by SSB protein than are the activities of the wild-type protein. Additionally, the RecA441 protein is more capable of using ssDNA that has been precoated with SSB protein as a substrate for ATPase and protease activities, implying that RecA441 protein is more proficient at displacing SSB protein from ssDNA. The enhanced SSB protein displacement ability of the RecA441 protein is dependent on elevated temperature. These observations are consistent with the hypothesis that the RecA441 protein competes more efficiently with SSB protein for limited ssDNA sites and can be activated to cleave repressors at elevated temperature by displacing SSB protein from the limited ssDNA that occurs naturally in *Escherichia coli*. Neither the ssDNA binding characteristics of the RecA441 protein nor the rate at which it transfers from one DNA molecule to another provides an explanation for its enhanced activities, leading us to conclude that kinetics of RecA441 protein association with DNA may be responsible for the properties of the RecA441 protein.

1. Introduction

The RecA protein of *Escherichia coli* has two distinct activities that contribute to its role in the repair of DNA damage. It acts as a recombinase, promoting recombinational repair (for reviews, see Cox & Lehman, 1987; Kowalczykowski, 1987; Radding, 1982), and it stimulates the cleavage of the LexA-repressor, thereby derepressing the set of unlinked genes that comprise the SOS system (for a review, see Little & Mount, 1982). The *cI* repressor of lambda phage is also a target of this protease activity† of the RecA protein, accounting for the RecA-dependent induction of prophage (Roberts *et al.*, 1978a). *In vivo*, the RecA protein is activated to its protease form by treatments that cause gross DNA damage (Witkin, 1976). *In vitro*, it is activated when RecA protein binds ssDNA and ATP or dATP, suggesting that activation *in vivo*

results from the binding of RecA protein to single-stranded regions or gaps in damaged DNA (Craig & Roberts, 1980; Phizicky & Roberts, 1981). ATPγS†

† Little (1984) has shown that the *lexA* and phage repressors undergo autodigestion at alkaline pH, indicating that the RecA protein is not a classical protease. Our use of the term “protease activity” is not meant to imply that the RecA protein is a classical protease, but rather that the RecA protein catalyzes the repressor cleavage reactions under physiological conditions.

‡ Abbreviations used: ATPγS, adenosine-5'-O'-(3-thiotriphosphate); ssDNA, single-stranded DNA; dsDNA, double-stranded DNA; etheno M13 DNA, ssM13 DNA with 1,N⁶-ethenoadenosine and 3,N⁴-ethenocytosine; poly(dT), polydeoxythymidilic acid; RecAwt protein, the wild-type RecA protein; SSB protein, *E. coli* single-stranded DNA binding protein.

can replace ATP in this reaction (Roberts *et al.*, 1978b; Little *et al.* 1980), indicating that the mere binding of ATP to RecA protein is sufficient, i.e. hydrolysis of ATP is not necessary (Craig & Roberts, 1981).

The *recA441* mutant strain of *E. coli* was first identified by its ability to induce prophage at elevated temperatures in the absence of DNA damage (Goldthwait & Jacob, 1964). It was subsequently shown to have thermally induced filamentous growth (Kirby *et al.*, 1967). Such filamentation is seen in wild-type strains when the cells are subjected to DNA damaging treatment and results from derepression of the *sulA* gene upon LexA protein inactivation (Huisman *et al.*, 1980). Additionally, if the synthesis of RecA441 protein is constitutively derepressed by the *lexA51* mutation, prophage induction occurs at all physiological temperatures in the absence of DNA damage (Mount, 1977). These properties are the result of constitutive protease activity either at elevated temperature or upon amplification of the RecA441 protein.

Craig & Roberts (1980) have shown that the RecA441 protein requires ATP and polynucleotide to cleave *cI* repressor *in vitro*. Cleavage can be supported by a variety of single-stranded polynucleotides, including poly(rC), poly(rA) and oligonucleotides as short as six nucleotides when ATP γ S is present. Based both on K_m values for ATP and dATP derived from the hydrolysis reaction and on the enhanced ability of small oligonucleotides to activate RecA441 protein to cleave the *cI* repressor, Phizicky & Roberts (1981) suggested that the RecA441 protein forms the proteolytically active complex more efficiently than RecAwt protein, and can utilize polynucleotide present in undamaged cells that is insufficient for the activation of the wild-type protein to the protease form. This polynucleotide could include single-stranded regions present at replication forks, oligonucleotides resulting from normal DNA degradation, and single-stranded regions produced from the melting of weakly paired regions of chromosomal DNA. McEntee & Weinstock (1981) offered further *in vitro* evidence that RecA441 protein is activated to cleave repressors by polynucleotides that are incapable of activating the wild-type protein, and proposed that the mutation in RecA441 protein alters polynucleotide recognition. While these studies offer insight into the *recA441* phenotype, they provide little explanation for the temperature-sensitive behavior observed in these mutant strains.

In this paper, we compare *in vitro* characteristics of the RecA441 protein to those of the wild-type RecA protein, in hopes of offering explanations for the constitutive protease activity observed in *recA441* strains *in vivo*. An examination of ATPase activity and LexA-protease activity, suggests that RecA441 protein is more efficient than RecAwt protein at competing with SSB protein for sites on ssDNA, particularly at low MgCl₂ concentration.

This enhanced ability of RecA441 protein is not explained by either its ssDNA binding affinity or the rate at which it transfers from one DNA molecule to another, leading us to conclude that kinetics of RecA protein association with ssDNA contribute to the RecA441 protein advantage. We propose that the constitutive protease activity of RecA441 protein can be ascribed to the protein's enhanced ability to displace SSB protein from the limited single-stranded regions that naturally occur in *E. coli*. This ability is improved by elevated temperature which would shift the equilibrium toward displacement of SSB protein.

2. Materials and Methods

(a) Chemicals and buffers

All chemicals were reagent grade and solutions were made using glass-distilled water. ATP and ADP were purchased from Pharmacia P-L Biochemicals and were dissolved as concentrated stocks at pH 7.5. Unless otherwise noted, the standard buffer (TD buffer) used in all experiments consisted of 20 mM-Tris·HCl (pH 7.5), 0.1 mM-dithiothreitol, and contained MgCl₂ as indicated in the Figure legends.

(b) Proteins

Wild-type RecA protein was purified from strain JC12772 (Uhlen & Clark, 1981) using a preparative procedure (S. C. Kowalczykowski, unpublished results) based on spermidine precipitation (Griffith & Shores, 1985). RecA441 protein was purified from strain BEU397, using the procedure described by Cox *et al.* (1981). Strain BEU397 is strain JC10289 containing pBEU54, a derivative of pBEU28 (Uhlen *et al.*, 1983), into which the *Bam*HI fragment with the *recA441* gene has been inserted. SSB protein was purified from strain RLM727 using a preparative protocol provided by Dr Roger McMacken of The Johns Hopkins University. Protein concentrations were determined using molar extinction coefficients of $2.7 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ for RecA protein and $3 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ for SSB protein, both at 280 nm (Ruyechan & Wetmur, 1976). LexA protein was purified from strain JL652 (Little, 1984) using the procedure described by Schnarr *et al.* (1985). Protein concentration was determined using a molar extinction coefficient of $7300 \text{ M}^{-1} \text{ cm}^{-1}$ at 280 nm.

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

(c) DNA

Single and double-stranded DNA were prepared from bacteriophage M13mp7 using the procedure described by Messing (1983). Molar concentrations were determined using extinction coefficients of $6500 \text{ M}^{-1} \text{ cm}^{-1}$ for duplex DNA and $8780 \text{ M}^{-1} \text{ cm}^{-1}$ for single-stranded DNA at 260 nm. The duplex DNA was linearized by digesting with *Eco*RI. Etheno M13 DNA was prepared from the phage DNA as described (Menetski & Kowalczykowski, 1985); its concentration was determined using an extinction coefficient of $7000 \text{ M}^{-1} \text{ cm}^{-1}$ at 260 nm (Menetski & Kowalczykowski, 1987a). Poly(dT) was

purchased from P-L Biochemicals and dissolved as a concentrated stock in TE buffer (10 mM-Tris·HCl, 1 mM-EDTA (pH 7.5)); its concentration was determined using an extinction coefficient of $8540 \text{ M}^{-1} \text{ cm}^{-1}$ at 259 nm.

(d) ATPase assay

The hydrolysis of ATP was monitored using a spectrophotometric assay that couples the production of ADP to the oxidation of NADH (Kreuzer & Jongeneel, 1983) and has been adapted for use with RecA protein by Kowalczykowski & Krupp (1987). Reactions were carried out in TD buffer containing 0.2 mg NADH/ml, pyruvate kinase and lactate dehydrogenase, at approximately 25 units/ml, 2 mM-phosphoenolpyruvate and 0.5 mM-ATP.

(e) LexA-protease assay

Reactions were performed in TD buffer containing 50 mM-NaCl, 1 mM-ATP and an ATP regenerating system consisting of 8 mM-phosphoenolpyruvate and 12.5 units pyruvate kinase/ml. RecA protein and SSB protein were added, as indicated, to the assay buffer containing DNA, and the mixture was incubated as described in the text. LexA protein was added last to start the cleavage reaction.

Portions of the assay mixture were removed and the cleavage reaction was stopped by the addition of an equal volume of sample buffer (2% (w/v) SDS, 125 mM-Tris·HCl (pH 6.7), 1.4 mM- β -mercaptoethanol, 20% (v/v) glycerol, 0.48 g urea/ml and 0.5 mg bromophenol blue/ml) and placement on ice. The samples were heated at 90°C for 5 min and then subjected to SDS/polyacrylamide gel electrophoresis in 15% (w/v) gels run at constant voltage.

Gels were stained for approximately 3 h with 2 mg Coomassie brilliant blue R/ml in 47.5% (v/v) ethanol, with 7 ml glacial acetic acid added per 100 ml. Gels were destained in destain solution 1 (47.5% ethanol, 10% glacial acetic acid) for 2 h and in destain solution 2 (4.75% ethanol, 7% glacial acetic acid) overnight.

Extent of LexA cleavage was determined spectrophotometrically using a Zeineh scanning densitometer with a tungsten lamp and a 595 nm filter. The percentage of intact LexA protein was computed using a Hewlett-Packard 3390A integrator. The ratio of the area of the intact LexA peak to the sum of the areas of the 3 peaks corresponding to intact LexA and the 2 LexA fragments was determined. The rate of LexA cleavage was computed from the slope of the least-squares fit of the linear portion of the reaction time-course.

(f) DNA binding assay

The binding of RecA protein to etheno M13 DNA was monitored fluorimetrically as described (Menetski & Kowalczykowski, 1985). RecA protein titrations of etheno M13 DNA were carried out by adding portions of RecA protein stock to 3 μM -etheno M13 DNA in TD buffer. Salt titrations were carried out by adding portions of concentrated NaCl to complexes of RecA protein and etheno M13 DNA in TD buffer. The salt titration midpoint is defined as the salt concentration required to dissociate one-half of the protein-DNA complex present (Menetski & Kowalczykowski, 1985). An ATP regenerating system consisting of 2 units pyruvate kinase/ml and 2 mM-phosphoenolpyruvate was present in all reactions that contained ATP.

(g) Transfer reaction

RecA protein-etheno M13 DNA complexes were formed in TD buffer at 25°C. At time zero, poly(dT) was added to induce transfer and the associated fluorescence decrease was monitored and analyzed as described (Menetski & Kowalczykowski, 1987a). Reactions contained 0.56 μM -RecA protein, 4.2 μM -etheno M13 DNA and 25 μM -poly(dT). An ATP regenerating system consisting of 2 units pyruvate kinase/ml and 2 mM-phosphoenolpyruvate was present in all reactions that contained ATP.

3. Results

(a) RecA protein concentration dependence of ssDNA-dependent ATPase activity

Phizicky & Roberts (1981) showed that RecA441 protein forms a ternary complex with NTP and polynucleotide more efficiently than RecAwt protein, and suggested that this is the basis of its enhanced proteolytic activity. Since ATP hydrolysis requires the formation of this ternary complex, further comparison of the ATPase activity of RecA441 and RecAwt proteins should provide additional insight into the differences between these two proteins. To compare the DNA binding stoichiometry and the turnover number of the two proteins during ATP hydrolysis, ssDNA-dependent ATPase activity was measured as a function of RecA protein concentration. Figure 1 shows that, in the absence of SSB protein (open symbols), the apparent stoichiometry and the saturation rate of ATP hydrolysis for RecA441 protein are the same as for RecAwt protein at all temperatures tested. The apparent site sizes for both proteins are approximately 7.5, 6 and 5 nucleotides per RecA protein monomer at 25°C, 37°C and 42°C, respectively.†

When SSB protein is present, there are differences in the ATPase activity of RecA441 protein and the wild-type protein. At 10 mM-MgCl₂ (see MgCl₂ dependence below), the addition of SSB protein stimulates the ssDNA-dependent ATPase activity of RecAwt protein, by allowing RecA protein to access regions of the ssDNA that were involved in secondary structures (Kowalczykowski & Krupp, 1987). SSB protein has a similar effect on RecA441 protein ATPase activity at 37°C and 42°C (Fig. 1(b) and (c)). At each of these temperatures both RecA proteins achieve a rate of ATP hydrolysis at saturation that is at least twice that observed in the absence of SSB protein. In addition, they achieve this rate with the same apparent

† Apparent site sizes were determined from the intersection of the horizontal line that the maximum rate of hydrolysis asymptotically approaches and the slope of the initial points; this is a different method from that employed by Kowalczykowski & Krupp (1987), who used the intersection of the lines defined by the initial and limiting slopes. Therefore, different apparent site sizes are reported here (Menetski & Kowalczykowski, unpublished results).

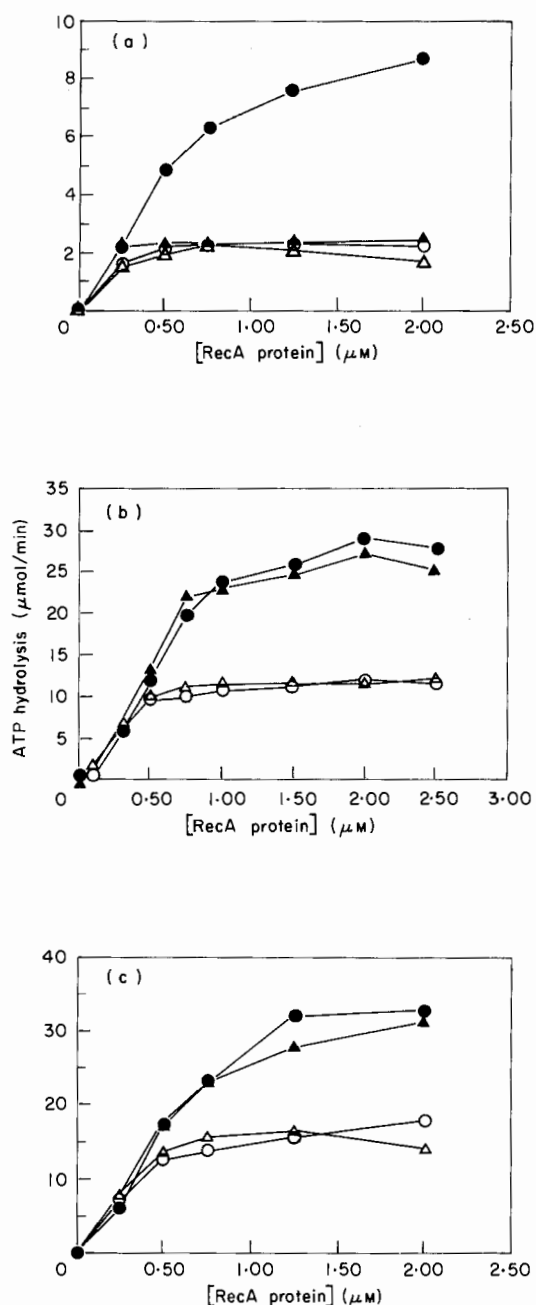


Figure 1. RecA protein concentration dependence of ssDNA-dependent ATPase activity. Reactions were performed in TD buffer containing 10 mM-MgCl₂, 3 μM -ssM13 DNA and 0.6 μM -SSB protein added, as indicated, after RecA protein had attained a steady-state rate of ATP hydrolysis. Panels (a), (b) and (c) represent reactions carried out at 25°C, 37°C and 42°C, respectively. Circles represent RecAwt protein and triangles RecA441 protein. Open symbols are reactions without SSB protein and filled symbols are reactions containing SSB protein.

stoichiometry of approximately three nucleotides per RecA protein monomer (see footnote to p. 863). However, at 25°C, the ATPase activity of RecA441 protein is not stimulated by SSB protein and is, in fact, slightly inhibited (Fig. 1(a)). This lack of

stimulation is not observed for RecAwt protein and, interestingly, is observed for RecA441 protein only at low temperature where the constitutive protease phenotype is not exhibited.

(b) *Effect of MgCl₂ concentration on ssDNA-dependent ATPase activity*

The effect of SSB protein on the ssDNA-dependent ATPase activity of RecAwt protein is dependent on MgCl₂ concentration and temperature, with the effect being stimulatory at elevated MgCl₂ concentrations and temperatures (Kowalczykowski & Krupp, 1987). Since the ATPase activity of RecA441 protein is not stimulated by SSB protein at 25°C and 10 mM-MgCl₂, we compared the effect of SSB protein on the rate of ATP hydrolysis by RecA441 and RecAwt proteins at various MgCl₂ concentrations and temperatures. In the absence of SSB protein, the rate of ATP hydrolysis by both proteins is essentially the same at all conditions tested, and it increases as temperature increases and/or MgCl₂ concentration decreases (Fig. 2, open symbols).

In the presence of SSB protein, there are once again differences in the behavior of RecA441 and RecAwt proteins (Fig. 2, filled symbols). Most notable is the 1.5-fold stimulation of the ATPase activity of RecA441 protein by SSB protein at 1 mM-MgCl₂, which is in sharp contrast to the essentially complete inhibition of RecAwt protein under these same conditions. In fact, at all three temperatures tested, the two proteins show somewhat of an opposite response to changes in MgCl₂ concentration in the presence of SSB protein. The rate of ATP hydrolysis by RecAwt protein increases, while that by RecA441 decreases slightly, as MgCl₂ concentration increases. At 25°C, this decrease in rate by RecA441 protein is most pronounced and culminates in a rate that is less than that observed in the absence of SSB protein, i.e. SSB protein somewhat inhibits the ATPase activity of RecA441 protein at 25°C and 10 mM-MgCl₂ (Fig. 1(a)).

At 4 mM-MgCl₂, the effect of SSB protein on the ssDNA-dependent ATPase activity of RecAwt protein is dependent on RecA protein concentration; at 0.3 μM -RecA protein, SSB protein inhibits the ATPase activity (Kowalczykowski & Krupp, 1987). Since SSB protein does not inhibit the ATPase activity of RecA441 protein at 1 mM-MgCl₂, we wondered if SSB protein would inhibit the ATPase activity of low concentrations of RecA441 protein at 4 mM-MgCl₂. In the absence of SSB protein, the ATPase activity of 0.2 μM -RecA441 protein was comparable to that observed for RecAwt protein at this protein concentration (data not shown). However, the ATPase activity of the wild-type protein at this concentration was inhibited by 0.6 μM -SSB protein at all temperatures tested, while that of RecA441 protein was unaffected by the addition of SSB protein (data not shown). Thus, a major difference between the

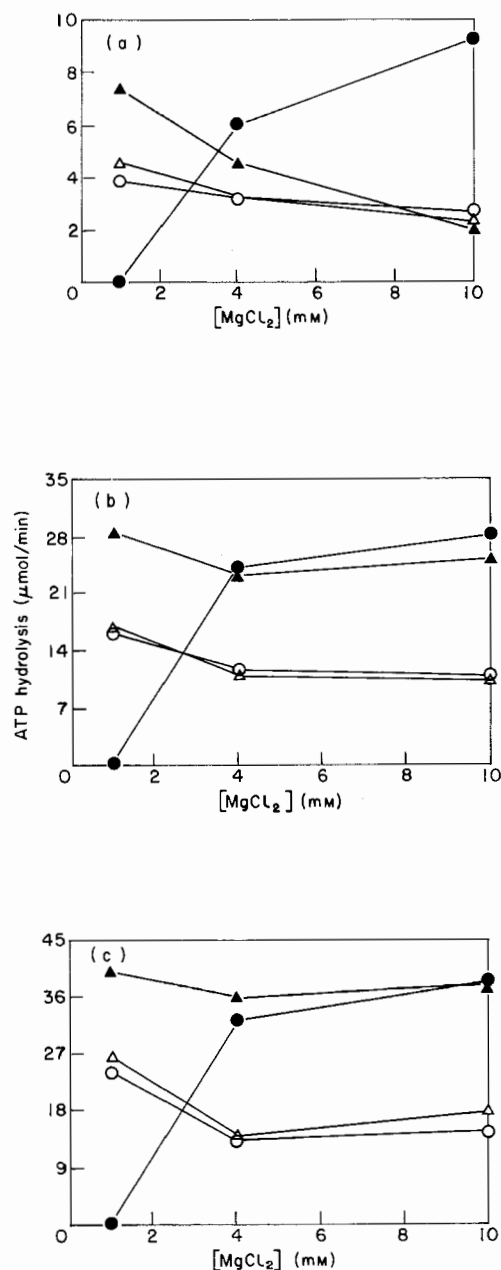


Figure 2. The effect of MgCl_2 concentration on ssDNA-dependent ATPase activity. Reactions were performed in TD buffer containing MgCl_2 as indicated, $3 \mu\text{M}$ -ssM13 DNA, $1.5 \mu\text{M}$ -RecA protein and $0.6 \mu\text{M}$ -SSB protein added, as indicated, after RecA protein had attained a steady-state rate of ATP hydrolysis. Panels (a), (b) and (c) represent reactions carried out at 25°C , 37°C and 42°C , respectively. Circles represent RecAwt protein and triangles RecA441 protein. Open symbols are reactions without SSB protein and filled symbols are reactions containing SSB protein.

ssDNA-dependent ATPase activity of the two RecA proteins is that RecA441 protein is more resistant to inhibition by SSB protein at lower MgCl_2 concentrations and at lower RecA protein concentrations.

(c) *Effect of NaCl concentration on ssDNA-dependent ATPase activity*

In the presence of SSB protein, the ssDNA-dependent ATPase activity of RecA441 protein is inhibited slightly by increases in MgCl_2 concentration, with the effect being more pronounced at low temperature. To determine if this sensitivity to MgCl_2 represents a non-specific salt sensitivity, the rate of ATP hydrolysis was examined at increasing NaCl concentrations. The results in Figure 3 show that the ATPase activity of RecA441 protein is less sensitive to increases in salt concentration than that of the wild-type RecA protein. At 25°C , there is nearly complete inhibition of the ATPase activity of RecAwt protein by 300 mM -NaCl (Fig. 3(a)). In contrast, the ATPase activity of RecA441 protein, in the absence of SSB protein, is essentially unaffected by increases in NaCl concentration up to 400 mM . In the presence of SSB protein, the rate for RecA441 protein increases with increasing NaCl concentration up to 200 mM and then decreases gradually as the NaCl concentration rises further, resulting in a rate at 400 mM -NaCl that is still greater than that observed when no NaCl is present.

At higher temperatures, the rate of ATP hydrolysis by the wild-type RecA protein, both in the absence and presence of SSB protein, is essentially unaffected by increasing NaCl concentration up to 200 mM ; beyond that the rate is inhibited, with the inhibition being more gradual at 42°C than it is at 37°C (Fig. 3(b) and (c)). For RecA441 protein, in the absence of SSB protein, the rate is greater at 100 mM -NaCl than it is when no NaCl is present. Beyond 100 mM -NaCl, there is a very gradual decrease in the rate of hydrolysis, but once again the rate at 400 mM -NaCl is greater than that observed when no NaCl is present. In the presence of SSB protein at 37°C , the rate of hydrolysis by RecA441 protein is again greater at 100 mM -NaCl than it is when no NaCl is present. As the NaCl concentration increases further, the rate decreases, but more gradually than that by RecAwt protein (Fig. 3(b)). At 42°C in the presence of SSB protein, increases in NaCl concentration have an effect on the rate of hydrolysis by RecA441 protein that essentially parallels that observed for the wild-type RecA protein (Fig. 3(c)). Thus, the effects of MgCl_2 on RecA441 protein activity must be specific to the magnesium ion, since RecA441 is not more sensitive to increases in NaCl concentration than RecAwt protein, but is in fact less sensitive.

(d) *RecA protein can displace SSB protein from ssM13 DNA*

Kowalczykowski & Krupp (1987) have shown that SSB protein, added to ssDNA prior to the addition of RecA protein, dramatically inhibits the ATPase activity of RecAwt protein. Since the ATPase activity of RecA441 protein is resistant to inhibition by SSB protein at conditions where the wild-type protein is not, it was of interest to

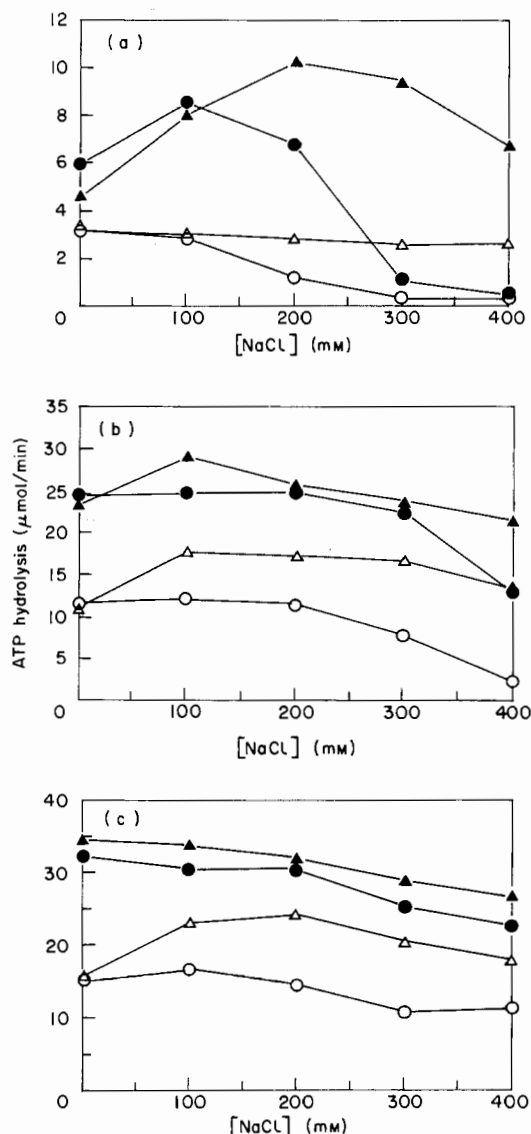


Figure 3. The effect of NaCl concentration on ssDNA-dependent ATPase activity. Reactions were performed in TD buffer containing 4 mM-MgCl₂, NaCl as indicated, 3 μM -ssM13 DNA, 1.5 μM -RecA protein and 0.6 μM -SSB protein added, as indicated, after RecA protein had attained a steady-state rate of ATP hydrolysis. Panels (a), (b) and (c) represent reactions carried out at 25°C, 37°C and 42°C, respectively. Circles represent RecAwt protein and triangles RecA441 protein. Open symbols are reactions without SSB protein and filled symbols are reactions containing SSB protein.

examine ATPase activity when SSB protein is bound to the ssDNA prior to the addition of RecA protein. At 25°C there is essentially a complete inhibition of the ATPase activity of both RecA441 and RecAwt proteins, regardless of MgCl₂ concentration (Table 1). Thus, at this temperature, neither RecA protein appears to be proficient at displacing SSB protein from ssDNA.

At 37°C and 42°C, both RecA proteins are able to

displace SSB protein from the ssDNA, which can be inferred from their ability to hydrolyze ATP. This hydrolysis occurs following a lag, which varies in length with experimental conditions and can be assumed to parallel the kinetics of SSB protein displacement by RecA protein. Increases in MgCl₂ concentration significantly improve the ability of RecAwt protein to displace SSB protein from ssDNA, as evidenced by decreases in the lag times and increases in the rates of ATP hydrolysis following the lag times (Table 1). At 1 mM-MgCl₂, RecAwt protein is unable to displace SSB protein from ssDNA and hydrolyze ATP, regardless of temperature. At 4 mM-MgCl₂, RecAwt protein is able to displace SSB protein from the ssDNA at both 37°C and 42°C. However, the rates of ATP hydrolysis are notably less than the rates seen when SSB protein is added to the reaction mix after the addition of RecA protein (compare rates in Table 1 with those in Fig. 2), suggesting that RecAwt protein cannot displace all of the SSB protein. As MgCl₂ concentration is increased further to 10 mM, RecAwt protein displaces SSB protein from ssDNA more quickly and more completely than it does at 4 mM-MgCl₂.

In contrast to what is observed for RecAwt protein, the ability of RecA441 protein to displace SSB protein from ssDNA is not dramatically affected by MgCl₂ concentration (Table 1). At both 37°C and 42°C, there is only a slight decrease in the length of the lag and a slight increase in the rate of hydrolysis as MgCl₂ concentration increases from 1 mM to 10 mM. Additionally, at all MgCl₂ concentrations, the mutant RecA protein displaces SSB protein from ssDNA more quickly and more completely than the wild-type RecA protein.

At any given MgCl₂ concentration, increasing the temperature from 37°C to 42°C significantly improves the ability of RecA441 protein to displace SSB protein from the DNA (Table 1). At 42°C, RecA441 protein achieves a rate of hydrolysis that is nearly 90% of that observed when the SSB protein is added to the DNA after the addition of RecA protein, and it achieves this rate after a relatively short lag (460 s). While both RecAwt and RecA441 proteins show this increased ability to displace SSB protein from ssDNA as temperature increases, the results in Figure 4 show that the ability of RecA441 protein improves more impressively as temperature increases. Thus, the results demonstrate that RecA441 protein displaces SSB protein from ssDNA more effectively than the wild-type RecA protein, with the difference being most pronounced at low MgCl₂ concentrations and at elevated temperatures.

(e) *Etheno M13 DNA-dependent ATPase activity*

Kowalczykowski & Krupp (1987) have shown that SSB protein inhibits the etheno M13 DNA-dependent ATPase activity of RecAwt protein under all conditions. Etheno M13 DNA is a

Table 1
Displacement of SSB protein and ATP hydrolysis

<i>t</i> (°C)	Protein	1 mM-MgCl ₂		4 mM-MgCl ₂		10 mM-MgCl ₂	
		Lag†	Rate‡	Lag	Rate	Lag	Rate
25	RecAwt	—	0	—	0	—	0
25	RecA441	—	0	—	0	—	0
37	RecAwt	—	0	2070	7.1	1200	11.7
37	RecA441	1200	15.3	1100	15.3	1020	16.6
42	RecAwt	—	0	1050	13.6	615	20.0
42	RecA441	560	27.0	460	31.8	475	32.1

All reactions were performed in TD buffer and contained 3 μ M-ssM13 DNA, 1.5 μ M-RecA protein, and 0.6 μ M-SSB protein added to ssDNA 1 min prior to the addition of RecA protein.

† Lag represents the time (in s) required to attain a steady-state rate of ATP hydrolysis. The lag time is determined by extending the linear portion of the kinetic time-course back to zero absorbance change and determining the time at which they intersect.

‡ The steady-state rate of ATP hydrolysis (μ mol ATP/min) following the lag is reported. 0 indicates less than 0.5 μ mol ATP hydrolyzed/min.

chloroacetaldehyde-modified ssM13 DNA that is devoid of secondary structure as a result of the modification. Since there are conditions under which RecA441 protein is more successful than RecAwt protein at competing with SSB protein for sites on ssDNA, we wondered if the etheno M13-stimulated ATPase activity of RecA441 protein was also resistant to inhibition by SSB protein. The rate of ATP hydrolysis by RecA441 and RecAwt proteins on etheno M13 DNA was therefore measured and the effect of SSB protein on this rate was determined. As can be seen in Figure 5, the rate of etheno M13 DNA-dependent ATP hydrolysis by RecA441 protein is unaffected by changes in MgCl₂ concentration. Although this result apparently contradicts what is observed on unmodified ssDNA, where an increase in MgCl₂ concentration results in a decrease in the rate of ATP hydrolysis, it is not surprising. The effect of MgCl₂ on the rate of ATP hydrolysis on unmodified ssDNA is presumably due to an indirect effect on the stability of DNA

secondary structure which limits RecA protein's access to it, and as previously mentioned, etheno M13 contains no secondary structure. While the ATPase activity of RecA441 protein is not affected by changes in MgCl₂ concentration, that of RecAwt protein is, somewhat surprisingly, reduced at 1 mM-MgCl₂ (Fig. 5).

The etheno M13 DNA-dependent ATPase activity of both the wild-type and the mutant RecA proteins is inhibited by SSB protein at all conditions tested (data not shown). This inhibition is gradual and the half-time with which the rate of hydrolysis decays has been determined. The half-time of decay for RecAwt protein tends to increase slightly as MgCl₂ concentration increases or temperature decreases (Table 2). The half-time of decay for RecA441 protein shows no such orderly tendency, yet is substantially greater than that for RecAwt protein under all conditions, implying that RecA441 protein is more resistant to displacement by SSB protein.

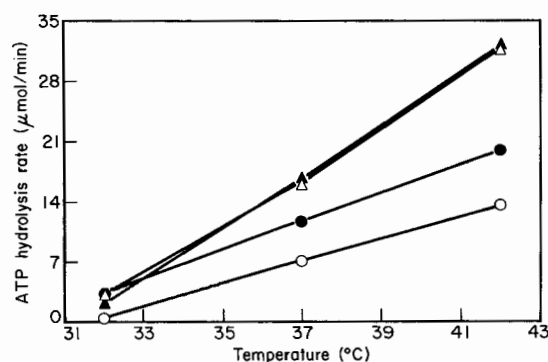


Figure 4. The effect of temperature on the rate of ATP hydrolysis following SSB protein displacement. Reactions were performed in TD buffer containing 3 μ M-ssM13 DNA, 1.5 μ M-RecA protein and 0.6 μ M-SSB protein added to ssDNA 1 min prior to the addition of RecA protein. Circles represent RecAwt protein and triangles RecA441 protein. Open symbols are reactions at 4 mM-MgCl₂ and filled symbols are reactions at 10 mM-MgCl₂.

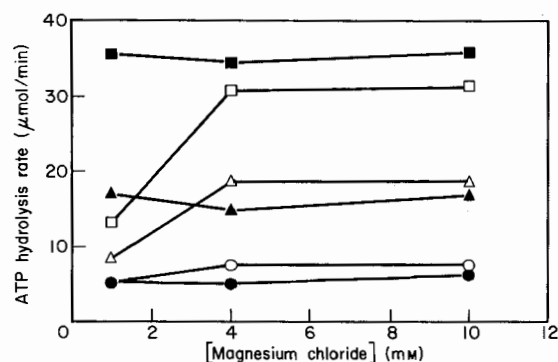


Figure 5. The effects of MgCl₂ concentrations and temperature on etheno M13 DNA-dependent ATPase activity. Reactions were performed in TD buffer containing 3 μ M-etheno M13 DNA and 1.5 μ M-RecA protein. Open symbols represent RecAwt protein and filled symbols RecA441 protein. Circles, triangles and squares are reactions at 25°C, 37°C and 42°C, respectively.

Table 2
Inhibition of etheno M13 DNA-dependent ATP hydrolysis by SSB protein

t (°C)	Protein	Half-time decay of ATP hydrolysis rate (min)†		
		1 mM-MgCl ₂	4 mM-MgCl ₂	10 mM-MgCl ₂
25	RecAwt	1.4	2.1	3.1
25	RecA441	7.6	4.6	6.5
37	RecAwt	0.76	1.2	2.5
37	RecA441	4.2	3.4	4.3
42	RecAwt	0.41	0.70	1.5
42	RecA441	2.2	2.4	11

All reactions were performed in TD buffer and contained 3 μ M-etheno M13 DNA, 1.5 μ M-RecA protein, and 0.6 μ M-SSB protein added after RecA protein had attained a steady-state rate of ATP hydrolysis.

† Half-times for decay of hydrolysis rate were determined from a non-linear least-squares fit of the raw data to a single exponential decay.

(f) *dsDNA-dependent ATPase activity*

The constitutive protease activity of RecA441 protein could possibly be attributed to an enhanced ability to invade duplex DNA, thereby supplying a required polynucleotide cofactor in the absence of DNA damage (Phizicky & Roberts, 1981). The steady-state rate of ATP hydrolysis by RecA protein on dsDNA is preceded by a lag that has been proposed to represent the time required to transiently denature the dsDNA and form single-stranded regions that can then support ATP hydrolysis (Kowalczykowski *et al.*, 1987). If RecA441 protein does indeed have an enhanced ability to invade duplex DNA, then the length of the lag in its dsDNA-dependent ATPase activity would be reduced. Therefore, the ATPase activity of RecA441 and RecAwt protein was examined with dsDNA under a variety of conditions. At 25°C, there is effectively no hydrolysis of ATP by either RecA protein (Table 3). At 37°C and 42°C, the activity of both RecA441 and RecAwt proteins is dependent on MgCl₂ concentration. At 10 mM-MgCl₂, the length of time required to attain a steady-state rate of ATP hydrolysis, or "lag time",

is greater for RecA441 protein than for RecAwt protein, with the difference being more pronounced at the lower temperature. Additionally, the steady-state rate of hydrolysis by RecA441 protein is somewhat less than that by RecAwt protein under these conditions.

At 1 mM-MgCl₂, the lag time for both proteins is less than at 10 mM-MgCl₂ (Table 3). The reduction in MgCl₂ concentration also affects the steady-state rate of ATP hydrolysis by both proteins; however, each is affected in an opposite way. The rate by RecA441 protein is increased, while that by RecAwt protein is decreased at the lower MgCl₂ concentration. At 42°C and 1 mM-MgCl₂, RecA441 protein achieves a steady-state rate of ATP hydrolysis that is twice that of RecAwt protein, after a lag that is half as long as that of RecAwt protein. Thus, these are conditions under which RecA441 protein does appear to have an enhanced ability to invade dsDNA and utilize this substrate for the stimulation of ATP hydrolysis.

(g) *The stimulation of LexA-repressor cleavage by RecA protein*

The preceding examination of ATPase activity suggests that RecA441 protein, when compared to RecAwt protein, competes more efficiently with SSB protein for sites on ssDNA and invades dsDNA more proficiently at elevated temperature and/or when the MgCl₂ concentration is low. In hopes of explaining the temperature-inducible constitutive protease activity of RecA441 protein and in light of these results, the *in vitro* cleavage of LexA protein by RecA441 and RecAwt protein was compared. At both 25°C (data not shown) and 42°C, in the absence of SSB protein, the rate of ssDNA-dependent repressor cleavage by RecA441 protein is significantly greater than that by RecAwt protein, with the difference being most pronounced at 1 mM-MgCl₂ (Table 4). The rate of cleavage by both proteins is greater at 1 mM-MgCl₂ than at either 4 mM or 10 mM-MgCl₂, which is most likely the result of an indirect effect on the secondary structure of the ssDNA.

Table 3
Double-stranded DNA-dependent ATP hydrolysis

t (°C)	Protein	1 mM-MgCl ₂		10 mM-MgCl ₂	
		Lag†	Rate‡	Lag	Rate
25	RecAwt	—	0	—	0
25	RecA441	—	0	—	0
37	RecAwt	500	10.6	800	17.1
37	RecA441	340	23.9	1115	12.5
42	RecAwt	300	24.4	360	34.8
42	RecA441	140	43.9	400	32.2

All reactions were performed in TD buffer and contained 8.4 μ M-linear dsM13 DNA and 3 μ M-RecA protein.

† Lag represents the time (in s) required to attain a steady-state rate of ATP hydrolysis. The lag time was determined as described for Table 1.

‡ The steady-state rate of ATP hydrolysis (μ M-ATP/min) following the lag is reported. 0 indicates less than 0.5 μ M-ATP hydrolyzed/min.

(h) *LexA-protease activity of RecA protein in the presence of SSB protein*

In the presence of SSB protein, the difference between the protease abilities of RecA441 and RecAwt proteins becomes more pronounced. Palmer & Kowalczykowski (unpublished results) have shown that SSB protein (0.6 μM) inhibits the LexA-protease activity of RecAwt protein on ssM13 DNA (3 μM), when RecA protein concentrations are less than 1 μM , but stimulates this activity when RecA protein concentrations are greater than 1 μM . In contrast, the protease activity of RecA441 protein is more resistant to this inhibition by SSB protein (Table 4). The addition of SSB protein to a LexA cleavage reaction containing 1 μM -RecA441 protein has no effect on the rate of repressor cleavage at 4 mM or 10 mM-MgCl₂, and only a slight inhibition of the rate at 1 mM-MgCl₂. This inhibition is minor when compared to the no observable cleavage of LexA protein by RecAwt protein under the same conditions.

When SSB protein is added to ssDNA prior to the addition of 1 μM -RecA protein, similar trends are obtained (Table 4). There is no observable cleavage of LexA protein by RecAwt protein, while RecA441 protein is able to cleave the LexA protein. These experiments were performed in two ways: SSB first(L), (L=lager), designates reactions in which SSB protein was added to the reaction buffer containing ssDNA, after one minute RecA protein was added, and after one more minute LexA protein was added to start the cleavage reaction. SSB first(SS), (SS=steady state), designates reactions in which SSB protein was again added to the reaction buffer containing ssDNA, after one minute RecA protein was added, this mixture was then incubated at 42°C for sufficient time to allow RecA protein to achieve a steady-state rate of ATP hydrolysis (at least 30 min for RecAwt protein and at least 15 min for RecA441 protein), and then LexA protein was added to start the cleavage reaction. When the SSB first(L) protocol is followed, a lag is observed in the cleavage of LexA

protein by RecA441 protein. The rate reported in Table 4 does not represent a steady-state rate, but rather the maximum rate attained before the 10 μM -LexA protein is completely cleaved into its two fragments, i.e. it represents a rate of cleavage that is occurring within the lag phase. When the SSB first(SS) protocol is followed, no lag is observed in the cleavage reaction and the rate of cleavage is about 80% of that observed when SSB is added second. Either protocol leads to no observable cleavage of LexA protein by the wild-type RecA protein.

At higher RecA protein concentration (1.5 μM), where the LexA-protease activity of the wild-type RecA protein is not inhibited by SSB protein added second, there is still a dramatic inhibition of the protease activity of RecAwt when SSB protein is added to ssDNA prior to the addition of RecA protein (Table 4, values in parentheses). In contrast, the rate of repressor cleavage by 1.5 μM -RecA441 in the SSB first(SS) reactions approaches that observed when SSB protein is added second (Table 4, values in parentheses).

To address the temperature sensitivity of RecA441 protein, the SSB protein displacement experiments were also performed at 25°C. At this temperature, the *lexA* protease activity of both the wild-type and the mutant RecA proteins is completely inhibited by SSB protein added to ssDNA prior to the addition of RecA protein (data not shown). Thus, as was seen with ATPase activity, the ability of RecA441 protein to displace SSB protein from ssDNA and stimulate the cleavage of LexA protein is dependent on elevated temperature.

(i) *LexA-protease activity of RecA protein with dsDNA*

RecA441 protein appears to have an enhanced ability to invade dsDNA at elevated temperature and low MgCl₂ concentration, i.e. lags are reduced and rates are increased in dsDNA-dependent ATPase activity. This fact and the observation of

Table 4
The cleavage of LexA protein

[MgCl ₂]	Protein	Rate of cleavage (μmol LexA/min)			
		No SSB	SSB 2nd†	SSB 1st(L)‡	SSB 1st(SS)‡
1 mM	RecAwt	1.37	0	0	0
1 mM	RecA441	2.83	2.3	0.55	1.86
4 mM	RecAwt	1.14	0	0	0
4 mM	RecA441	1.63	1.7	0.86	1.45
10 mM	RecAwt	1.1	0 (2.6)§	0 (0)	0 (0.4)
10 mM	RecA441	1.76	1.8 (2.8)	0.81 (1.0)	1.48 (2.6)

Reactions were performed at 42°C in TD buffer containing 50 mM-NaCl, and 3 μM -ssM13 DNA, 1 μM -RecA protein and 0.6 μM -SSB protein added as indicated. 0 indicates less than 0.03 μmol LexA cleaved/min.

†SSB 2nd designates reactions where SSB protein is added to ssDNA 1 min after the addition of RecA protein.

‡See the text for an explanation of SSB 1st(L) and SSB 1st(SS) designations.

§ Values in parentheses represent rates of cleavage obtained with 1.5 μM -RecA protein.

Lu & Echols (1987) that RecA441 can cleave LexA protein in the presence of dsDNA led us to examine the rates of LexA protein cleavage by RecA441 and the wild-type RecA protein when dsDNA is present as a cofactor. At 25°C and all MgCl₂ concentrations tested (1 mM, 4 mM and 10 mM), there is no appreciable cleavage of LexA protein by either RecA protein (data not shown). The same is true of RecAwt protein at 42°C. However, at 42°C, dsDNA does support cleavage of LexA protein by 1 μM-RecA441 protein at a rate that is about 10% of that observed on ssDNA (0.17 μmol LexA/min). The attainment of this rate is not preceded by a lag and is surprisingly independent of MgCl₂ concentration (data not shown). Since MgCl₂ concentration is known to affect the length of the lag and the rate of ATP hydrolysis on dsDNA, yet has no effect on dsDNA-supported LexA protein cleavage by RecA441 protein, we conclude that RecA441 protein is not invading the duplex during this reaction, but is perhaps just binding to the dsDNA, and thereby activated to cleave LexA protein at an albeit slow rate.

(j) *Salt titrations of RecA protein-etheno M13 DNA complexes*

The preceding results suggest that there are conditions under which RecA441 protein is more successful than the wild-type protein at competing with SSB protein for sites on ssDNA. To determine whether this could be attributed to differences in the ssDNA binding characteristics of RecA441 and RecAwt proteins, we measured their binding to etheno M13 DNA, a modified ssM13 DNA whose fluorescence increases upon RecA protein binding (Menetski & Kowalczykowski, 1985). RecA441 and RecAwt protein bind etheno M13 DNA with

virtually the same stoichiometry, saturating the DNA with a site size of about eight nucleotides per RecA protein monomer (data not shown). Since the salt concentration required to dissociate a RecA protein-DNA complex has been shown to be related to the equilibrium affinity of the protein for ssDNA (Menetski & Kowalczykowski, 1985), salt titrations of RecAwt protein and RecA441 protein-DNA complexes were carried out. Surprisingly, the salt titration midpoints for RecA441 protein are lower than those for RecAwt protein, both in the absence of any nucleotide cofactor and in the presence of ATP (Fig. 6). In the absence of nucleotide cofactor, the salt titration midpoint for RecA441 protein is lower, by 110 mM-NaCl, than that for RecAwt protein at both 1 mM and 10 mM-MgCl₂.

In the presence of 500 μM-ATP, both RecA proteins show a marked increase in salt titration midpoint and a high relative fluorescence increase, which are indicative of a high affinity binding state (Menetski & Kowalczykowski, 1987b). However, the salt titration midpoint for RecA441 protein is still lower than that for the wild-type protein, with the difference being greater at 10 mM-MgCl₂ than at 1 mM-MgCl₂ (Fig. 6). These lower salt titration mid-points for RecA441 protein suggest that the explanation for the enhanced ability of RecA441 protein in the competition with SSB protein is not an increased equilibrium or steady-state affinity for ssDNA.

ADP has been shown to decrease the equilibrium binding affinity of RecA protein for ssDNA (Menetski & Kowalczykowski, 1985). The results in Figure 6 show that, in the presence of 200 μM-ADP, a salt titration midpoint of about 160 mM-NaCl is observed for both RecA441 protein and RecAwt protein, regardless of MgCl₂ concentration. In the case of the wild-type protein, this value corresponds

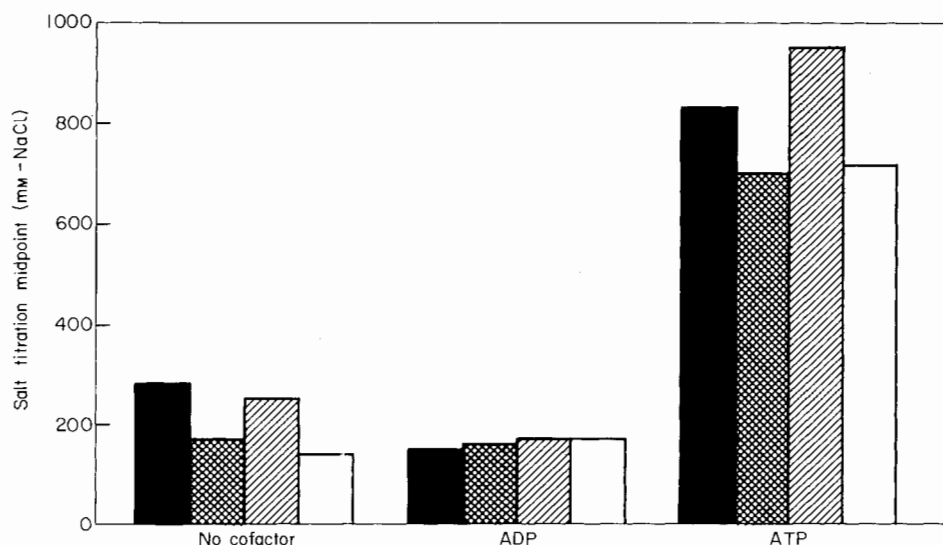


Figure 6. Salt titration midpoints of RecA protein-etheno M13 DNA complexes. Reactions were performed at 37°C in TD buffer, and contained 1.1 μM-RecA protein, 3 μM-etheno M13 DNA and 200 μM-ADP or 500 μM-ATP as indicated. Filled and hatched bars are RecAwt at 1 mM and 10 mM-MgCl₂, respectively. Cross-hatched and open bars are RecA441 at 1 mM and 10 mM-MgCl₂, respectively.

to a decrease in affinity relative to that observed in the absence of nucleotide cofactor. However, in the case of RecA441 protein, the salt titration midpoint observed in the presence of ADP is equivalent to that observed in the absence of nucleotide cofactor.

(k) *Transfer of RecA protein from etheno M13 DNA to poly(dT)*

The observation that the salt titration midpoint for RecA441 protein is never greater, and often less, than that for RecAwt protein, is inconsistent with the idea that RecA441 protein binds ssDNA more tightly than RecAwt protein, and as a result can compete more efficiently with SSB protein for limited sites. However, this inconsistency could be due to differences in the salt sensitivity of the equilibrium binding properties of the two proteins, or it may imply that some kinetic property of the RecA protein interaction with ssDNA is important. Therefore, the lifetime of RecA protein-DNA complexes was addressed by measuring the rate at which RecA protein transfers from etheno M13 DNA to poly(dT). Menetski & Kowalczykowski (1987a) have shown that the transfer reaction does not occur by a simple dissociation mechanism, but rather occurs *via* the formation of an intermediate consisting of etheno M13 DNA, RecA protein and poly(dT). Additionally, the transfer reaction consists of a fast transferring component and a slow transferring component, whose rates and relative proportions vary with experimental conditions. In the presence of ATP, the transfer of RecA441 protein is similar to that of RecAwt protein (Table 5). In the absence of nucleotide cofactor, the rate of each of the components and the amplitude of the fast component of RecAwt protein both increase as MgCl₂ increases, presumably due to an effect on the aggregation state of the protein-DNA complexes (Menetski & Kowalczykowski, 1987a,b). Interestingly, the no cofactor transfer of RecA441 protein is much less sensitive to changes in MgCl₂ concentration, and at both 1 mM and 4 mM-MgCl₂, RecA441 protein transfers like RecAwt protein does at 4 mM-MgCl₂.

In the presence of ADP, there are differences in

the transfer of RecA441 and RecAwt protein (Table 5). The fast component of RecAwt protein is essentially non-existent at 1 mM-MgCl₂ and too fast to be measured at 4 mM-MgCl₂. Also, the rate of the slow component of RecAwt protein is much greater in the presence of ADP than it is in the absence of nucleotide cofactor. In contrast, the characteristics of RecA441 protein transfer in the presence of ADP are not much different from those observed in the absence of nucleotide cofactor. Two components are clearly defined at all conditions tested and, most significantly, the rates are much slower than the rates observed for the wild-type protein.

4. Discussion

The *recA441* mutant strain of *E. coli* expresses constitutive protease activity at elevated temperature or upon amplification of the mutant RecA protein. Our examination of the biochemical properties of the wild-type and the mutant RecA proteins finds them to be similar in the absence of SSB protein. They bind ssDNA with the same stoichiometry and hydrolyze ATP at the same rate when no NaCl is present. Additionally, the salt titration midpoint in the presence of ADP is the same for both RecA proteins, and the characteristics of their transfer from one DNA molecule to another, in the presence of ATP, are the same. Differences do exist between the two RecA proteins in the absence of SSB protein and, in general, RecA441 protein displays enhanced activity relative to RecAwt protein. The ssDNA-dependent ATPase activity of RecA441 protein shows a lower sensitivity to the presence of NaCl. The rate of LexA protein cleavage by RecA441 is greater than that by RecAwt. At low MgCl₂ concentration, the etheno M13 DNA-dependent and dsDNA-dependent ATPase activities of RecA441 protein are also greater than those of RecAwt protein. Additionally, the transfer of RecA441 protein from one DNA molecule to another, in the presence of ADP, occurs at a much slower rate than does that of RecAwt protein. However, one area where the wild-type RecA protein shows an advantage is in the salt stability of the RecA protein-etheno M13

Table 5
Transfer of RecA protein from etheno M13 DNA to poly(dT)

[MgCl ₂]	Protein	No cofactor				ADP				ATP			
		<i>k</i> 1†	<i>A</i> 1‡	<i>k</i> 2§	<i>A</i> 2	<i>k</i> 1	<i>A</i> 1	<i>k</i> 2	<i>A</i> 2	<i>k</i> 1	<i>A</i> 1	<i>k</i> 2	<i>A</i> 2
1 mM	RecAwt	7.1	0.30	0.77	0.70	—	—	19	1.0	7.9	0.81	0.95	0.19
1 mM	RecA441	57	0.46	1.1	0.54	12	0.34	0.64	0.66	6.8	0.70	1.7	0.30
4 mM	RecAwt	50	0.72	7.5	0.28	—	0.40	36	0.60	14	0.53	3.1	0.47
4 mM	RecA441	73	0.53	3.5	0.47	47	0.50	2.8	0.50	12	0.32	2.4	0.68

All reactions were performed in TD buffer and contained 0.56 μM-RecA protein, 4.2 μM-etheno M13 DNA, 25 μM-poly(dT) added at *t*=0, and 200 μM-ADP or 500 μM-ATP as indicated.

† *k*1 is the rate of the fast component (10⁻³ s⁻¹).

‡ *A*1 is the relative amplitude of the fast component.

§ *k*2 is the rate of the slow component (10⁻³ s⁻¹).

|| *A*2 is the relative amplitude of the slow component.

DNA complex; namely, the salt titration midpoints for RecA441 protein, both in the presence of ATP and in the absence of nucleotide cofactor, are lower than those for RecAwt protein. While these differences are interesting, they offer little insight into the temperature-inducible phenotype observed in *recA441* strains *in vivo*.

In contrast, the differences that exist in the presence of SSB protein are conducive to an hypothesis that explains the temperature-sensitive behavior observed in *recA441* strains. Both the ssDNA-dependent ATPase and LexA-protease activities of RecA441 protein are more resistant to inhibition by SSB protein at low MgCl₂ and RecA protein concentrations. Additionally, an examination of ATPase and LexA-protease activities utilizing ssDNA, which has been precoated with SSB protein, suggests that RecA441 protein can displace SSB protein from ssDNA more quickly and more completely than RecAwt protein. This enhanced ability to displace SSB protein is contingent upon elevated temperature. Finally, the half-time with which the rate of etheno M13 DNA-dependent ATP hydrolysis decays after addition of SSB protein is greater for RecA441 protein than for RecAwt protein. These observations are consistent with the hypothesis that RecA441 protein competes more efficiently with SSB protein for limited ssDNA sites and can be activated to cleave repressors by displacing SSB protein from the single-stranded regions that occur naturally in *E. coli*. Our hypothesis to explain the constitutive protease activity in *recA441* strains is dependent on the involvement of SSB protein during SOS/prophage induction. Consistent with this idea, *SSB* mutant strains have been shown to be sensitive to ultraviolet light, defective in phage induction (Vales *et al.*, 1980), and unable to induce RecA protein to normal levels (Meyer *et al.*, 1982). Additionally, Roberts *et al.* (1982) have proposed a model for SOS induction that suggests that RecA protein association with single-stranded gaps in damaged DNA might involve the displacement of SSB protein.

It is conceivable that the enhanced protease activity observed in some *recA* mutant strains can be attributed to the fact that the mutant proteins are more effective at invading secondary structure in DNA and therefore more efficiently activated to cleave repressors. However, after an examination of ATPase activity, which has been shown to parallel LexA-protease activity under a variety of conditions (Palmer & Kowalczykowski, unpublished results), we conclude that this is not the case for RecA441 protein. The rates of hydrolysis by both RecA441 and RecAwt proteins saturate at the same point on ssM13 DNA in the absence of SSB protein (see Fig. 1) and are stimulated to the same extent by the addition of SSB protein. This suggests that RecA441 protein does not have a heightened ability to invade secondary structure.

Despite the similarities in the ssDNA-dependent ATPase activity of RecA441 and RecAwt proteins,

differences are observed when SSB protein is present. Under most conditions, the ssDNA-dependent activities of RecA441 protein are less susceptible to inhibition by SSB protein. At 1 mM-MgCl₂, the ATPase activity of the wild-type RecA protein is completely inhibited by the addition of SSB protein. In contrast, that of RecA441 protein is not inhibited, but rather stimulated, by this addition of SSB protein. Additionally, at the intermediate MgCl₂ concentration of 4 mM, SSB protein has an inhibitory effect on the ATPase activity of low concentrations of the wild-type RecA protein, while no such inhibition of RecA441 protein activity is observed. The inhibition of the ATPase activity of RecAwt protein by SSB protein is presumed to be due to the fact that, at low MgCl₂ concentrations and low RecA protein concentrations, the competition for limited sites on ssDNA favors SSB protein binding, and RecA protein is displaced from the DNA (Kowalczykowski & Krupp, 1987). Thus, at both low protein concentrations and low MgCl₂ concentrations, RecA441 protein appears to be more competent than RecAwt protein at competing with SSB protein for limited sites on ssDNA.

When SSB protein is present on ssDNA, and RecA protein must displace it in order to hydrolyze ATP, there is a lag prior to the onset of ATP hydrolysis, which corresponds to the time required for RecA protein to displace the SSB protein from the DNA (Kowalczykowski & Krupp, 1987). Also, the rate of ATP hydrolysis following the lag is less than that observed when SSB protein is added to ssDNA after the addition of RecA protein, signifying that RecA protein cannot displace all of the SSB protein; i.e. there appears to be a kinetic block to saturation of the ssDNA. RecA441 displaces SSB protein from ssDNA more quickly and more completely than RecAwt protein at all conditions tested, except at 25°C where neither RecA protein is proficient at displacing SSB protein. Both RecA proteins displace SSB protein from ssDNA more quickly and more completely as temperature increases. However, the ability of RecA441 protein improves more dramatically with increasing temperature (see Fig. 4), as might be expected from a mutation that yields temperature-sensitive behavior. At 42°C, the RecA441 protein appears to be able to overcome the aforementioned kinetic block and approaches saturation of the ssDNA. Thus, these biochemical properties of the RecA441 protein parallel the observed *in vivo* phenotype, i.e. there is an enhancement of activity at elevated temperature.

The etheno M13-dependent ATPase activity of both RecA441 and RecAwt proteins is inhibited by the addition of SSB protein. However, consistent with the above observations that the ATPase activity of RecA441 protein is more resistant to inhibition by SSB protein, the kinetics of inhibition for RecA441 protein are much slower than those for the wild-type RecA protein. This suggests that, compared to the RecAwt protein, RecA441 protein

has a longer lifetime on the DNA before relinquishing sites to SSB protein. This could imply that RecA441 protein is more processive in its hydrolysis of ATP (see Menetski & Kowalczykowski, 1987b).

Although most of the characterization of the RecA441 protein was done *via* an examination of ATP hydrolysis properties, it is important to note that the increased resistance to inhibition by SSB protein, observed in the ATPase activity of RecA441 protein, is paralleled in LexA-protease activity. At low RecA protein concentrations and low MgCl₂ concentrations, the addition of SSB protein to the cleavage reaction inhibits the ability of the wild-type RecA protein to cleave LexA protein. This inhibition is not observed for RecA441 protein (Table 4), and cleavage of LexA protein by RecA441 protein occurs at 42°C regardless of when SSB protein is added to the reaction mix. When SSB protein is added after RecA protein (1 μM), it has essentially no effect on the rate of repressor cleavage by RecA441 protein, while inhibiting that by RecAwt protein. If SSB protein is added to the ssDNA prior to the addition of RecA protein, the behavior of RecA441 during repressor cleavage is similar to that observed in ATPase activity. The ability of RecA441 protein to stimulate the cleavage of LexA protein, when it is added to ssDNA after the addition of SSB protein, is dependent on elevated temperature, i.e. no cleavage occurs at 25°C. Thus, as was seen with ATPase activity, the ability of RecA441 protein to compete with SSB protein for limited ssDNA sites, and thereby stimulate the cleavage of LexA protein, is enhanced relative to that of the wild-type RecA protein.

The constitutive protease activity of RecA441 can be explained in light of the preceding observations. By displacing SSB protein from the forks of replicating chromosomes, RecA441 protein could be activated to cleave repressors in the absence of DNA damage. Since RecA441 protein is not able to efficiently displace SSB protein from ssDNA at 25°C, and is able to do so at 42°C, this activation is dependent on elevated temperature, i.e. it is thermally induced. Since RecAwt protein is less competent at displacing SSB protein from ssDNA, wild-type strains do not display this constitutive protease activity. Compatible with this notion, D'Ari *et al.* (1979) have shown that the constitutive protease activity of *recA441* strains can be suppressed at 42°C by the *dnaB252* mutation, which inhibits initiation of replication, without affecting elongation, and thus eliminates these replication forks.

At elevated temperature, RecA441 protein cleaves LexA protein very slowly in the presence of dsDNA. This cleavage is not preceded by a lag or affected by changes in MgCl₂ concentration, suggesting that RecA441 protein is not denaturing the duplex to be activated to its protease form. Nonetheless, the slow rate of repressor cleavage observed by RecA441 protein in the presence of dsDNA *in vitro* may have some significance to the

activation of RecA441 protein to cleave repressors *in vivo* (Lu & Echols, 1987). While *dna* mutations, which affect the existence of the replication fork, suggest that a dsDNA pathway alone is not sufficient for activation of the constitutive protease activity of RecA441 protein, dsDNA-dependent cleavage of LexA protein could result in a decreased level of LexA repressor and an increased level of RecA441 protein in *recA441* strains, contributing to the ease with which induction occurs. Increased levels of RecA441 protein would give it an added advantage in the competition with SSB protein for single-stranded regions of DNA, and decreased levels of LexA protein would allow SOS genes to be derepressed more readily.

If the above hypotheses are correct, then our *in vitro* data could suggest that effective *in vivo* MgCl₂ concentration is quite low. The reasoning behind this view is that the differences between the abilities of RecA441 protein and the wild-type protein are most pronounced at low MgCl₂ concentrations, and the RecA441 protein is presumed to display "hyper-activity" relative to RecAwt protein *in vivo*. The following activities of RecA441 protein are enhanced relative to RecAwt protein at 1 mM-MgCl₂: the etheno M13 DNA-dependent ATPase activity, the dsDNA-dependent ATPase activity, and finally the ability to compete with SSB protein for ssDNA sites and be activated to hydrolyze ATP or cleave LexA protein. Thus, the *in vitro* conditions at which the RecA441 protein is most proficient intimate that *in vivo* MgCl₂ concentrations might be low.

Efforts to explain why the RecA441 protein has an enhanced ability to compete with SSB protein have been less positive. Despite a greater resistance to increasing NaCl concentration and a decreased sensitivity to inhibition by SSB protein in ssDNA-dependent activities, the affinity of RecA441 protein for ssDNA, as deduced from salt titration midpoints, is somewhat less than that of RecAwt protein. Additionally, the rate of RecA441 protein dissociation from ssDNA in the presence of ATP, as inferred from the rate at which it transfers from one DNA molecule to another, is the same as that of RecAwt protein. Since RecA441 protein does not appear to have an increased equilibrium affinity for ssDNA or a decreased rate of dissociation from ssDNA, we conclude that the RecA441 protein advantage may lie in a step involving the association (i.e. polymerization) of RecA441 protein with ssDNA. The observation that the etheno M13 DNA-dependent ATPase activity of RecA441 protein is inhibited more slowly than that of RecAwt protein implies that the RecA441 protein has a longer lifetime on ssDNA before dissociating. However, it may also be indicative of an increased rate of RecA441 protein association with the DNA. If the two RecA proteins display the same kinetics of dissociation, as the transfer reaction suggests, yet RecA441 protein reassociates with the DNA more rapidly than RecAwt protein (i.e. preventing the binding of SSB protein), then the net result would

be to observe an apparently longer lifetime on the ssDNA for the RecA441 protein. More rapid association of RecA441 protein with ssDNA would allow the protein to compete more efficiently with SSB protein for limited ssDNA sites, and would also account for the more rapid displacement of SSB protein observed in the ATP hydrolysis reaction.

In summary, as an explanation for the temperature-inducible phenotype of *recA441* strains we offer that the RecA441 protein can, by virtue of its enhanced ability to compete with SSB protein for limited ssDNA sites, displace SSB protein from the ssDNA that occurs naturally in *E. coli* and be activated to cleave repressors at elevated temperature in the absence of DNA damage. The difference between the abilities of the wild-type and the mutant RecA proteins are most pronounced at low magnesium concentration, hinting that *in vivo* the MgCl₂ concentration may be quite low. Despite the fact that RecA441 protein can displace SSB protein from ssDNA more quickly and more completely than RecAwt protein and is displaced from etheno M13 DNA by SSB protein more slowly than RecAwt protein, the equilibrium binding affinity of RecA441 protein for ssDNA is not greater than that of RecAwt protein and the rate of dissociation does not appear to be slower. This suggests that the kinetics of RecA protein association with ssDNA are more significant to the enhanced ability of RecA441 protein to compete with SSB protein for limited ssDNA sites.

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