Interaction of the recA Protein of *Escherichia coli* with Adenosine 5'-*O*-(3-Thiotriphosphate)*

George M. Weinstock‡, Kevin McEntee§, and I. R. Lehman

From the Department of Biochemistry, Stanford University School of Medicine, Stanford, California 94305

Incubation of the recA protein of *Escherichia coli* with the ATP analog adenosine 5'-*O*-(3-thiotriphosphate) (ATP(γS)) in the presence of DNA produces an irreversible inhibition of ATPase activity, although in the presence of ATP, ATP(γS) shows an initial competitive inhibition. ATP(γS) is not appreciably hydrolyzed by recA protein and the inhibition of ATPase activity is due to the formation of stable complexes which contain equimolar amounts of ATP(γS) and recA protein. Formation of stable complexes requires DNA, which is also stably bound to recA protein in the presence of ATP(γS), at a ratio of 5 to 10 nucleotides/recA protein monomer. The DNA requirement is satisfied by either single- or double-stranded DNA, and in the latter case, the pH dependence is comparable to that observed for ATP hydrolysis. Binding of ATP(γS) is inhibited by other nucleoside di- and triphosphates with efficiencies corresponding to their inhibitory effects on the ATPase activity of recA protein.

The ATP analog of adenosine 5'-*O*-(3-thiotriphosphate), ATP(γS)[1] (1), has proved an invaluable aid in studies of the recA protein of *Escherichia coli*. This derivative, in which a sulfur atom replaces one of the oxygen atoms of the γ-phosphate group of ATP, is a potent inhibitor of the ATP-dependent strand assimilation (2, 3), SS DNA annealing (4), and DNA-dependent ATPase (2, 4–6) activities of the recA protein. ATP(γS) has also been useful in studies of the ATP- and polynucleotide-dependent protease activity of recA protein (5, 6) and in analyzing the interaction of recA protein with SS and DS DNA (2, 7–9).

Because of the importance of ATP(γS) in mechanistic studies of the recA protein, we sought to define in detail the interaction between ATP(γS) and the recA protein. A previous study (2) showed that the inhibition of ATPase activity by ATP(γS) was competitive and that ATP(γS) was not

efficiently hydrolyzed by the recA protein. Here, we extend these observations and show that although inhibition is competitive, it is also essentially irreversible due to the formation of recA protein-ATP(γS) complexes that neither hydrolyze the ATP(γS) nor dissociate during the course of most recA protein-dependent reactions. Furthermore, we find that the complexes contain 1 ATP(γS) molecule/recA protein monomer and require DNA for their formation.

EXPERIMENTAL PROCEDURES

Materials—[^{35}S]ATP(γS) was a generous gift from Dr. F. Eckstein (Max Planck Institut, Göttingen, Germany) and was also purchased from New England Nuclear. Unlabeled ATP(γS) and GTP(γS) were from Boehringer Mannheim; UTP(γS) was also generously donated by Dr. Eckstein. In various preparations, between 60 and 90% of the radioactivity of [^{35}S]ATP(γS) was in ATP(γS), as judged by polyethyleneimine cellulose chromatography (1) and Norit adsorption (see below). Unlabeled NTP(γS) preparations showed a similar variation in purity, depending on their age, with the major contaminant being the nucleoside diphosphate. NTP(γS) concentrations were determined using the extinction coefficient of the corresponding NTP. Other nucleoside tri- and diphosphates were obtained from P-L Biochemicals and Sigma; [^{3}H]ATP was from Amersham; Na$_3$PO$_3$S·12H$_2$O was from Ventron (Alfa); Norit from Baker; nitrocellulose filters (HAWP, 45 nm, 24 mm diameter) from Millipore Corp.; calf thymus DNA from Sigma; poly(dT) and (dT)$_{12}$ from P-L Biochemicals; other DNA's were prepared as described previously (4, 10).

recA protein was Fraction II (10), purified through the phosphocellulose step, and was greater than 90% pure. Its concentration was determined from the absorbance at 280 nm using an extinction coefficient (an A_{280} of 1 equals 51 μM recA protein) calculated from the amino acid composition (11). Other reagents were as described previously (10).

Assay for Hydrolysis of ATP—ATP hydrolysis was assayed by thin layer chromatography on polyethyleneimine cellulose plates as described previously (10). The standard reaction mixture contained 20 mM buffer, 10 mM MgCl$_2$, 1 mM dithiothreitol, and DNA, ATP, and recA protein as indicated.

Assay for Hydrolysis of ATP(γS). Formation of inorganic thiophosphate (PO$_3$S) by the hydrolysis of [^{35}S]ATP(γS) was monitored by Norit adsorption. An aliquot of the reaction mixture was mixed with an equal volume of Na$_3$PO$_3$S and 2% Sarkosyl, and then 5 volumes of 50 mM Na$_3$PO$_3$S containing 10% (w/v) Norit was added. After mixing, the Norit was removed by centrifugation and radioactive PO$_3$S in the supernatant fluid was measured. The standard reaction mixture was the same as for the ATPase assay.

Filter-binding Assay—Following incubation of the standard reaction mixture (described above), an aliquot was filtered with suction through a nitrocellulose filter previously soaked in 20 mM Tris-HCl (pH 8.0), 10 mM MgCl$_2$, 1 mM dithiothreitol, and 30 mM NaCl (B buffer). The filter was washed once with 10 volumes of either B buffer (low salt wash) or B buffer containing 1 M NaCl (high salt wash) followed by a second wash with 10 volumes of B buffer. When all liquid had passed through, the filter was dried and the bound radioactivity determined.

Filtration was routinely carried out at 2 ml/min; slower rates did not increase retention. At least 100 μg of recA protein could be retained by the filters and no difference in efficiency of retention was found between filtration of 20-μl or 90-μl aliquots of a 27 μM solution

[1] The abbreviations used are: ATP(γS), adenosine 5'-*O*-(3-thiotriphosphate); DS, double-stranded; SS, single-stranded; GTP(γS), guanosine 5'-*O*-(3-thiotriphosphate); UTP(γS), uridine 5'-*O*-(3-thiotriphosphate); SDS, sodium dodecyl sulfate; NTP, nucleoside triphosphate; NDP, nucleoside diphosphate.

of recA protein. Filters pretreated with 0.5 M KOH (9) showed a similar capacity. However, pretreatment of the protein with either 1% SDS or Sarkosyl for 2 min at 30 °C completely eliminated its capacity to bind to filters. Protein binding to filters did not require Mg^{2+} and was stable to repeated washing with B buffer.

RESULTS

Irreversible Inhibition of ATPase by ATP(γS)—ATP(γS) inhibits annealing of complementary single strands of DNA, single strand assimilation into homologous DS DNA, and the DNA-dependent ATPase activities associated with the recA protein. Inhibition of the SS DNA-dependent ATPase by ATP(γS) appeared competitive (Ref. 2 and Fig. 1) with a $K_i^{ATP(γS)}$ of approximately 0.6 μM as contrasted with a K_m for ATP of approximately 20 μM (12). ATP(γS) also inhibited the DS DNA-dependent ATPase and the UTPase activities of recA protein (data not shown).

ATP(γS) inhibited the extent as well as the initial rate of ATP hydrolysis, suggesting that in the presence of ATP(γS) the enzyme was irreversibly altered. To test this possibility, recA protein was preincubated with varying concentrations of ATP(γS) in the presence of SS DNA, then ATP was added, and the ATP(γS) concentration was adjusted so that it remained constant and ATPase activity was measured. As shown in Fig. 2, preincubation with increasing concentrations of ATP(γS) led to a corresponding increase in inhibition of the ATPase. The degree of inhibition was directly proportional to the amount of ATP(γS) present during preincubation. Thus, since the ATP(γS) concentration was constant during ATP hydrolysis, inhibition must have occurred during the preincubation. Approximately 70% of the ATPase activity could be inhibited under these conditions, although >99% of the ATPase activity was due to recA protein.[2] Maximum inhibition occurred when there were equimolar concentrations of ATP(γS) and recA protein. Thus, ATP(γS) causes an irreversible inhibition of the ATPase activity of the recA protein.

The irreversible inhibition required DNA. As shown in Fig. 3, preincubation of recA protein with excess ATP(γS) in the presence of varying amounts of SS DNA led to a progressive inhibition of ATPase activity, dependent on the ratio of DNA nucleotides to recA protein. Maximum inhibition occurred at a ratio of about 9 nucleotides/recA protein monomer. When recA protein was preincubated with ATP(γS) in the presence of DS DNA at pH 6.2, inhibition of both DS and SS DNA-dependent ATPase activities occurred, whereas at pH 8.0, there was no inhibition, consistent with the pH optimum for DS DNA-dependent ATP hydrolysis and DS DNA binding by the recA protein (8, 10). Preincubation in the absence of DNA gave no inhibition of DNA-independent ATPase activity at either pH 6.2 or pH 7.5.

These findings indicate that the irreversible inhibition of ATPase activity by ATP(γS) has requirements similar to those for ATP hydrolysis, *i.e.* a DNA cofactor and the appropriate pH in the presence of DS DNA. These observations suggest 3 possible mechanisms for the inhibition: (i) ATP(γS) is hydrolyzed, but the thiophosphate produced is tightly bound to the enzyme and dissociates very slowly; (ii) hydrolysis is initiated and a covalent recA protein-ATP(γS) or recA protein-PO₃S intermediate accumulates; or (iii) no bonds are broken, but a stable noncovalent recA protein-ATP(γS) complex is formed. The experiments described below favor the 3rd alternative.

Lack of Hydrolysis of ATP(γS) by recA Protein—Incuba-

---

[2] The residual ATPase activity may result from enzyme molecules which bind the ADP contaminating the ATP(γS) preparation and are thus protected from ATP(γS) inhibition.

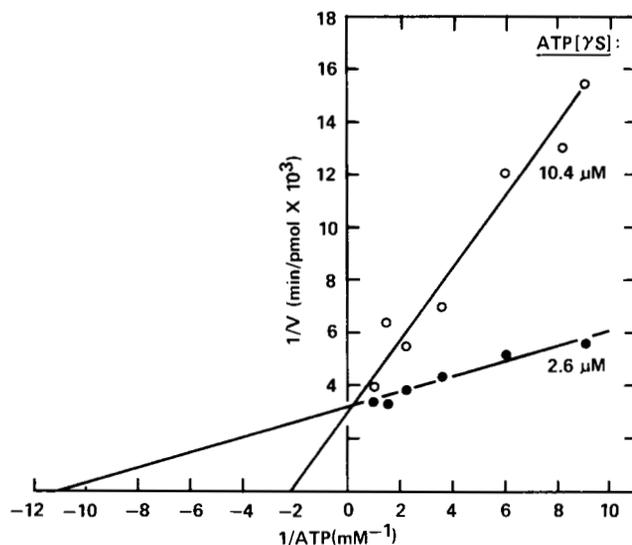FIG. 1. **Inhibition of ATPase of recA protein by ATP(γS).** The standard reaction (60 μl) contained 20 mM Tris-HCl (pH 8.1), 250 μM calf thymus SS DNA, 0.7 μM recA protein, either 2.6 or 10.4 μM ATP(γS), and the indicated concentrations of [³H]ATP. Initial velocities were determined from a time course performed at 30 °C.

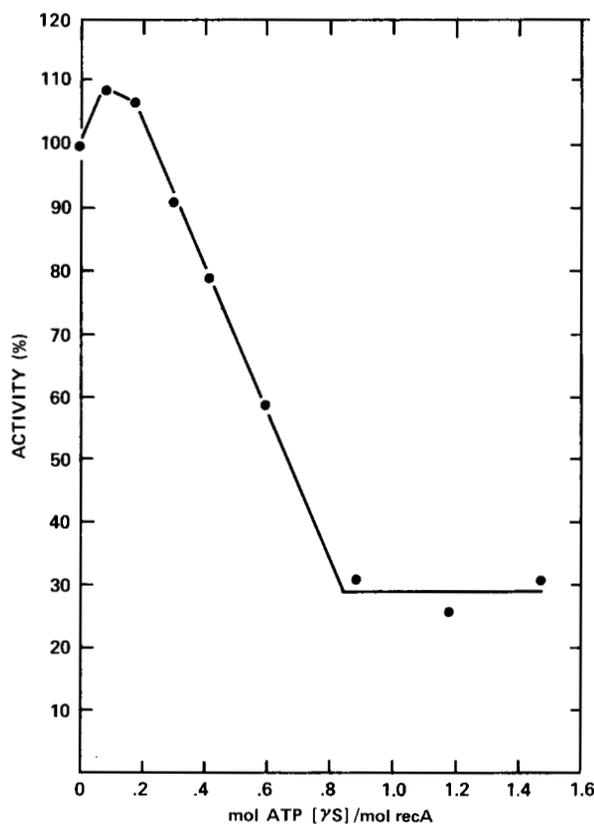FIG. 2. **Inhibition of ATPase activity of recA protein by preincubation with ATP(γS).** recA protein (17.6 μM) was incubated with varying concentrations of ATP(γS) in a 60-μl preincubation mixture containing 20 mM Tris-HCl (pH 8.0), 1 mM dithiothreitol, 30 mM NaCl, and 210 μM φX174 SS DNA. After 30 min at 30 °C, an aliquot (3 μl) was diluted into the assay mixture (57 μl) containing 20 mM Tris-HCl (pH 8.0), 10 mM MgCl, 1 mM dithiothreitol, 30 mM NaCl, 930 μM [³H]ATP, 84 μM φX174 SS DNA, and varying amounts of ATP(γS) to give a final concentration of 1.3 μM. Initial velocities were determined from a time course.

tion of [³⁵S]ATP(γS) with recA protein and either SS or DS DNA resulted in an extremely low rate of [³⁵S]PO₃S formation. At either recA protein or ATP(γS) excess, PO₃S was produced at a rate of approximately 1 mol of ATP(γS) hydrolyzed/mol of recA protein/1500 min, the limit of detection and several orders of magnitude below the rate of ATP hydrolysis (data not shown). Treatment of the reaction with either SDS or Sarkosyl did not produce an increase in the amount of PO₃S formed. Thus, it is unlikely that PO₃S, produced by hydrolysis of ATP(γS) and bound noncovalently to the enzyme, is responsible for inhibition of ATPase activity.

Detection of recA Protein-ATP(γS) Complexes—Following incubation of recA protein with [³⁵S]ATP(γS), the ³⁵S was converted to a form that was stably retained by nitrocellulose filters even after washing with 1 M NaCl (Table I). The
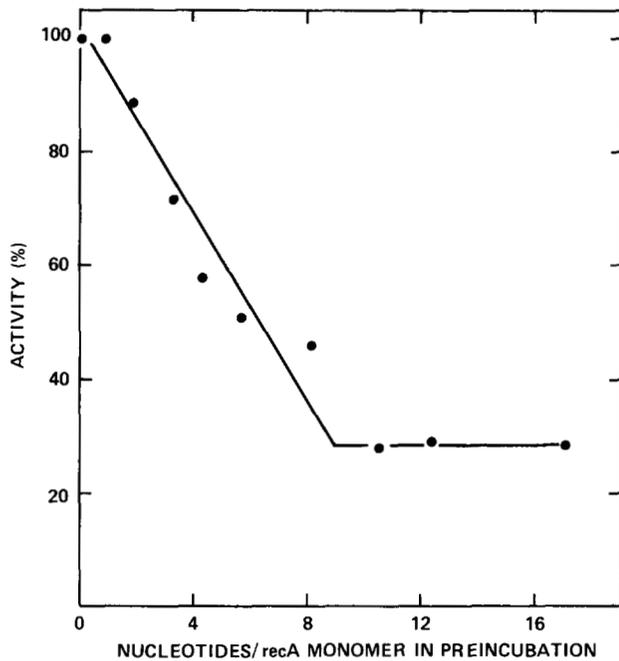

FIG. 3. **Dependence on DNA of ATP(γS) inhibition of ATPase activity.** The preincubation mixture was the same as in Fig. 2 except that the ATP(γS) concentration was 26 μM, and the concentration of φX174 SS DNA was varied. Aliquots were diluted 20-fold into an assay mixture as described in Fig. 2 except that ATP(γS) was omitted and the φX174 SS DNA concentration was 168 μM. Initial velocities were determined from a time course.

TABLE I

Filter-binding assay for recA protein-ATP(γS) complexes

The standard reaction (50 μl) contained 20 mM buffer (either Tris-HCl (pH 8.0) or, where indicated, sodium maleate (pH 6.2)), 4.3 μM [³⁵S]ATP(γS), either 101 μM φX174 SS DNA or 77 μM pZ6b DS DNA, and 2.6 μM recA protein. Incubation was for 25 min at 30 °C at which time 20-μl aliquots were filtered and washed as described under "Experimental Procedures."

	Mol ATP(γS) retained/mol recA protein	
	Low salt wash	High salt wash
Complete (SS DNA)	0.55	0.52
−recA protein	0.003	0.005
−DNA	0.010	0.008
pH 6.2	0.59	0.53
KOH-treated filter	0.54	0.50
Complete (DS DNA)	0.011	0.013
pH 6.2	0.27	0.26
KOH-treated filter	0.19	0.20

TABLE II

Dissociation of recA protein-ATP(γS) complexes at 60 °C

The standard reaction (250 μl) with 20 mM Tris-HCl (pH 8.0) contained 2.1 μM [³⁵S]ATP(γS), 121 μM φX174 SS DNA, and 5.3 μM recA protein. After incubation for 30 min at 30 °C, 30-μl aliquots were assayed by filter binding (low salt wash) or Norit adsorption as described under "Experimental Procedures" before and after a 2-min incubation at 60 °C.

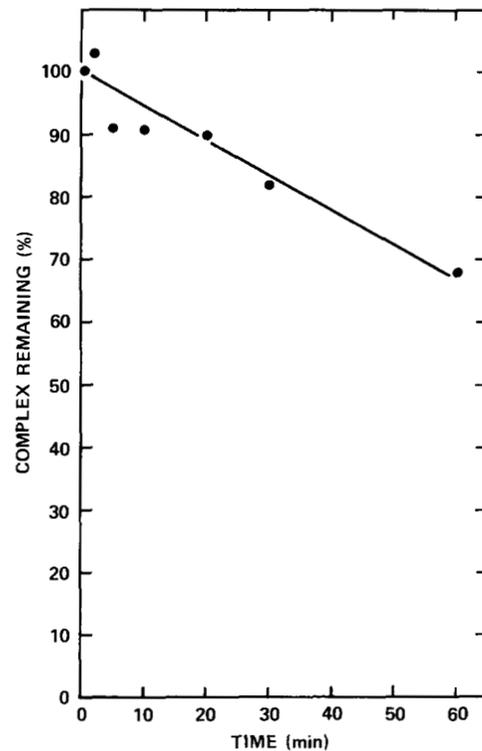|	Per cent of total radioactivity		
	Complete	−SS DNA	−recA protein
Initially bound to filter	53	2.0	0.4
Bound to filter after 2 min, 60 °C	0.9	1.5	0.8
Initially Norit nonadsorbable	9.5	9.4	5.6
Norit nonadsorbable after 2 min, 60 °C	11.5	12.1	6.3

FIG. 4. **Dissociation of recA protein-ATP(γS) complexes.** The standard reaction (100 μl) with 20 mM Tris-HCl (pH 7.5) contained 10.6 μM recA protein, 616 μM calf thymus SS DNA, and 5 μM [³⁵S] ATP(γS). After incubation at 37 °C for 15 min, 10 μl of unlabeled ATP(γS) was added to give a final concentration of 1 mM. Ten-μl aliquots were assayed by filter binding (high salt wash) at the indicated times after addition of unlabeled ATP(γS).

conversion required recA protein and either SS or DS DNA, although DS DNA was effective only at a pH at which the DS DNA-dependent ATPase was active. Retention on nitrocellulose filters was due to binding of recA protein to the filter since alkali-treated filters that have lost the capacity to bind DNA (9) were equally effective in this assay. Thus, the ³⁵S in [³⁵S]ATP(γS) exists in a complex with recA protein that can be retained on nitrocellulose filters in the presence of 1 M NaCl. Furthermore, the conditions for formation of this complex parallel those required for irreversible inhibition of ATPase activity.

Incubation of the complexes at 60 °C for 2 min prior to filtration resulted in loss of the bound ³⁵S (Table II), although recA protein was still efficiently retained by the filters under these conditions (data not shown). The treatment at 60 °C did not produce inorganic PO₃S as judged by Norit adsorption.

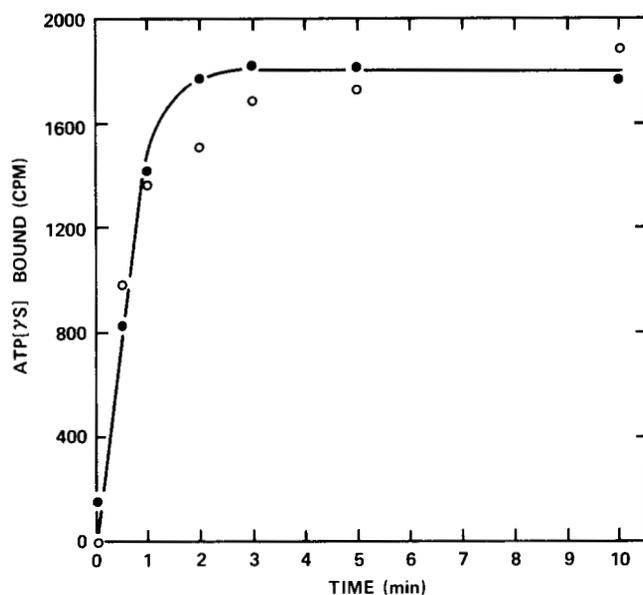FIG. 5. **Kinetics of formation of stable ATP(γS)-recA protein complexes at 37 °C.** The standard reaction (200 μl) with 20 mM Tris-HCl (pH 7.5) also contained 5 μM [³⁵S]ATP(γS), 308 μM calf thymus SS DNA, and 1.1 μM recA protein. Twenty-five μl aliquots were assayed by filter binding (high salt wash) after the indicated times of incubation at 37 °C. O——O, Reaction was preincubated for 12 min at 37 °C without ATP(γS); ●——●, no preincubation.
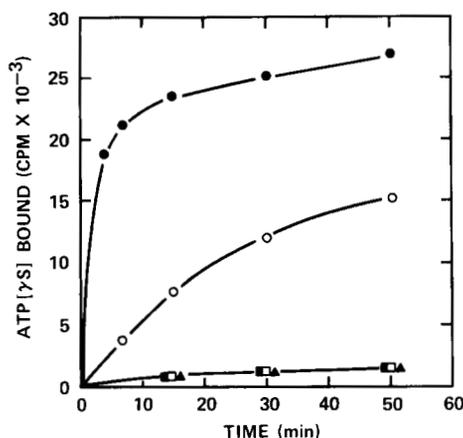

FIG. 6. **Kinetics of ATP(γS) binding to recA protein at 30 °C.** The standard reaction (300 μl) with either 30 mM Tris-HCl (pH 8.0) or sodium maleate (pH 6.2) contained 4.3 μM [³⁵S]ATP (γS), 2.6 μM recA protein, and either 50 μM φX174 SS DNA, 51 μM pZ6b DS DNA, or no DNA. Aliquots (30 μl) were assayed by filter binding (low salt wash). ●——●, SS DNA, pH 8.0; O——O, DS DNA, pH 6.2; ▲——▲, DS DNA, pH 8.0; ■——■, no DNA, pH 8.0; □——□, no DNA, pH 6.2.

Thus, we conclude that ATP(γS) is not hydrolyzed by recA protein; furthermore, there is no accumulation of a covalent recA protein-PO₃S intermediate. Rather, the complexes consist of ATP(γS) tightly bound to recA protein. The rather mild treatment required for complete dissociation of the [³⁵S] ATP(γS) suggests a noncovalent association.

Characteristics of the Reaction Leading to Tight Binding of ATP(γS) to recA Protein—Although sensitive to heat treatment, the recA protein-ATP(γS) complexes were otherwise extremely stable, being resistant to exhaustive washing with 1 M NaCl and exposure to 30 mM EDTA. As shown in Fig. 4, the complexes had a half-life of about 90 min at 37 °C. Their

remarkable stability accounts for the irreversibility of the inhibition of ATP hydrolysis by ATP(γS).

Although the turnover number for SS DNA-dependent ATP hydrolysis at 37 °C is about 10 ADP produced/min/ recA protein monomer, 1 to 2 min were required for complete binding of ATP(γS) to recA protein (Fig. 5). At 30 °C, binding of ATP(γS) in the presence of SS DNA was slightly slower, while in the presence of DS DNA (pH 6.2), the rate was much reduced (Fig. 6). In the presence of 20 μM recA protein and 30 μM ATP(γS), the DS DNA-dependent reaction showed kinetics similar to the reaction in the presence of SS DNA (data not shown). These results indicate that the high affinity of recA protein for ATP(γS) is not a consequence of a rapid rate of formation of recA protein-ATP(γS) complexes, but is rather a result of their very slow rate of dissociation and, in fact, the rate of formation of these complexes is slower than the rate of turnover of ATP during hydrolysis.

Incubation in the absence of DNA at pH 6.2 or 8.0 produced

TABLE III
Requirements for tight binding of ATP(γS) to recA protein

The standard reaction (50 μl) with 20 mM Tris-HCl (pH 8.0) contained 4.3 μM [³⁵S]ATP(γS), 2.6 μM recA protein, and either 101 μM φX174 SS DNA, 125 μM (dT)₁₂, or 108 μM (dT)₁₀₀₀. Incubation was for 20 min at 30 °C at which time 30 μl was filtered (low salt wash) as described under "Experimental Procedures." In the complete reaction, 0.6 mol of ATP(γS) was bound/mol of recA protein.

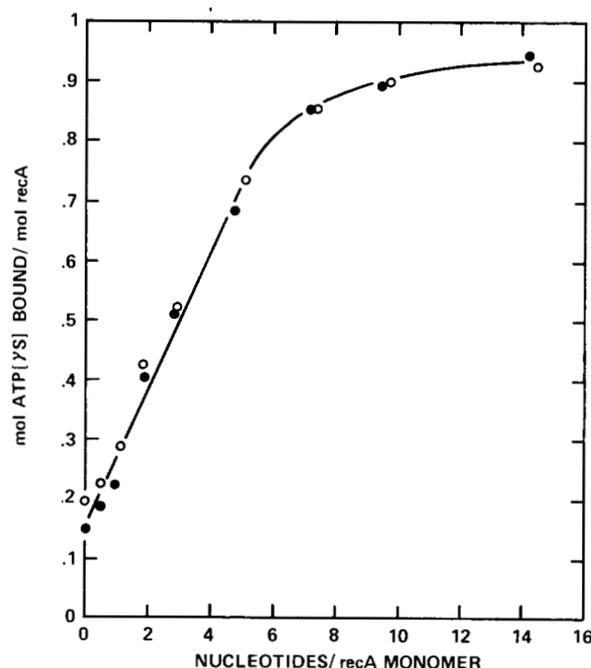|	Relative binding
	%
Complete	100
−Mg²⁺	0.4
+200 mM NaCl	3.9
+500 mM NaCl	1.9
+10 mM *N*-ethylmaleimide	67.3
−φX174 SS + (dT)₁₂	2.0
−φX174 SS + (dT)₁₀₀₀	85.3

FIG. 7. **Formation of recA protein-ATP(γS) complexes: titration of DNA.** The standard reaction (100 μl) with either 20 mM Tris-HCl (pH 8.0) for SS DNA (O——O) or 20 mM sodium maleate (pH 6.2) for DS DNA (●——●) contained 21.7 μM recA protein, 34 μM [³⁵S]ATP(γS), and the indicated amount of φX174 SS DNA. Incubation was for 30 min at 30 °C at which time 90 μl was filtered (low salt wash) as described under "Experimental Procedures".

no increase in ATP(γS) binding over a period of at least 120 min, consistent with the failure of ATP(γS) to inhibit irreversibly the DNA-independent ATPase activity of recA protein. Furthermore, no stable binding of ATP(γS) was observed in the presence of DS DNA at pH 8.0.

As shown in Table III, the formation of stable recA protein-ATP(γS) complexes required Mg^{2+}. As noted earlier, once formed they were resistant to EDTA. Formation of the stable complex was also sensitive to salt, although once formed, the final complexes were stable to 1 M NaCl. Unlike the ATPase reaction (4), formation of complexes was not inhibited by N-ethylmaleimide. The polynucleotide requirement for complex formation showed the same specificity as for ATP hydrolysis (10); in particular, $(dT)_{12}$ did not promote complex formation but $(dT)_{1000}$ did (Table III), indicating a similar polynucleotide size requirement as for ATP hydrolysis. As shown in Fig. 7, tight binding of ATP(γS) depended on the ratio of recA protein to DNA, and saturation occurred at about 6 nucleotides/recA protein monomer with either SS or DS DNA, a value similar to that observed for the irreversible inhibition of ATPase activity. At pH 8.0, DS DNA (29 nucleotides/recA protein monomer) in the presence of subsaturating amounts of SS DNA (either 2 or 5 nucleotides/recA protein monomer) caused no additional binding of ATP(γS) over the SS DNA level, although under these conditions, binding of recA protein to DS DNA is stimulated by SS DNA (2, 9).

Stoichiometry of ATP(γS) Binding to recA Protein—Titration of recA protein in the presence of excess ATP(γS) (Fig. 8) indicated that about 1.3 mol of ATP(γS) were bound/mol of recA protein monomer at 37 °C. Eighty per cent of the labeled ATP(γS) was bound when recA protein was in excess over ATP(γS). When the titration was performed at 30 °C, 1.0 mol of ATP(γS) was bound/mol of recA protein monomer (data not shown). Titration of ATP(γS) in the presence of a constant amount of recA protein (Fig. 9) gave a ratio of 1.6 mol of ATP(γS) bound/mol of recA protein monomer, although in this case, only 60% of the labeled ATP(γS) was bound. In similar titrations, at 30 °C, ratios of 1.3 and 0.7 were observed with 2.6 and 22 μM recA protein, respectively. Finally, when recA protein was preincubated with varying amounts of unlabeled ATP(γS) in the presence of DNA and
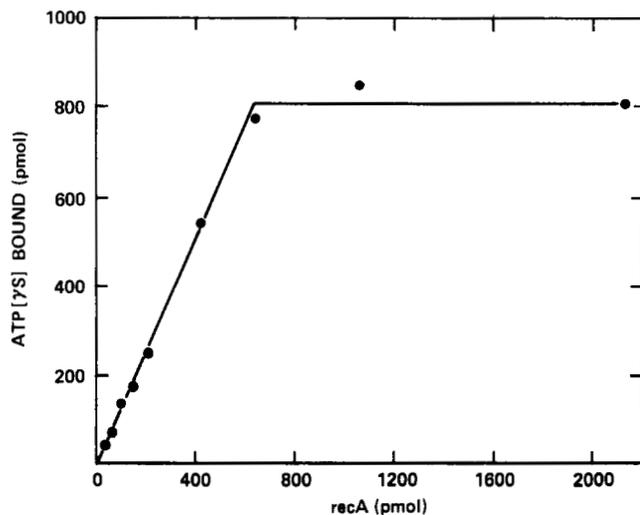

FIG. 9. **Formation of recA protein-ATP(γS) complexes: titration of ATP(γS).** The standard reaction (100 μl) with 20 mM Tris-HCl (pH 7.5) contained 616 μM calf thymus SS DNA, 2.1 μM recA protein (●——●) or 10.6 μM recA protein (○——○), and the indicated concentration of [³⁵S]ATP(γS). Incubation and assay were as in Fig. 8.
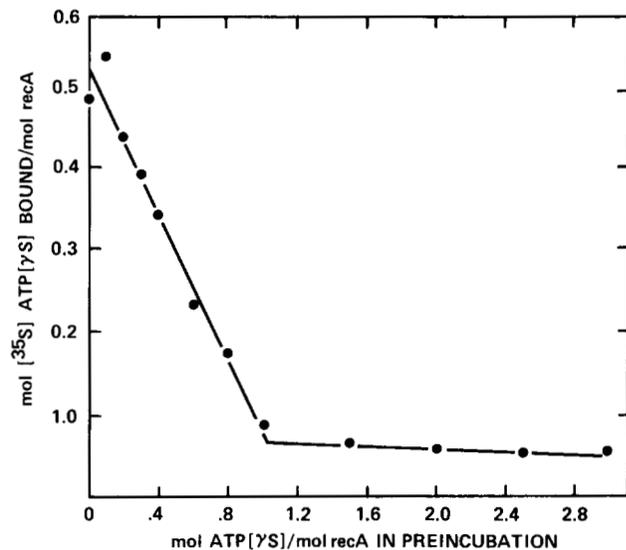
FIG. 10. **Inhibition of [³⁵S]ATP(γS) binding to recA protein by preincubation with unlabeled ATP(γS).** The standard reaction (50 μl) with 20 mM Tris-HCl (pH 8.0) contained 101 μM φX174 SS DNA, 2.6 μM recA protein, and varying concentrations of unlabeled ATP(γS). After 30-min incubation at 30 °C, 9 μl of [³⁵S]ATP(γS) (320 pmol) were added and incubation was continued for an additional 30 min. Aliquots (50 μl) were filtered (low salt wash) as described under "Experimental Procedures."

FIG. 8. **Formation of recA protein-ATP(γS) complexes: titration of recA protein.** The standard reaction (100 μl) with 20 mM Tris-HCl (pH 7.5) contained 616 mM calf thymus SS DNA, 10 μM [³⁵S]ATP(γS), and the indicated amount of recA protein. Incubation was for 20 min at 37 °C at which time samples were filtered (high salt wash) as described under "Experimental Procedures".
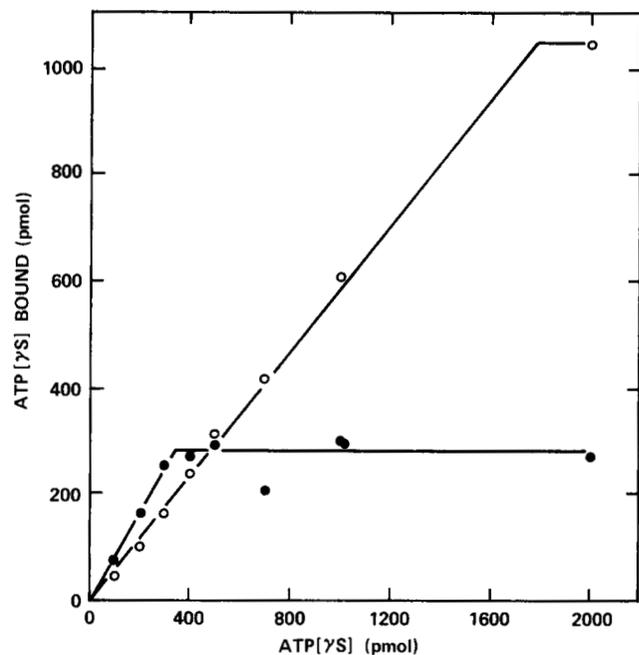
then excess [³⁵S]ATP(γS) added, binding of the labeled ATP(γS) was maximally inhibited when the ratio during preincubation was ≥1 mol of ATP(γS)/mol of recA protein monomer (Fig. 10). Under these conditions, maximal binding of labeled ATP(γS) was only 0.5 mol/mol of recA protein monomer. These results indicate that approximately 1 molecule of ATP(γS) is tightly bound/recA protein monomer. Furthermore, since this ratio was also observed for the irreversible inhibition of ATPase activity by ATP(γS) (Fig. 2),

TABLE IV

Inhibition of ATP(γS) binding to recA protein by NTP's and NDP's

The standard reaction (50 μl) with 20 mM Tris-HCl (pH 8.0) contained 4.3 μM [^{35}S]ATP(γS), 101 μM φX174 SS DNA, 2.6 μM recA protein, and the indicated NTP or NDP. Incubation was for 20 min at 30 °C, at which time 30-μl aliquots were filtered (low salt wash) as described under "Experimental Procedures." In the absence of added NTP or NDP, 0.5 mol of ATP(γS) was bound/mol of recA protein monomer. This value represents 100% binding.

NTP or NDP	Concentration	ATP(γS) bound
	μM	%
None		100
ATP	930	33.4
ADP	950	2.0
UTP	790	67.3
UDP	790	5.9
UTP(γS)	150	4.6
dTTP	1220	3.5
GTP	870	64.4
GTP(γS)	200	31.1

the filter-binding measurements quantitatively detect all stably bound ATP(γS).

Effect of Other NTP and NDPs on Binding of ATP(γS) to recA Protein—ADP was more effective than ATP as an inhibitor of the binding of ATP(γS) to recA protein (Table IV). Since hydrolysis of ATP must have occurred during the incubation, the inhibition observed in the presence of ATP may have been due to ADP rather than ATP. Although UTP showed a weak inhibition of ATP(γS) binding, UTP(γS) and UDP were effective inhibitors. UTP(γS) is also an inhibitor of the ATPase activity of recA protein, a finding that is consistent with other observations that ATP and UTP hydrolysis involve common or overlapping binding sites (12, 13). dTTP, although not hydrolyzed to a significant extent by recA protein, was an inhibitor of ATP(γS) binding in addition to being an inhibitor of ATPase activity (12). GTP and GTP(γS), which are poorly hydrolyzed by the recA protein and are weak inhibitors of ATPase activity, had a correspondingly weak effect on binding of ATP(γS) to recA protein.

DISCUSSION

Tight Binding of ATP(γS) to recA Protein—Our main conclusion is that in the presence of DNA, ATP(γS) binds essentially irreversibly to the recA protein. Although the anhydride linkage between the β and γ phosphates in ATP(γS) could, in principle, be hydrolyzed in a manner analogous to ATP, we find little hydrolysis of ATP(γS) by recA protein. Thus, stable recA protein-ATP(γS) complexes are formed which inhibit the ATPase, UTPase, and single strand annealing and assimilation activities of recA protein.

The stable binding of ATP(γS) to recA protein is not inconsistent with its competitive inhibition of ATPase activity (2). At the ATP(γS) concentrations used, stable complex formation is a relatively slow process when compared with ATP hydrolysis. Thus, when ATP is present in excess over ATP(γS), initially at least, competitive inhibition is to be expected. However, binding of ATP(γS) will ultimately block

all ATP binding sites, limiting the extent of hydrolysis. Consistent with this, we find that ATP (and ADP) inhibit the binding of ATP(γS) to recA protein. Similar results have been observed for UTP hydrolysis which shares part or all of the ATP binding site (10).

It is noteworthy that the stable binding of ATP(γS) to recA protein requires DNA. Binding of ATP(γS) to recA protein does occur in the absence of DNA, resulting in an altered oligomeric form of the protein (8), but these recA protein-ATP(γS) complexes are less stable than those formed in the presence of DNA. Since ATP(γS) also stabilizes recA protein-DNA complexes (8), the stable complexes most likely contain DNA as well as ATP(γS). Presumably, when ATP replaces ATP(γS), ATP hydrolysis alters the recA protein-ATP-DNA complexes to allow DNA annealing and assimilation to occur.

Stoichiometry of ATP(γS) Binding to recA Protein—Approximately 1 ATP(γS) molecule is bound stably/monomer of recA protein at saturation. The values from different experiments range from 0.5 to 1.7. This ratio is subject to several sources of uncertainty. These include lack of knowledge of the true extinction coefficient for recA protein, the fraction of active enzyme molecules, and the presence of impurities in the ATP(γS) preparations, for example, ADP. Despite these uncertainties, our best estimate is that there is a 1:1 molar ratio of recA protein to ATP(γS) in the stable complexes.

Since ATP(γS) irreversibly inhibits ATPase activity, and ATP(γS) binding is inhibited by both NTPs and NDPs in a manner analogous to their effects on ATPase activity, it is likely that the tight binding site for ATP(γS) is also the binding site for ATP in the ATPase reaction. In view of the Hill coefficient of 3 for ATP hydrolysis (12), the actual form of the protein in ATP hydrolysis is thus at least a trimer. However, this does not rule out the existence of other NTP binding sites on the enzyme.

REFERENCES

1. Goody, R. S., and Eckstein, F. (1971) *J. Am. Chem. Soc.* **93**, 6252–6257
2. Shibata, T., Cunningham, R. P., DasGupta, C., and Radding, C. M. (1979) *Proc. Natl. Acad. Sci. U. S. A.* **76**, 5100–5104
3. McEntee, K., Weinstock, G. M., and Lehman, I. R. (1979) *Proc. Natl. Acad. Sci. U. S. A.* **76**, 2615–2619
4. Weinstock, G. M., McEntee, K., and Lehman, I. R. (1979) *Proc. Natl. Acad. Sci. U. S. A.* **76**, 126–130
5. Roberts, J. W., Roberts, C. W., Craig, N. L., and Phizicky, E. M. (1978) *Cold Spring Harbor Symp. Quant. Biol.* **43**, 917–920
6. Craig, N. L., and Roberts, J. W. (1980) *Nature* **283**, 26–30
7. Cunningham, R. P., Shibata, T., DasGupta, C., and Radding, C. M. (1979) *Nature* **281**, 191–195
8. McEntee, K., Weinstock, G. M., and Lehman, I. R. (1981) *J. Biol. Chem.* **256**, 8835–8844
9. McEntee, K., Weinstock, G. M., and Lehman, I. R. (1980) *Proc. Natl. Acad. Sci. U. S. A.* **77**, 857–861
10. Weinstock, G. M., McEntee, K., and Lehman, I. R. (1981) *J. Biol. Chem.* **256**, 8829–8834
11. Horii, T., Ogawa, T., and Ogawa, H. (1980) *Proc. Natl. Acad. Sci. U. S. A.* **77**, 313–317
12. Weinstock, G. M., McEntee, K., and Lehman, I. R. (1981) *J. Biol. Chem.* **256**, 8845–8849
13. Weinstock, G. M., McEntee, K., and Lehman, I. R. (1981) *J. Biol. Chem.* **256**, 8856–8858