The protein encoded by the *Escherichia coli* recA gene has two activities that involve a polynucleotide and a nucleoside triphosphate such as ATP: it promotes the pairing of single-stranded DNA to its homologous sequence in a DNA duplex, and it catalyzes a specific proteolytic cleavage of repressors, a reaction that requires both a nucleoside triphosphate and a polynucleotide (3-5). recA protein also has a DNA-dependent nucleoside triphosphatase activity (6). These activities reflect various roles of recA function in certain cellular processes (sometimes called SOS functions) that promote the repair of damaged DNA (7, 8). The ability of recA protein to destroy repressors by proteolytic cleavage underlies its regulatory role in directing the expression of genes that encode DNA repair-related functions (3, 4, 9-11). A primary target of this proteolytic activity is the *E. coli* lexA gene product (9), which probably acts as a repressor of both the recA gene itself (9, 10, 12) and other genes involved in DNA repair (11). recA protein also directs the cleavage of the immunity repressors of temperate bacteriophages such as λ and P22 (4, 13), the critical event in recA-dependent prophage induction in response to DNA damage. The ability of recA protein to promote the pairing of DNA strands reflects its direct role in homologous recombination (14, 15), including recombinational repair of damaged DNA (16).

We present evidence here that recA protein is activated to cleave repressors by binding to single-stranded DNA in the presence of a nucleoside triphosphate. The ATP analogue ATP$^3S$ (17) substitutes for ATP in two reactions of recA protein: repressor cleavage (4, 5, 9), and the partial unwinding of duplex DNA in the presence of single-stranded DNA (18), the latter a reaction that presumably represents the initiation of DNA strand exchange. Despite being active in these reactions, ATP$^3S$ is a potent inhibitor of both the complete DNA strand exchange reaction (2, 18) and the recA DNA-dependence ATP$\text{ase}$ activity (4). We show here that ATP$^3S$ binds in a tight complex with a recA protein in the presence of single-stranded DNA, and is hydrolyzed only very slowly by the recA triphosphatase activity. These results support our presumption (4) that a ternary complex of recA protein, DNA, and nucleoside triphosphate is the active species in proteolytic cleavage, and probably also in the reaction that initiates DNA strand exchange. We also determine that further purification of recA protein does not identify any other macromolecular components required for repressor cleavage, and we show that small oligonucleotides efficiently support recA repressor cleavage.

## EXPERIMENTAL PROCEDURES

1. Materials

- DNA was prepared by phenol extraction ofophage by heating at 100°C for 5 min and quick chilling. It was assumed that a solution of 50 mg DNA has an absorbance at 260 nm of 1.3. Oligonucleotides were obtained from P-L Biochemicals.

- ATP$^3S$ was obtained from Boehringer-Mannheim. ATP$^3S$ was a generous gift of L. Roberts and was purified by the method of Sandy et al. (19).

The abbreviations used are: ATP&$^3S$, ATP$^3S$; ATP, adenosine-5'-0-(3-thiotriphosphate) (3-5). recA protein is a purified fraction produced by the Phage Lambda lysogenic strain of *Escherichia coli* (18).

1. Lambda repressor was purified from *E. coli* strain 44277(20), which contains repressor as tetramer per cent of the total cell protein; purification was followed by polyacrylamide gel analysis. Cells were grown in 10 liter batch to new Bacterium formation fermentor at 37°C in medium containing 0.5% NaCl, 10 gm yeast extract and 16 gm tripton per liter. When the OD$_600$ of the culture reached 0.75, the cells were harvested at a Shilke continuous flow centrifuge, frozen, and stored at -20°C until use. The purification method was modified from Lander and Anderson (21). All steps were carried out at 4°C.

- Cells were thawed and suspended in 1.6 ml per gm cells of lysate buffer (0.1 M Tris HCl, pH 7.5; 0.5 M NaCl, 1 M DTA, 0.5 M CaCl$_2$, 0.1 M EDTA, 0.1 M GSH, 0.1 M glycerol and 3 MM mercaptoethanol). After cell lysis by sonication and addition of 5 volumes buffer (16 gm Tris HCl, pH 7.5, 1.0 EDTA, 2.0 M GSH, 0.18 M glycerol) containing also 1.2 M NaCl and 3.0 M mercaptoethanol, cell debris was removed by centrifugation at 45 minutes at 1000 g. Polymin P (200 mg) adjusted to pH 7.9 with HCl was added with stirring to the supernatant to 1.5 times the concentration required for clearing by centrifugation (22). The mixture was stirred 15 minutes and centrifuged 15 min at 8000 g, and the pellet was resuspended in 1/2 the original supernatant volume of 8 M buffer containing 0.2 M NaCl and 3 M 2-hydroxyethanoic acid.

2. The abbreviations used are: ATP$^3S$, adenosine-5'-O-(3-thiotriphosphate); NTP, nucleotide triphosphate.
The concentration of recA protein was calculated from the measured extinction coefficient, \( \epsilon_{280} = 5.9 \) (Table 1). The reductant (thiophosphate from [35S]ATP-S).
Like repressor cleavage and ATPase activity, ATPyS hydrolysis activity co-sediments with recA protein on a sucrose gradient (data not shown). Table II shows that at 2.3 μM ATPyS, the rate of hydrolysis is about 1/5000 of that of ATP at 5 mM. Since 2.3 μM is near or above the $K_m$ for ATPyS hydrolysis, we assume that the hydrolysis rate is negligible at any ATPyS concentration. The turnover number for ATPyS hydrolysis is one per several hours, versus one per several seconds for ATP. This result strongly suggests that a complex of recA protein with ATPyS cleaves repressor, and that hydrolysis of the nucleotide is not required for cleavage activity.

If hydrolysis of ATPyS were required for repressor cleavage,

![Graph](image)

**Fig. 1.** Repressor cleavage activity co-sediments with highly purified recA protein. Reaction mixtures of 60 μl contained 12 mM Tris-HCl, pH 7.5, 1 mM potassium phosphate, 0.25 mM EDTA, 0.4 mM CaCl$_2$, 2.4 mM dithiothreitol, 0.045 mM NaCl, 0.015 μM KCl, 5% (w/v) sucrose, 4% (v/v) glycerol, 1 mM ATPyS, 2 mM MgCl$_2$, 10 μg/ml (dA)$_8$, 4 μg of repressor, and recA protein as indicated. Fractions of equal size were taken across the peak of a sucrose gradient of recA protein. The purification of λ repressor included chromatography on hemoglobin agarose. After incubation for 105 min at 37 °C, reaction mixtures were analyzed by polyacrylamide gel electrophoresis. □, micrograms of recA protein per reaction; ○, micrograms of λ repressor cleaved; △, nanograms of recA cleaved/min/μg of recA protein.

![Graph](image)

**Fig. 2.** The rate of repressor cleavage is independent of the extent of ATPyS hydrolysis. Reaction mixtures containing 11 mM Tris-HCl, pH 7.5, 0.4 mM EDTA, 1.6 mM dithiothreitol, 6% (w/v) sucrose, 1 mM Tris-HCl, pH 7.9, 2% (v/v) glycerol, 0.2 mM CaCl$_2$, 3.7 μM [$^3$P]ATPyS, 2.6 mM MgCl$_2$, 1 mM potassium phosphate, pH 7.0, 0.02 μM NaCl, 0.02 μM KCl, 0.015 M MgCl$_2$, 0.015 M KCl, and 1.5 mg/ml of denatured λ DNA, and 113 μg/ml of recA protein were incubated at 37 °C for 5, 10, 30, or 45 min. One-tenth of a volume of 10 mM Tris, pH 7.9, 20% (v/v) glycerol, 1 mM EDTA, 10 mM dithiothreitol, 2 mM CaCl$_2$, 0.2 mM KCl, and 1.5 mg/ml of λ repressor was added, and incubation was continued at 37 °C. At 5-min intervals, aliquots were removed and chilled, and ATPyS hydrolysis and repressor cleavage were assayed, from 25- and 50-μl portions, respectively. A, time course of repressor cleavage after different times of preincubation. The rate was determined by the slope of the line in the interval 5-15 min (except at 45 min when the interval 5-10 min was used). Because the cleavage reaction is inhibited by high salt concentration upon addition of repressor, B, extent of ATPyS hydrolysis and the rate of repressor cleavage. The ATPyS hydrolysis represents incubation both before and after addition of repressor, but repressor did not affect the rate of ATPyS hydrolysis. The rate of repressor cleavage is plotted against the time at which repressor was added.

![Graph](image)

**Table II**

| Reactions | Rate nmol/min/mg recA protein |
|-----------|-----------------------------|
| ATP       | 350                         |
| ATPyS     | 0.967                       |

It is possible that some species resulting from this hydrolysis would accumulate during incubation, causing the rate of repressor cleavage to increase continuously. In the experiment of Fig. 2, we examined ATPyS hydrolysis and repressor cleavage in the same reaction, by incubating recA protein and ATPyS together in conditions of the cleavage reaction and removing samples at intervals to measure both their ability to cleave repressor and the extent of ATPyS hydrolysis. Fig. 2 shows that the rate of repressor cleavage is independent of the time of preincubation of recA protein with ATPyS and with the extent of ATPyS hydrolysis. This experiment also shows that stoichiometric hydrolysis of ATPyS is not required for repressor cleavage: in the 15 min following addition of repressor after the 5-min preincubation, 8 × 10$^{-11}$ mol of repressor were cleaved while 0.6 × 10$^{-11}$ mol of ATPyS were hydrolyzed per 50-μl sample, more than 10 cleavage events...
per molecule of ATPγS hydrolyzed. We conclude that if any species dependent upon ATPγS hydrolysis is required for repressor cleavage, it exists in very small concentration and does not accumulate during the reaction. Since ATPγS promotes repressor cleavage more efficiently than do nucleoside triphosphates that are hydrolyzed much faster (34), it seems most likely that a complex of recA protein with the triphosphate is the active species in repressor cleavage, and that hydrolysis of the triphosphate is not required.

**ATPγS Binds Tightly to recA Protein in the Presence of DNA**—The finding that ATPγS at micromolar concentrations efficiently stimulates recA protein to cleave repressors, even though it is hydrolyzed extremely slowly, suggests that ATPγS forms a relatively stable complex with recA protein. The experiment of Table III shows that radioactive ATPγS incubated with recA protein in the presence of DNA is retained by a nitrocellulose filter. Neither ATPγS itself nor ATPγS incubated with recA protein in the absence of DNA is bound in these conditions, although recA protein alone is retained by nitrocellulose (data not shown). Thus, incorporation of ATPγS into this complex requires both recA protein and polynucleotide. The DNA concentration dependence of ATPγS binding is similar to that of ATPase activity, in that bound ATPγS reaches a plateau at higher DNA concentrations (data not shown), and it is unlike the DNA concentration response of repressor cleavage, which is inhibited by higher than optimal DNA concentrations (see Ref. 4 and below). The binding of ATPγS is apparently irreversible, since 1) the half-life of the complex is in the order of hours, similar to the turnover number for ATPγS hydrolysis; and 2) bound radioactivity of the recA protein is not exchanged for nonradioactive ATPγS added after the complex is formed. We presume that the trappable species is a ternary complex of recA protein, polynucleotide, and ATPγS, although we have not measured bound DNA directly. Others have shown that DNA binds tightly to recA protein in the presence of ATPγS (2, 35).

There is evidence of three sorts that ATPγS is bound without hydrolysis or other covalent modification. 1) Direct examination by polyethyleneimine chromatography of material retained by the filter showed that more than 90% of the radioactivity was in ATPγS (data not shown). 2) We detect trapping of either ring-labeled [14C]ATPγS or thiophosphate-labeled [32P]ATPγS. 3) ATPγS hydrolysis is so slow that little could occur during the time in which complex formation is observed.

**The Stoichiometry of ATPγS Binding**—The binding of ATPγS to recA protein saturates at high concentrations of ATPγS, as shown below (Fig. 4). To determine the stoichiometry of ATPγS binding to recA protein in the presence of single-stranded DNA or (dA)16. The assay was performed as described in the legend to Table III, except that bovine serum albumin was omitted; the concentration of [32P]ATPγS was varied as indicated; the concentration of recA441 protein was 85 µg/ml; either 20 µg/ml of heat-denatured λ DNA or 10 µg/ml of (dA)16 was present; and incubation was for 5 min. Because a significant fraction of the ATPγS is bound at low concentration, the unbound concentration is plotted. The ordinate is the ratio of moles of ATPγS bound to moles of recA protein in the filtered sample. O, heat-denatured λ DNA; C, (dA)16.

**Table III**

| Additions                               | ATPγS bound |
|-----------------------------------------|-------------|
| None                                    | 0.5         |
| Heat-denatured λ DNA                    | 1.0         |
| recA protein                            | 2.5         |
| recA protein and heat-denatured λ DNA  | 115         |

**Fig. 3. Stoichiometry of ATPγS binding to recA protein.** The assay was performed as described in the legend to Table III, except with 5.3 µM [14C]ATPγS and recA441 protein as indicated. The amounts are calculated for 1 ml of binding reaction.

**Fig. 4. Binding of ATPγS to recA protein in the presence of single-stranded DNA or (dA)16.** The assay was performed as described in the legend to Table III, except that bovine serum albumin was omitted; the concentration of [32P]ATPγS was varied as indicated; the concentration of recA441 protein was 85 µg/ml; either 20 µg/ml of heat-denatured λ DNA or 10 µg/ml of (dA)16 was present; and incubation was for 5 min. Because a significant fraction of the ATPγS is bound at low concentration, the unbound concentration is plotted. The ordinate is the ratio of moles of ATPγS bound to moles of recA protein in the filtered sample. O, heat-denatured λ DNA; C, (dA)16.
ometry of bound ATPγS and recA protein, we measured ATPγS bound with increasing concentrations of recA protein, using a saturating concentration of ATPγS (Fig. 3). The slope of this curve gives 0.9 molecules of ATPγS bound per recA protein monomer. It is a reasonable presumption that each monomer has one binding site for ATPγS (and for other NTP's), although this measurement obviously is consistent with some different configuration such as two ATPγS molecules bound to half of the recA monomers.

Both DNA and oligonucleotides such as (dA)19 support the binding of ATPγS to recA protein (Fig. 4). The concentration of ATPγS bound at saturation in the presence of this oligonucleotide is identical with that with single-stranded DNA, so that the stoichiometry is an intrinsic property of the recA polypeptide. However, a higher concentration of ATPγS is required to saturate the binding supported by (dA)16, suggesting that the oligonucleotide interacts with recA protein less efficiently than does DNA.

**Stimulation of Proteolytic Activity of recA Protein by Oligonucleotides**—The proteolytic activity of recA protein toward repressors, like its ATPase and ATPγS-binding activities, requires polynucleotide. Both long denatured DNA molecules, such as phage λ DNA, and small oligonucleotides satisfy the requirement (4). In reactions using ATPγS, the maximum rate of repressor cleavage occurs at a concentration of DNA that is proportional to the recA protein concentration, suggesting that a stoichiometric complex between these components is the active complex in repressor cleavage (4). Higher than optimal concentrations of single-stranded λ DNA inhibit cleavage, as shown in the experiment of Fig. 5. We have found two differences between the activity of DNA and the activity of a series of deoxyadenosine oligonucleotides: 1) Small oligonucleotides do not inhibit repressor cleavage at high concentration. 2) The smaller oligonucleotides promote cleavage only at much higher concentrations than are required of DNA, suggesting that they do not interact stoichiometrically with recA protein in these conditions. Fig. 6 shows that (dA)9, (dA)16, and (dA)19-24 are similar to λ DNA in promoting repressor cleavage at low concentrations, and thus presumably also bind efficiently to recA protein. In contrast, a higher concentration of (dA)9 is required to saturate the cleavage rate, and (dA): shows only slight activity at much higher concentration. The oligonucleotide (dT)9 is also active at somewhat higher concentrations than single-stranded DNA.

Although (dA)19-24 inhibits cleavage at higher concentrations, (dA)16 and smaller oligonucleotides do not. This result shows that the inhibition of cleavage by high concentrations of DNA is separable from the stimulation, and is thus probably an interaction distinct from that which stimulates repressor cleavage. If recA protein is preincubated with a saturating concentration of (dA)19 in the presence of ATPγS, and excess single-stranded DNA is then added, repressor cleavage is inhibited (data not shown); thus, the site or sites responsible for inhibition are not irreversibly bound by excess oligonucleotide.

**DISCUSSION**

We have shown that recA protein binds ATPγS tightly in the presence of DNA, probably in a ternary complex of all three components. Presumably, an equivalent complex forms with natural triphosphates such as ATP before they are hydrolyzed. We have inferred that binding of recA protein into this initial complex, without hydrolysis of the NTP, invokes its proteolytic activity toward repressors. ATPγS stimulates recA protein to cleave repressors more efficiently than does ATP (4, 34), yet the rate of ATPγS hydrolysis by recA protein is several thousand-fold less than the rate of ATP hydrolysis. Furthermore, less than one ATPγS molecule is hydrolyzed for each repressor monomer cleaved, indicating that ATPγS hydrolysis is not directly coupled to repressor cleavage. ATPγS also stimulates recA protein to bind and unwind duplex DNA (18), a reaction that may represent the initiation of DNA strand exchange; we presume that ATPγS or NTPPγS hydrolysis also is not essential to this initial reaction.

Since ATPγS strongly inhibits both ATP hydrolysis at the completion of strand exchange catalyzed by recA protein, ATP hydrolysis probably is required for subsequent steps in strand exchange that involve the alignment of homologous...
sequences during strand pairing.

Besides their response to ATPγS, a second property common to the repressor cleavage reaction and the unwinding of duplex DNA by recA protein is that oligonucleotides satisfy the polynucleotide requirement for both (4, 18). The affinity of recA protein for longer oligonucleotides is apparently high, because the longer deoxyadenosine oligonucleotides and single-stranded DNA are active at similar low concentrations. The activity of oligonucleotides suggests that these reactions may not require the extended polymerization of recA protein that occurs on a long polynucleotide strand (36).

Excess single-stranded DNA inhibits both repressor cleavage and DNA strand exchange (4, 1), suggesting that the target for inhibition is recA protein, not λ repressor. One explanation of this inhibition follows from the observation that binding of recA protein to (limiting) single-stranded DNA in the presence of NTP activates it to melt duplex DNA: excess single-stranded DNA could inhibit both strand exchange and cleavage by occupying secondary sites that would otherwise bind duplex DNA. Cleavage could be inhibited in several ways: because a recA protein-DNA network impenetrable by repressor is formed, or the cleavage site is directly obscured, or a structural change is induced in the enzyme. Smaller oligonucleotides might not inhibit at high concentrations either because they bind too weakly to occupy the secondary sites, or because they cannot form an extended network of recA protein and DNA.

Because an excess of smaller oligonucleotides does not inhibit repressor cleavage, the rate of repressor cleavage is proportional to the concentration of recA protein in these conditions. Thus it is convenient to use oligonucleotides to assay the cleavage activity of recA protein, as we have done in the experiment of Fig. 1.

Activation of recA protein to cleave repressors in vivo occurs when cellular DNA is damaged or its replication is interrupted, for example by ultraviolet irradiation. We have suggested that one pathway of activation is the binding of recA protein to single-stranded DNA in gaps that result from these treatments (4). Any single-stranded polynucleotide appears to promote repressor cleavage in vitro, even though the different activities of (dT)$_n$ and the dA series suggest that there is some effect of base composition. We interpret this generalized activity of polynucleotide to represent the ability of recA protein to interact with any sequence of exposed single-stranded chromosomal DNA, simultaneously invoking repressor cleavage activity and engaging this DNA in strand exchange. The finding that small oligonucleotides also activate repressor cleavage is consistent with suggestions that degradation fragments of damaged DNA (37) also could provide a pathway of recA activation.

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