"Activated"-RecA protein affinity chromatography of LexA repressor and other SOS-regulated proteins

(Nucleoprotein complex/lexA-SA119 mutant/recA430 mutant/α repressor/UmuC and UmuD* proteins)

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ABSTRACT We have developed an affinity column to study the interaction of LexA repressor and other substrates with the activated form of RecA protein. Nucleoprotein complexes of RecA protein, (dT)$_{25-30}$, and adenosine 5'-[γ-thio]triphosphate were formed in solution and bound to RecA protein-agarose columns. These "activated"-RecA nucleoprotein complexes were retained by strong hydrophobic interactions. Purified LexA protein bound tightly to these activated RecA columns, whereas the LexA protein bound poorly to RecA-agarose alone. Once bound, LexA protein underwent specific proteolysis, and the fragments were released from the complex. The mutant LexA protein, LexA-SA119, which cannot carry out self-cleavage or RecA-mediated cleavage in solution, bound efficiently to the activated RecA column but was not cleaved, indicating that these columns can be used to identify residues involved in RecA-LexA binding. As an example of this use, nucleoprotein complexes were prepared using the RecA430 protein. In vivo the recA430 mutation blocks induction of the SOS response. LexA protein was not efficiently retained on the immobilized RecA430 complexes, suggesting that Gly-204 is required for efficient repressor binding. These results show that activated RecA affinity columns can be used to investigate the binding and cleaving properties of mutationally altered RecA and LexA proteins. Additionally, these activated RecA columns have been used to investigate binding interactions of phage λ repressor, as well as the UmuC protein, which is required for chemical mutagenesis.

The bacterium Escherichia coli expresses a complex response to DNA damage stress or replication blockage that includes increased expression of DNA repair and mutagenesis functions, as well as interruption of normal cell division (1). The fundamental mechanism controlling expression of these genes is the cellular LexA repressor, which, after treatments that damage DNA or arrest replication, is proteolytically cleaved into two nonrepressing fragments. In vivo, cleavage of LexA as well as lambda phage repressors requires an activated form of the RecA protein. In recA− mutant cells, there is no proteolysis of LexA protein or phage repressors and no expression of the SOS response. Recently, it has been shown that RecA protein not only regulates the levels of expression of two genes required for chemical mutagenesis, umuC and umuD, but that activated RecA protein proteolytically processes UmuD protein to an active form (2–4). Thus, UmuD protein is another substrate for activated RecA. Additionally, genetic evidence suggests RecA or activated RecA protein also functions directly in mutagenesis (4).

Although the precise biochemical nature of the activated RecA protein has not been determined, RecA protein can be readily converted to a form in vitro that can direct cleavage of LexA repressor, lambdoid phage repressors, and UmuD protein (3, 5, 6). This "activation" results from stoichiometric binding of RecA protein to a single-stranded polynucleotide in the presence of certain nucleoside triphosphates (ATP, dATP) or analogs such as adenosine 5'-[γ-thio]triphosphate ATP[yS] (7). The binding of RecA protein to polynucleotide produces a nucleoprotein or ternary complex that is capable of promoting proteolysis of the target proteins in vitro. Recently, it has been demonstrated that the LexA and phage λ repressors, as well as UmuD protein, are capable of self-cleavage at alkaline pH (3, 8), a result indicating that the activated RecA protein facilitates the autoproteolysis of LexA repressor (and other substrates) by a mechanism that involves placement of catalytic lysine (Lys-156) and serine (Ser-119) residues close to the scissile bond (4, 9).

We have recently shown that RecA ternary complexes bind tightly but noncovalently to RecA protein affinity columns (10). The RecA-agarose serves as a support for RecA nucleoprotein complexes and provides a convenient means for preparing activated RecA protein columns for investigating the binding and proteolytic cleavage of LexA repressor as well as other SOS-regulated proteins. In this report we show that LexA repressor did not bind to RecA protein-agarose columns but bound tightly to immobilized activated RecA complexes. After binding, LexA repressor underwent proteolytic cleavage on the affinity column, and the two repressor fragments were released from the bound complex. The mutationally altered LexA repressor, LexA-SA119, which is not cleaved in vivo or in vitro (9) bound tightly to the activated RecA column but did not undergo cleavage, showing that binding and cleavage can be mutationally separated and readily distinguished by using the affinity column. Moreover, when nucleoprotein complexes were prepared with the RecA430 protein, LexA repressor binding was dramatically reduced, and no cleavage products were formed. In vivo the recA430 mutation blocks efficient expression of the SOS response (11). These activated RecA protein affinity columns can also be used to investigate the interaction of RecA protein with other components of the SOS response.

EXPERIMENTAL PROCEDURES

Proteins and Reagents. RecA protein was prepared from strain KM1842 as described (12). LexA and LexA-SA119 (9) repressors were the gift of J. Little (University of Arizona, Tucson). λ repressor from a wild-type strain and a hypercleavable mutant were provided by F. Gimble and R. Sauer (Massachusetts Institute of Technology). The RecA430 protein was prepared from strain DM1187Δ21 containing plasmid Yrp12-recA430 (13) as described for RecA protein. Oligodeoxythymidylic acid, (dT)$_{25-30}$, was purchased from Pharma-
RESULTS

Binding and Proteolytic Cleavage of LexA Repressor on Activated RecA Protein Affinity Columns. RecA protein affinity columns were originally prepared to investigate the binding of cellular proteins to RecA protein (10). During characterization of these columns, we examined the interaction of purified LexA protein with these affinity columns. Fig. 1A shows that LexA protein did not bind efficiently to the RecA protein column, and most of the repressor was eluted in the buffer wash fractions. This column also contained noncovalently bound RecA protein, which was removed from the column by washing with urea (8 M). These results demonstrated that LexA protein did not bind to the

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ACM; ATP[γS] was obtained from Boehringer Mannheim. Activated agarose, Affi-Gel 10, was obtained from Bio-Rad.

Bacterial Strains and Plasmids. E. coli strain CSR603 containing plasmids pGW2123 or pGW2122 were used for maxicell labeling experiments. Strain Gw6756, a lacF mutant, was used for preparing labeled UmuC protein and carries two compatible plasmids, pAC-T17 and pGW2030. The pAC-T7 plasmids encode the rifampicin-resistant T7 RNA polymerase (14). Plasmid pGW2030 contains the umuC gene controlled by a T7 promoter. The strains for labeling UmuC and UmuD* proteins were the gift of J. Battista and G. Walker (Massachusetts Institute of Technology).

Preparation of RecA Protein and Activated RecA Protein Affinity Columns. RecA protein was covalently attached to activated agarose as described (10). Nucleoprotein complexes were prepared by incubating RecA protein (50 μg) with [dT]25-30 (13 μM) and ATP[γS] (1 mM) at 37°C for 30 min in C buffer [20 mM Tris-HCl, pH 7.5/20 mM KCl/10 mM MgCl2/0.5 mM dithiothreitol]. Reactions were diluted with 250 μl of R buffer [20 mM Tris-HCl, pH 7.5/0.2 mM EDTA/0.5 mM dithiothreitol/10 mM MgCl2/5% (vol/vol) glycerol], loaded onto RecA-agarose columns (1 ml) and incubated an additional 30 min at room temperature. RecA430 nucleoprotein complexes were prepared, as described above except that purified RecA430 protein (50 μg) was used for complex formation.

Affinity Chromatography of LexA, LexA-SA119, and A Repressors. LexA protein (50 μg) or LexA-SA119 repressor (30 μg) was loaded onto RecA affinity columns or columns containing prebound RecA nucleoprotein complexes (activated RecA affinity columns) and incubated at 25°C for the indicated times. Columns were washed with R buffer (5 vol) followed by stepwise increases in NaCl concentration in R buffer (see figures for details), and fractions (0.5 ml) were collected. Aliquots (40 μl) were removed, mixed with an aliquot (20 μl) of loading buffer [62.5 mM Tris-HCl, pH 6.8/2% NaDodSO4/10% (vol/vol) glycerol] and heated to 100°C for 3 min. Samples were fractionated by NaDodSO4/15% PAGE and stained with silver (15). The RecA-agarose columns were stable to repeated washing with 1 M NaCl.

Maxicell Labeling, Extract Preparation, and Affinity Chromatography. Maxicell labeling with [35S]methionine was done with strain CSR603 containing plasmid pGW2122 or pGW2123 encoding the UmuD* and UmuD*UmuC proteins, respectively (ref. 4; and J. Battista, personal communication). After labeling, crude soluble cell extracts were prepared by Brij lysis (12), dialyzed in R buffer, and applied to affinity columns. Labeling of UmuC protein alone was also done with a T7 RNA polymerase-directed system, essentially as developed by J. Battista (Massachusetts Institute of Technology). Strain Gw6756 was grown to an OD600 = 0.5 in M9 minimal medium, T7 polymerase was induced by isoprropyl β-d-thiogalactoside (1 mM) for 30 min, rifampicin (Sigma) was added to a final concentration of 400 μg/ml, and the culture was incubated for an additional 60 min. [35S]Methionine (1300 Ci/mmol, Amersham; 1 Ci = 37 GBq) was added to a final concentration of 1 μCi/ml for 25 min followed by addition of unlabeled methionine (500 μg/ml). Labeled cells were lysed on ice, and labeled UmuC protein was solubilized from the cell extract by using a procedure developed by J. Battista; the details of this procedure will be published elsewhere. The solubilized UmuC protein was loaded on 7-fold with R buffer and loaded onto affinity columns. Labeled proteins were eluted in a step gradient as described above. Aliquots of column fractions were analyzed by electrophoresis in NaDodSO4/polyacrylamide gels and visualized by sodium salicylate-enhanced fluorography (16).

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Fig. 1. Binding and cleavage of LexA protein on immobilized RecA-nucleoprotein complexes. LexA protein was loaded onto RecA protein-agarose (A) or activated RecA protein-agarose columns containing prebound nucleoprotein complexes (B–E) as described. Nucleoprotein complexes were formed using 26 μM RecA protein and 13 μM [dT]25-30 in 50 μl reaction volumes. Columns were incubated for the indicated time, and protein was eluted in a step gradient. Column fractions were analyzed as described. (A) LexA protein incubated 30 min on RecA-agarose affinity column; (B–E) LexA protein incubated on activated RecA-agarose columns for 5 min (B); 15 min (C); 30 min (D); and 60 min (E). In A the RecA-agarose column contained additional RecA protein that was not covalently attached to agarose.
immobilized RecA protein on these columns, a result that was consistent with our earlier observations (10).

The binding behavior of LexA protein on columns containing prebound RecA nucleoprotein complexes was markedly different. As shown in Fig. 1B, LexA protein bound to these columns, and a substantial fraction was eluted in the 1 M NaCl wash. After allowing 5 min for binding, >50% of the LexA repressor was found in these high-salt fractions. The remaining LexA protein was recovered in the pass-through fractions—either as full-length LexA protein or migrating at the position of cleavage fragments. (The amount of full-length LexA protein found in the buffer wash was variable from experiment to experiment, and our results suggested that the column was slightly overloaded with LexA protein.) When LexA repressor was incubated on the activated RecA protein affinity column for 15 min before starting the gradient wash, a substantial amount of protein eluted in the 1 M NaCl wash; however, there was a significant increase in the cleavage fragments that eluted with buffer only (Fig. 1C). Further incubations of 30 min (Fig. 1D) and 60 min (Fig. 1E) resulted in reduced amounts of LexA protein eluting in the 1 M NaCl wash and a concomitant increase in the amount of cleavage fragments that eluted in the buffer wash.

The LexA-SA119 Repressor Binds to Activated RecA Protein Affinity Columns but Is Not Cleaved. The results shown in Fig. 1 suggested that the binding of LexA repressor to activated RecA protein could be kinetically distinguished from cleavage using the affinity columns. To investigate this in more detail, we examined the binding of LexA-SA119 repressor to the RecA nucleoprotein affinity columns. The LexA-SA119 protein contains an alanine residue at position 119 instead of serine. This residue has been shown to be necessary for autodigestion of LexA at alkaline pH, as well as for RecA protein-dependent cleavage in vitro (9). As shown in Fig. 2, the LexA-SA119 protein bound tightly to the activated RecA protein affinity column and was eluted in 1 M NaCl. However, unlike wild-type LexA repressor, the mutationally altered LexA-SA119 protein did not undergo cleavage on the affinity column, even after prolonged incubation. These results demonstrate that Ser-119 is important for cleavage of LexA repressor but is not required for binding to activated RecA protein complexes.

RecA430 Nucleoprotein Complexes as Deficient in Binding LexA Protein. In addition to their utility in characterizing mutations in the lexA gene, the RecA nucleoprotein columns were used to examine the effects of mutations in the recA gene that alter the ability of the RecA protein to recognize LexA repressor. The recA430 mutation is completely deficient in prophase λ induction in vivo (17, 18), and the purified RecA430 protein does not promote cleavage of phage λ repressor (19). The recA430 mutation also blocks expression of SOS functions in E. coli after DNA damage (11), indicating that the activated RecA430 protein does not recognize LexA protein efficiently in vivo. Nucleoprotein complexes containing purified RecA430 protein, (dT)$_{25-30}$ and ATP[$\gamma$S] were prepared as described for the RecA protein and applied to RecA-agarose columns. Binding and elution properties of these RecA430 nucleoprotein complexes were indistinguishable from those of wild-type RecA ternary complexes. Chromatography of LexA repressor on these RecA430 nucleoprotein columns indicated that there was little interaction between these proteins (Fig. 3) with >90% of the LexA protein eluting in the buffer wash. No increase in binding was detected after 30 min of incubation on the column (Fig. 3A) or 60 min (Fig. 3B). Moreover, no cleavage products were detected in the flow-through fractions from any of the experiments. The RecA430 complexes were removed from the column by treatment with 8 M urea consistent with their tight binding to the RecA support.

One possible explanation for the failure of LexA protein to bind immobilized RecA430 nucleoprotein complexes was that the RecA430 enzyme used to prepare complexes was inactivated during purification. We tested this possibility by examining the DNA-dependent ATPase activity of this enzyme preparation as well as the ability to promote cleavage of LexA protein in solution. Normal levels of single-stranded DNA-dependent ATP hydrolysis activity were detected. Furthermore, in contrast to the results obtained with the activated RecA430 affinity columns, RecA430–oligonucleotide ternary complexes promote efficient cleavage of LexA protein in solution (data not shown). We conclude that the RecA430 protein used to prepare the affinity column was active and, significantly, the biochemical properties of immobilized RecA430 nucleoprotein complexes accurately reflect the behavior of the recA430 mutation in vivo (see Discussion).

Binding of Other SOS Proteins to RecA and Activated RecA Affinity Columns. Activated RecA protein interacts with the phage λ repressor in vivo and in vitro and promotes its protolytic cleavage at a rate that is ~40- to 50-fold slower than that of LexA protein (20). As seen with LexA protein, purified λ repressor did not bind to the RecA protein-agarose columns and ~95% of the protein was recovered in the flow-through fractions (Table 1). Unlike LexA protein, how-

![Fig. 2](image1.png)

**Fig. 2.** The LexA-SA119 repressor binds to activated RecA protein agarose but is not cleaved. The LexA-SA119 repressor (30 μg) was loaded onto an activated RecA-agarose column as in Fig. 1 and incubated for 30 min. Protein was eluted by a step gradient as shown. Fractions were collected and analyzed as described.

![Fig. 3](image2.png)

**Fig. 3.** LexA protein binds poorly to immobilized nucleoprotein complexes containing RecA430 protein. Nucleoprotein complexes containing purified RecA430 protein, (dT)$_{25-30}$ and ATP[$\gamma$S] were prepared as described and were prebound to a RecA-agarose column. LexA protein (50 μg) was loaded onto the affinity column and incubated for 30 min (A) or 60 min (B) before washing. Columns were washed and fractions were analyzed as described.
ever, λ repressor bound weakly to the activated RecA column and ≈50% of the λ repressor eluted from the column at NaCl concentrations of 50 mM or greater. This elution profile was reproducible, and similar elution behavior was seen with the wild-type λ repressor, as well as with a hypercleavable mutant (21). Although we detected a relatively weak binding interaction with λ repressor on the activated RecA column, no cleavage products were seen, even after incubating λ repressor on the column for up to 2 hr.

Recent evidence suggests that RecA protein or activated RecA protein is required directly for induced mutagenesis in addition to regulating expression of the SOS response. Two genes, umuC and umuD, encode proteins that are necessary for chemical mutagenesis, and the UmuD protein is proteolytically processed by activated RecA protein to form an UmuD* active in mutagenesis in vivo (2–4). Using radiolabeled UmuC and UmuD* proteins prepared in maxicells or an in vivo T7 expression system (see Materials and Methods) we examined binding of these proteins to RecA and activated RecA protein affinity columns. The results are presented in Table 1. UmuC protein, bound tightly to both affinity columns, although twice as much protein bound to the activated RecA column as to the RecA-agarose column alone (62% versus 31%). Complete removal of UmuC protein required washing the columns with 8 M urea. Under comparable conditions, no binding of UmuD* to either column was detected. However, in the presence of UmuC protein, ≈25% of the UmuD* loaded onto the columns was eluted in 8 M urea, suggesting that UmuD* interacts with the columns by first binding UmuC protein.

**DISCUSSION**

During the characterization of RecA protein-binding interactions using affinity chromatography, we observed that RecA protein–oligonucleotide complexes formed in the presence of the nonhydrolyzable analog, ATPγS, were efficiently retained on the affinity columns and could be completely removed by treatments with chaotropic agents, such as guanidine hydrochloride or urea. This result suggested that these RecA nucleoprotein complexes were bound through a combination of strong hydrophobic and ionic interactions between the covalently coupled RecA protein and RecA protein at the ends of the protein–DNA filaments (10). RecA nucleoprotein complexes have been shown to promote protolytic cleavage of both LexA and λ repressors and to participate in the processing of UmuD protein to a form needed for induced mutagenesis. By immobilizing these complexes on a RecA protein-agarose column, we have been able to investigate binding of activated RecA to LexA repressor and other SOS-regulated proteins.

The results presented in Figs. 1 and 2 show that RecA protein affinity chromatography provides a means for examining separately the binding and cleavage of LexA repressor by activated RecA complexes. Our evidence indicates that LexA protein does not recognize RecA protein alone but binds tightly to ternary complexes of RecA protein and DNA. Furthermore, the amount of bound LexA protein decreases with incubation time in parallel with the appearances of cleavage products. We conclude from these experiments that (i) LexA protein discriminated between free RecA protein and activated RecA protein with respect to binding; (ii) binding of RecA protein to activated RecA was very tight and was stable to treatment with 200 mM NaCl (but is dissociated with 1 M NaCl); (iii) LexA protein that was bound to RecA nucleoprotein complexes underwent cleavage to two fragments; and (iv) these product fragments did not remain bound to the complexes but were released. This last conclusion is consistent with the observation that the cleavage fragments do not inhibit RecA-dependent cleavage of LexA protein in solution (9). Furthermore, the tight binding of LexA to the activated RecA complexes is consistent with a model in which binding of LexA protein to activated RecA protein induces or stabilizes a conformation of the repressor that more readily undergoes autodigestion.

The LexA-SA119 repressor, which lacks a critical serine residue (Ser-119) needed for self-cleavage, was, nevertheless, capable of binding tightly to activated RecA protein complexes. This result demonstrated that this serine residue did not participate in binding to activated RecA but was important for subsequent bond cleavage. The affinity columns should be useful for identifying additional mutations in the lexA gene that alter the ability of LexA protein to recognize and bind activated RecA protein.

By preparing activated affinity columns using RecA430 nucleoprotein complexes, we observed that this mutation greatly reduced binding of LexA repressor. Although this behavior is qualitatively in agreement with the in vivo results that the recA430 mutation severely blocks expression of SOS functions, it is nevertheless contrary to results obtained in solution where RecA430–polynucleotide complexes efficiently promote LexA repressor cleavage. One possible explanation for this difference is that RecA430–polynucleotide complexes can aggregate in solution, whereas such associations would be greatly disfavored when the complexes are immobilized on the affinity column. This aggregation may favor binding of LexA repressor (as well as λ repressor) and promote higher levels of cleavage. Alternatively, the structure of the RecA430 nucleoprotein complex may be different when bound to the column compared with its structure in solution. Nonetheless, our results argue that the region surrounding residue 204 of RecA protein plays an important role in the recognition and binding of LexA repressor. RecA protein affinity chromatography should be useful for identifying other residues in RecA protein that participate in LexA repressor recognition.

Phage λ repressor also showed preferential binding to activated RecA columns compared with columns that contain RecA protein alone. However, unlike LexA protein, λ repressor was eluted from the column at relatively low-salt concentration (50 mM). Moreover, we have been unable to detect any cleavage of λ repressor on the affinity columns. We have tried to maximize binding by extending the incubation period on the column before washing, by increasing the temperature of incubation and washing to 37°C, as well as by using repressor that had been purified from a hyperinducible mutant. Although these modifications improved binding, we were unable to observe a tight association of λ repressor with activated RecA columns. In vitro cleavage of λ repressor

### Table 1. Binding of λ repressor, UmuC, and UmuD* to RecA and activated RecA affinity columns

<table>
<thead>
<tr>
<th>RecA-agarose, % (activated RecA* -agarose, %)</th>
<th>R buffer +</th>
<th>R buffer +</th>
<th>R buffer +</th>
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<tbody>
<tr>
<td>NaCl</td>
<td>50 mM</td>
<td>+ 1 M</td>
<td>NaCl</td>
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| λ repressor | 95 (50) | 5 (45-50) | 0 (<5) | 0 (0) |
| UmuC | 69 (38) | 0 (0) | 0 (18) | 31 (44) |
| UmuD* | 100 (100) | 0 (0) | 0 (0) | 0 (0) |
| UmuD* + UmuC | ND (75) | ND (0) | ND (0) | ND (25) |

Nucleoprotein complexes containing 22 μM RecA protein and 128 μM (DT)5-30 in 100-μl reaction volumes were formed as described and loaded onto RecA-agarose columns (1 ml). Purified λ repressor (50 μg) or aliquots of cell extracts containing 23S-labeled UmuC and/or UmuD* proteins were loaded onto the columns and after 1 hr the protein was eluted by the step gradients shown. Fractions were collected and analyzed as described. ND, not done.

*Column incubation and wash steps performed at 37°C.
by RecA–nucleoprotein complexes is extremely slow, ≈ 1/50th the rate of LexA digestion. Moreover, it appears that monomers of λ repressor are the form that is cleaved by activated RecA and that the dimer dissociation constant is ≈ 2 × 10⁻⁸ M (20). These results suggest that under conditions of our affinity chromatography only ≈ 1% of λ repressor was present as monomers and would have been available to bind the RecA nucleoprotein affinity column.

Partially purified UmuC protein bound to both RecA protein columns as well as activated RecA protein columns (Table 1), although we detected a greater fraction of bound UmuC protein on the activated RecA protein affinity support. This result could mean that UmuC preferentially binds activated RecA protein or that it binds to a (unlabeled) factor in crude extracts that recognizes activated RecA. Although these results indicate a strong interaction between UmuC and RecA protein (as well as activated RecA protein), it will be necessary to examine these interactions in greater detail by using purified UmuC protein. Although the functional significance of these results remains to be shown, it is interesting to note that binding of UmuD* to the RecA supports required the presence of UmuC protein. It has been postulated that a protein complex of DNA polymerase, RecA, UmuC, and UmuD* is important at the site of a lesion in DNA to allow mutagenic bypass on a damaged template (22). Thus, activated RecA protein affinity chromatography may be useful in determining the structure and properties of protein assemblies needed for chemical mutagenesis in E. coli.

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