Interaction of the recA Protein of *Escherichia coli* with Single-stranded DNA*

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The interaction of recA protein with single-stranded (ss) \( \Phi X174 \) DNA has been examined by means of a nuclease protection assay. The stoichiometry of protection was found to be 1 recA monomer/\(~4\) nucleotides of ssDNA both in the absence of a nucleotide cofactor and in the presence of ATP. In contrast, in the presence of adenosine 5'-O-(thiotriphosphate) (ATP\(\gamma\)S) the stoichiometry was 1 recA monomer/\(~8\) nucleotides. No protection was seen with ADP. In the absence of a nucleotide cofactor, the binding of recA protein to ssDNA was quite stable as judged by equilibrium with a challenge DNA (\( t_\text{eq} \sim 30\) min). Addition of ATP stimulated this transfer (\( t_\text{eq} \sim 3\) min) as did ADP (\( t_\text{eq} \sim 0.2\) min). ATP\(\gamma\)S greatly reduced the rate of equilibration (\( t_\text{eq} > 12\) h). Direct visualization of recA-ssDNA complexes at sub saturating recA protein concentrations using electron microscopy revealed individual ssDNA molecules partially covered with recA protein which were converted to highly condensed networks upon addition of ATP\(\gamma\)S. These results have led to a general model for the interaction of recA protein with ssDNA.

The recA protein of *Escherichia coli* is essential for homologous recombination and for the postreplicative repair of damage to DNA. *In vitro*, recA protein will promote a variety of ATP-dependent DNA-pairing activities including the recombination of complementary single strands, assimilation of linear single strands into duplex DNA (D-loop formation), and the exchange of strands between linear duplex and homologous circular single-stranded DNAs. Although these pairing activities serve to illustrate the role of the recA protein in recombination, very little is known about their mechanism (1).

An understanding of how recA protein interacts with single-stranded DNA is essential to the development of a molecular description of recA protein-promoted reactions. The binding of recA protein to ssDNA\(^1\) has been analyzed using a nitrocellulose filter binding assay (2). However, this assay is of limited value because it is insensitive to the binding of recA protein in excess of that required for DNA retention. Recent studies employing fluorescent-derivatized DNA (dDNA) have provided interesting information on the binding of recA protein to ssDNA (3, 4). However, these studies too must be interpreted with caution. For example, the binding of recA proteins to dDNA is estimated to be \( >2000\) times tighter than the binding to the underderivatized ssDNA (4). We report here the use of a nuclease protection assay designed to quantitate directly the binding of recA protein to \( \Phi X \) ssDNA. This approach has been used to examine the stoichiometry of binding as well as the kinetics of transfer of recA protein among ssDNA molecules. The results of these studies combined with complementary electron microscopic observations have provided a general description of the interaction of recA protein with ssDNA.

**EXPERIMENTAL PROCEDURES**

**Materials**

recA protein was purified to homogeneity as described (5). Its concentration was calculated from the extinction coefficient \( \epsilon_{260} = 5.9 \) (6). DNase I and venom phosphodiesterase were from Worthington. Creatine phosphokinase, phosphocreatine, AMP, ADP, ATP, AMP-PNP, AMP-PCP, and calf thymus DNA were from Sigma. ATP\(\gamma\)S was from Boehringer Mannheim. Nitrocellulose filters (HAWP, 0.45-\(\mu\)m pore diameter) were from Millipore, and GF/C filters were from Whatman.

Unlabeled and \(^3\)H-labeled circular \( \Phi X \) ssDNAs were prepared as described (7). DNA concentrations were calculated using \( A_{260} \) of 1 as equivalent to 36 \( \mu\)g/ml of ssDNA. All DNA concentrations are expressed as nucleotides.

**Methods**

**Nuclease Protection Assay**—The standard nuclease digestion reactions contained 25 mM Tris HCl (pH 7.2), 10 mM MgCl\(_2\), 50 \( \mu\)M \( \Phi X \) (\(^3\)H)ssDNA, 5% glycerol, and the indicated amounts of recA protein. Separate reactions (500 \( \mu\)l) were performed in 1.5-ml Eppendorf tubes for each recA protein concentration. After the solutions were preincubated for 5 min at 25 °C, a 50-\(\mu\)l aliquot was removed to determine the 100% value. Then 10 \( \mu\)g of DNase I and 10 \( \mu\)g of venom phosphodiesterase were added, and the reactions were incubated at 37 °C. Aliquots (50 \( \mu\)l) were removed at various time intervals and added to 100 \( \mu\)l of ice-cold trichloroacetic acid (10%) along with 5 \( \mu\)l of heat-denatured calf thymus DNA (2 mg/ml). The aliquots were kept on ice for 30 min and then filtered on GF/C filters. The filters were washed with 10% trichloroacetic acid (3 times with 1 ml) and 95% ethanol (once with 1 ml), then dried, and the radioactivity determined. Alternatively, the protected DNA was assayed by filtration of the aliquots on KOH-treated nitrocellulose filters, washing with 1 ml of reaction buffer, drying, and determining radioactivity. (KOH-treated nitrocellulose filters were prepared as described (8)).

The amount of DNA remaining undigested in the absence of recA protein was measured by omitting recA protein from the reactions until after the nuclease digestion. These values were \(<1\%\) of the total DNA.

When nuclease protection was measured in the presence of a nucleotide cofactor, the nucleotide was included in the reaction solution during the preincubation step. When an ATP regeneration...
system was included, it consisted of 8 mM phosphocreatine and 40 
\(\mu\)g/ml creatine phosphokinase. Additional titrations employing higher 
or lower \(\phi X\) ssDNA concentrations were performed exactly as for the 
standard titration.

RecA Protein Transfer Kinetics—In the standard transfer reaction, 
RecA protein (1 \(\mu\)M) was preincubated with 30 \(\mu\)M \(\phi X\) [\(\text{H}\)]ssDNA in 
25 mM Tris-HCl (pH 7.2), 10 mM MgCl\(_2\), and 1 mM dithiothreitol 
for 5 min at 25 °C. This preincubation solution was then mixed with 
an equal volume of a challenge solution containing 25 mM Tris-HCl 
(pH 7.2), 10 mM MgCl\(_2\), 1 mM dithiothreitol, and 30 \(\mu\)M unlabeled 
\(\phi X\) ssDNA; the reaction mixture was kept at 25 °C. Aliquots (75 \(\mu\)l) 
were removed at various time intervals and quenched by mixing with 
2 \(\mu\)l of 20 mM ATP\(_7\)S (final concentration of ATP\(_7\)S, 0.5 mM). After 
5 min at 25 °C, 3 \(\mu\)l of DNase I (1 mg/ml) and 1.3 \(\mu\)l of venom 
phosphodiesterase (2.2 mg/ml) were added to each aliquot, followed 
by incubation at 37 °C for 20 min. The aliquots were then added to 
10 \(\mu\)l of trichloroacetic acid (10%) along with 15 \(\mu\)l of heat-denatured 
calf thymus DNA (2 mg/ml). After 20 min on ice, the aliquots were 
filtered using GF/C filters as described above. Zero time points were 
measured by: (i) mixing 37.5 \(\mu\)l of the preincubation solution with 2 
\(\mu\)l of ATP\(_7\)S (20 mM) for 1 min followed by mixing with 37.5 \(\mu\)l of 
the challenge solution; (ii) mixing 37.5 \(\mu\)l of the challenge solution 
with 2 \(\mu\)l of ATP\(_7\)S (20 mM), followed by mixing with 37.5 \(\mu\)l of 
the preincubation solution.

When transfer was measured in the presence of a nucleotide 
cofactor, the nucleotide was added at an equal concentration to both 
the preincubation and challenge solutions after the 5-min preincubu-
tion period. Following an additional 2-min incubation at 25 °C, the 
solutions were mixed and treated as described above. When an ATP 
regeneration system was used, it was added to both the preincubation 
and challenge solutions prior to mixing. When higher challenge DNA 
centralizations were employed, the concentration of challenge DNA 
was increased, but the volume of the challenge solution was kept the 
same. The complementary transfer reactions were performed exactly 
as described above except that the unlabeled \(\phi X\) ssDNA was in the 
preincubation solution and the \(\phi X\) [\(\text{H}\)]ssDNA was in the challenge 
solution.

Electron Microscopy—Individual samples were prepared as de-
scribed in the legend to Fig. 8. They were then diluted to 0.5 \(\mu\)g/ml 
DNA and mounted on grids for electron microscopy using either the 
polylysine method (9) or by spreads of formamide (40%-10%) cyto-
chrome c mixtures (10). Samples were fixed by the sequential addition 
of formaldehyde and glutaraldehyde (11) where indicated. The prep-
arations were rotary shadowed with tungsten and examined using a 
Philips EM301 electron microscope.

RESULTS

The nuclease protection assay was used to quantitate the 
binding of recA monomers to ssDNA. Various amounts of 
recA protein were added to a fixed concentration (30 \(\mu\)M) of 
\(\phi X\) [\(\text{H}\)]ssDNA to form recA-ssDNA complexes. ATP\(_7\)S was added 
to convert the complexes to a nondissociable form (2), 
and any DNA not associated with recA protein was digested 
by a combination of DNase I and venom phosphodiesterase.

The addition of recA protein to the \(\phi X\) [\(\text{H}\)]ssDNA rendered 
a portion of the DNA resistant to nuclease digestion, as judged 
by acid-precipitable radioactivity. The amount of DNA prote-
tected was proportional to the amount of recA protein added, 
suggesting that the mechanism of protection involves the 
binding of recA protein to the DNA. A typical time course of 
nuclease digestion for a given recA protein concentration is 
shown in Fig. 1. At sufficiently high recA protein concentra-
tions, >90% of the DNA could be protected from digestion. 
From the titration curve shown in Fig. 2, it can be estimated 
that approximately 7.5 nucleotides of ssDNA were protected 
per recA monomer in the presence of ATP\(_7\)S.

A second titration was performed in which \(\phi X\) [\(\text{H}\)]ssDNA was incubated with various amounts of recA protein without 
the addition of ATP\(_7\)S before nuclease digestion. Again, the 
amount of DNA protected was proportional to the amount of 
recA protein added. However, under these conditions, approxi-
mately 3.5 nucleotides of DNA were protected per recA 
monomer. Thus, recA protein protects twice as much ssDNA 
from nuclease digestion in the presence of ATP\(_7\)S as in its 
asbence.

The titration experiments were repeated using nitrocellulose 
filter binding to measure the amount of DNA protected 
from digestion. The results were very similar to those obtained 
by acid precipitation, with each recA monomer protecting 7.5 
nucleotides of \(\phi X\) [\(\text{H}\)]ssDNA in the presence of ATP\(_7\)S and 
3.7 nucleotides in its absence. Agreement between the two 
assays indicates that nuclease digestion does not result in 
cleavage of the ssDNA between recA monomers to produce 
fragments that are protected but too small to be acid precipi-
table.

Additional titrations were performed at both a higher (150 
\(\mu\)M) and a lower (15 \(\mu\)M) \(\phi X\) [\(\text{H}\)]ssDNA concentration, using 
acid precipitation to measure nuclease protection. At 150 \(\mu\)M 
\(\phi X\) DNA, the protection per recA monomer was 7.9 nucleo-
Effect of ATP and ADP on nuclease protection by recA protein. Reactions were carried out as described under "Experimental Procedures." Reaction solutions contained 30 µM 5X [H] ssDNA, 1.2 µM recA protein, O, no nucleotide; I, 500 µM ATP; 2, 500 µM ATP plus an ATP-regeneration system; 3, 500 µM ADP. The points represent the percentage of DNA remaining acid precipitable.

When complexes were formed between 5X [H] ssDNA (30 µM) and recA protein (1.2 µM) in the presence of a saturating concentration of ATP (based on ssDNA-dependent ATPase activity), there was no protection of the DNA from nuclease digestion (Fig. 3). Similarly, when the recA-ssDNA complexes were formed at saturating levels of ATP, there was no protection. However, when the recA-ssDNA complexes were formed in the presence of ATP and an ATP regeneration system, protection of the DNA was observed (Fig. 3). The amount of DNA protected was the same as in the absence of nucleotide; approximately 4 nucleotides/recA monomer.2 These results indicate that the lack of protection in the presence of ATP but in the absence of the ATP regeneration system is due to ongoing ATP hydrolysis leading to the buildup of ADP (kdiss = 8 min⁻¹ at 25°C) which in turn leads to a weakened interaction of recA protein with the ssDNA.

When complexes were formed between 5X [H] ssDNA (30 µM) and recA protein (1 µM) in the presence of AMP-PNP (500 µM), approximately twice as much DNA was protected from digestion (1 recA monomer/7.0 nucleotides) as in the absence of nucleotide. Thus, the 2-fold greater protection is not peculiar to ATPγS and may be a general property of nonhydrolyzable ATP analogs. The results with AMP-PNP, however, are more complex than those obtained with ATPγS in that the complexes required prolonged incubation (20 min) prior to digestion before protection was seen. In contrast, the protection stoichiometries obtained with ATPγS (or without nucleotide) were independent of the preincubation period, over a range of 2 to 20 min.

Transfer of recA Protein—The nuclease protection assay was used to measure the movement or transfer of recA protein from one ssDNA molecule to another and to determine the effect of various nucleotides on this process. The standard reaction contained 1 µM recA protein and 30 µM 5X [H] ssDNA. The ssDNA is in excess so that all of the recA protein should be bound to DNA under these conditions. Transfer is initiated by the addition of a second equivalent of unlabeled 5X ssDNA to the preformed complexes. The reaction is quenched after various time intervals by the addition of ATPγS, which converts the recA-ssDNA complexes to a nondissociable form (2). The reaction mixture is treated as before with DNase I and venom phosphodiesterase to determine the amount of 5X [H] ssDNA remaining resistant to digestion.

In these experiments it is important to establish that all of the recA protein is associated with the 5X [H] ssDNA at the time the unlabeled challenge 5X ssDNA is added. The amount of 5X [H] ssDNA protected at zero time was measured in two ways. One was to add ATPγS to the preformed recA-5X ssDNA complexes before addition of the unlabeled challenge DNA. This guarantees that all of the recA protein is fixed on the [H] ssDNA. The second way was to add ATPγS to the preformed complexes simultaneously with the challenge DNA. In this case, any unbound recA protein can be fixed to the challenge DNA as well as to the [H] ssDNA, resulting in a reduced amount of 5X [H] ssDNA protected from digestion. Unless otherwise indicated, all transfer experiments gave zero time points measured by the second method that were at least 94% of the values measured by the first method, indicating that all of the recA protein was associated with the 5X [H] ssDNA at the time of the challenge.

When the transfer reaction was performed in the absence of nucleotide, a slow time-dependent decay in the amount of 5X [H] ssDNA protected from digestion was observed (Fig. 4). The reaction was too slow to follow accurately to completion, but the half-time of equilibration could be estimated as >30 min. When the transfer reaction included ATP (200 µM), there was a rapid first-order decay in the amount of 5X [H] ssDNA protected from digestion, with a half-time of equilibration of 3.2 min (Fig. 4). The recA protein equilibrated equally among the two DNA populations as judged by the final reduction of 5X [H] ssDNA protected to 50% of the zero-time value. Thus, there is a substantial stimulation in the rate of recA protein transfer among ssDNA molecules by ATP. The inclusion of an ATP regeneration system in the
transfer reaction did not affect the kinetics, showing that the stimulation of transfer is due directly to the hydrolysis of ATP at the active site and not to the accumulation of ADP as a result of the ssDNA-dependent ATPase activity of recA protein. When ATPγS was substituted for ATP in the preincubation, there was no decrease in 4X [3H]ssDNA protection upon addition of the unlabeled challenge DNA (tₐ > 12 h), confirming that ATPγS causes the formation of a nondissociable complex between recA protein and ssDNA.

A complementary version of the transfer experiment was performed in which recA protein was first complexed with unlabeled φX ssDNA, and then 1 eq of φX [3H]ssDNA was added as a challenge. In this case, instead of measuring the movement of recA protein from the DNA to which it was initially bound, the assay measures the movement of recA onto the challenge DNA, as indicated by an increase in φX [3H]ssDNA protection as a function of time. As expected, first-order increases in φX [3H]ssDNA protection were seen with rate constants in good agreement with those measured by the original method (Fig. 5).

**Dependence of recA Protein Transfer on DNA Concentration**—The rates of recA protein transfer were somewhat dependent on the concentration of challenge DNA. recA protein (1 μM) was incubated with φX [3H]ssDNA (30 μM) in the presence or absence of ATP, and then transfer was initiated by the addition of increasing concentrations of unlabeled challenge DNA. In experiments with high concentrations of challenge DNA, denatured calf thymus DNA rather than φX ssDNA was used. Measurements using both φX ssDNA and denatured calf thymus DNA at lower challenge DNA concentration (≤5 eq) showed that the two DNAs behaved similarly.

The DNA concentration dependence of transfer reactions in the presence and absence of ATP were the same as judged by the slopes of plots of kₜ₁/₂ versus challenge DNA concentration (Fig. 6). Thus, there was about a 5-fold increase in rate in the absence of ATP and less than a 2-fold increase in its presence going from 1 to 25 eq of challenge DNA. As will be seen below, for reactions that were too slow to be easily measured with 1 eq of challenge DNA, higher concentrations (15 eq of ssDNA) were required.

![Fig. 5. Complementary assays of recA protein transfer reaction.](image)

**Effect of ADP on Transfer of recA Protein**—Addition of ADP (200 μM) to recA-φX ssDNA complexes resulted in a stimulation of transfer much greater than that seen with ATP. Transfer reactions measured using 1 eq of φX ssDNA as a challenge gave a half-time of equilibration of ~10 s, using the two complementary assay methods (Fig. 4). Addition of 200 μM ADP plus 200 μM P₆ gave a transfer rate equivalent to that seen with ADP alone, whereas P₆ caused no stimulation of recA protein transfer. Similarly, AMP (200 μM) had no effect on transfer. The effect of challenge DNA concentration on transfer in the presence of ADP was not determined because higher challenge DNA concentrations resulted in transfer rates too fast to be measured. Despite the high rate of transfer in the presence of ADP, a comparison of zero-time measurements indicated that >90% of the recA protein was associated with the φX [3H]ssDNA at the time the transfer reaction was initiated.

**Effect of Other Nonhydrolyzable ATP Analogs on Transfer of recA Protein**—AMP-PNP is a competitive inhibitor of the ssDNA-dependent ATPase activity and prevents transfer of recA protein from recA-φX DNA complexes to challenge DNA (12). AMP-PNP is also a competitive inhibitor of the ssDNA-dependent ATPase activity of recA protein, with a Kᵢ = 60 μM (data not shown). When recA-φX ssDNA complexes were formed with AMP-PNP at standard concentrations of recA protein and ssDNA, the transfer was too slow to measure accurately when 1 eq of φX ssDNA was used as the challenge. Consequently, transfer reactions were carried out using 15 eq of denatured calf thymus DNA (Fig. 7). Under these conditions, the transfer of recA protein in the absence of nucleotide proceeded with first-order kinetics, with a half-time of equilibration of 5.4 min, in good agreement with the data of Fig. 6. When the recA-φX ssDNA complexes were incubated with AMP-PNP (200 μM) for 2 min before addition of the challenge, transfer proceeded to about 60% of completion; no further transfer occurred. When the preincubation period was increased to 10 min, the reaction proceeded to 40% of completion; after a 20-min preincubation the reaction stopped after reaching 30% of completion (Fig. 7). Thus, there appears to be a time-dependent conversion of recA-φX ssDNA complexes to a nontransferable form in the presence of AMP-PNP.
AMP-PCP is not a significant competitive inhibitor of the ssDNA-dependent ATPase activity of recA protein (K_i > 300 μM); correspondingly, it did not have a significant effect on the rate of recA protein transfer (data not shown).

Examination of recA Protein-ssDNA Complexes by Electron Microscopy—Complexes of φX ssDNA with subsaturating concentrations of recA protein were prepared in the presence and absence of various nucleotides and examined by electron microscopy (Fig. 8).

When the φX ssDNA was mounted using the polyllysine procedure (9), it appeared as a uniform field of individual collapsed molecules. When sufficient recA protein to give ~15% protection was added to the φX ssDNA before mounting, the DNA appeared as a uniform field of individual collapsed molecules with nearly all molecules containing regions of bound recA protein. The regions of the molecule where recA protein was bound appeared rigid and extended. A survey of the field of φX ssDNA molecules revealed that nearly all molecules were partially covered with tracts of recA protein; no completely covered φX ssDNA molecules were seen. When recA protein was mounted alone, it appeared as a uniform field of short filaments. Thus, when subsaturating amounts of recA protein are added to φX ssDNA under standard conditions, nearly all of the φX DNA molecules acquire limited tracts of bound recA protein.

When ATPγS was added to the recA−φX ssDNA complexes, all of the φX ssDNA and recA protein molecules were concentrated dramatically into a number of large aggregated complexes. The addition of ATPγS to recA protein alone had no effect. Thus, addition of ATPγS to complexes of φX ssDNA and subsaturating recA protein results in an extreme concentration of the recA−φX DNA into higher order complexes. When saturating recA protein was added to φX DNA, the φX ssDNA molecules appeared as completely coated circles. The addition of ATPγS under these conditions failed to produce aggregation.

The complexes of φX ssDNA with subsaturating recA protein were also examined using the cytochrome c spreading technique (10). In this method, the samples are spread in formamide solution in order to unfold the ssDNA. Since this treatment results in removal of bound protein, all samples were fixed using a stepwise addition of 1% formaldehyde and 0.6% glutaraldehyde just prior to spreading (11).

When the φX ssDNA was spread using this technique, it appeared as a uniform field of individual molecules. When a subsaturating concentration of recA protein was added to the φX ssDNA before mounting, the complexes were indistinguishable from the φX ssDNA alone. The regions of bound recA protein were not distinguishable because all of the molecules were covered with a layer of denatured cytochrome c. recA protein alone was not visible using this technique, presumably because the filaments would not be stable to the formamide treatment. These results indicate that the fixing procedure does not chemically cross-link φX ssDNA molecules or unassociated recA−φX ssDNA complexes. Also, the fixing procedure had no effect on the appearance of recA−φX ssDNA complexes with or without ATPγS when visualized using the polyllysine technique, providing further evidence that intermolecular cross-linking did not occur.

When ATPγS was added to the recA−φX ssDNA complexes prior to fixation and spreading, all of the complexes were again concentrated into large networks of apparently interconnected complexes. The addition of ATPγS to recA protein alone had no effect. Similarly, addition of ATP to recA−φX ssDNA complexes prior to fixation was without effect. When AMP-PNP was added to recA−φX ssDNA complexes and incubated for 20 min prior to fixation, the DNA appeared both as collapsed individual molecules and as interconnected complexes, although the complexes were much smaller than those seen with ATPγS.

Discussion

We have used a nuclease protection assay to study the binding of recA protein to φX ssDNA. By titrating φX ssDNA with recA protein, we have determined a stoichiometry of protection of 1 recA monomer/3.7 ± 0.4 nucleotides of φX ssDNA. This value is in agreement with the earlier estimate of West et al. (13), of 1 recA monomer/4 nucleotides, based on the ratio of recA protein to φX ssDNA in saturated recA−φX ssDNA complexes isolated by ultracentrifugation (13). It is also in agreement with the value of 1 recA monomer/4 nucleotides obtained by Morrical and Cox using light-scattering measurements. This stoichiometry does differ significantly from the value of 1 recA monomer/6 nucleotides that has been recently reported by Silver and Fersht (3) and independently by Cazenave et al. (14), based on titrations of fluorescent c-DNA with recA protein.

In the presence of ATP and an ATP regeneration system, the stoichiometry of nuclease protection was the same as in the absence of nucleotide cofactor. However, in the presence of ATPγS, a nonhydrolyzable analog of ATP that binds tightly to the ATP active site (9), the stoichiometry of protection was 1 recA monomer/7.7 ± 0.5 nucleotides of φX ssDNA, twice that seen in the absence of ATPγS. A similar increase in protection stoichiometry was seen in the presence of AMP-PNP. The 2-fold difference in protection stoichiometry was independent of the φX ssDNA concentration, indicating that recA protein bound stoichiometrically to the φX ssDNA in the absence as well as in the presence of ATPγS and AMP-PNP under our experimental conditions.

Direct visualization of recA−φX DNA complexes at subsaturating recA protein concentrations using electron microscopy revealed significant differences between the complexes formed in the presence and in the absence of ATPγS. The recA−φX ssDNA complexes in the absence of ATPγS ap-

\(^{3}\) S. Morrical and M. M. Cox, personal communication.
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Fig. 8. Association of recA protein with single-stranded DNA. Samples a-d were prepared using the polylysine procedure; samples e and f were prepared by the cytochrome c spreading procedure. a, circular single-stranded DNA. Circular φX ssDNA (30 μM) was incubated in 25 mM Tris-HCl (pH 7.2), 10 mM MgCl₂, and 5% glycerol (reaction buffer) for 5 min at 25 °C prior to mounting. b, d, e, complexes of circular single-stranded DNA and recA protein. recA protein (1.0 μM) was incubated with 30 μM φX ssDNA in reaction buffer for 5 min at 25 °C prior to mounting. c and f, complexes of circular single-stranded DNA and recA protein in the presence of ATPγS. recA protein (1.0 μM) was incubated with 30 μM φX ssDNA and 500 μM ATPγS in reaction buffer for 5 min at 25 °C prior to mounting. The bar in a-f indicates 100 nM. In the samples prepared by the cytochrome c spreading procedure, triethanolamine HCl (pH 7.2) was substituted for Tris-HCl (pH 7.2) in the reaction buffer.
peared as individual φX ssDNA molecules that were partially covered with tracts of recA protein. Addition of ATP to the complexes caused no change in appearance. However, when ATPγS was added to the recA-φX ssDNA complexes, all of the complexes were condensed into large interconnected networks. Smaller but similarly interconnected networks were seen with AMP-PNP. The large complexes are consistent with the results of McEntee et al., who reported that addition of ATPγS to recA-φX ssDNA complexes converted them to large rapidly sedimenting forms (2).

A plausible explanation for our findings is that in the absence of a nucleotide cofactor each recA monomer binds to a single strand of φX ssDNA with a stoichiometry of 1 monomer/4 nucleotides. However, in the presence of ATPγS, recA protein is converted to a state in which each monomer can bind 2 strands of φX ssDNA for an overall stoichiometry of 1 recA monomer/8 nucleotides. If the 2 strands are derived from different individual φX ssDNA molecules, the population of molecules would become extensively interconnected. This type of binding mechanism would be analogous to the binding of recA protein to duplex DNA. recA protein is known to form stable complexes with duplex DNA only in the presence of ATPγS and in doing so causes significant unwinding of the duplex DNA (15). The stoichiometry of binding of recA protein to DNA in these complexes has been measured as 1 recA monomer/3 base pairs (or 1 recA monomer/6 nucleotides) in approximate agreement with the results obtained here with ssDNA.

The 2-strand binding model is only one interpretation of our results. We cannot preclude the possibility that ATPγS induces a protein-protein interaction between recA monomers that leads to interconnection of DNA molecules as well as a 2-fold increase in protection/monomer but does not require two formal ssDNA binding sites on each recA monomer. At the present time this distinction cannot be made either in the case of the binding of recA protein to duplex DNA. The important point, however, is that nonhydrolyzable ATP analogs induce or trap recA protein in a form in which ssDNA molecules are held together.

Kinetic measurements of the movement of recA protein from one φX ssDNA molecule to another reveal that in the absence of nucleotide cofactors, the binding of recA protein to φX ssDNA is quite stable with a half-time of equilibration with 1 eq of φX ssDNA of approximately 30 min. It had been reported previously on the basis of strand exchange challenge experiments that recA protein equilibrated rapidly (t0 < 17 s) between two populations of φX ssDNA molecules (16). However, at the initial recA protein and φX ssDNA concentrations used, it is likely that much of the recA protein was bound to the original φX ssDNA at the time of the challenge, thus complicating interpretation of the results.3

Addition of ATP to recA-φX ssDNA complexes stimulates the equilibration of the recA protein with added challenge DNA (t0 ~3 min). ADP causes an even greater stimulation of equilibration (t0 ~0.2 min). These results suggest that ADP stimulates the release of recA protein from ssDNA, leading to rapid equilibration with challenge DNA. The slower equilibration seen with ATP suggests that a slow step leading to or including the ATP hydrolysis step precedes a rapid ADP-induced release step. Control experiments show that even in the presence of ADP, under our conditions, the overall equilibrium still favors almost complete association of recA protein with the φX ssDNA.

Addition of ATPγS to recA-φX ssDNA complexes has an effect very different than that of ADP and completely prevents the movement of recA protein from φX ssDNA. This suggests that the binding of ATPγS converts recA protein to a high-affinity conformation in which it may interact with two strands of ssDNA. A similar effect on the kinetics is seen with AMP-PNP. It is striking that ADP, ATPγS, and AMP-PNP which are all competitive inhibitors of the ssDNA-dependent ATPase activity and presumably all bind to the ATP-binding site have completely different effects on the affinity of recA protein for ssDNA.

We suggest the following model to consolidate the results presented in this paper. recA protein initially interacts with φX ssDNA. Binding of ATP to the recA-φX ssDNA complex may transiently convert recA to a high-affinity conformation that can interact with a second strand of φX ssDNA. When ATP is hydrolyzed to ADP and P0, recA protein is released from the ssDNA and the complex breaks down. Rebinding of recA protein to ssDNA occurs, resulting in the equilibration with any added challenge DNA. The addition of ADP would cause rapid release of the recA protein without prior formation of the transiently two-stranded intermediate, resulting in rapid equilibration. ATPγS, on the other hand, would bind to the recA-φX ssDNA complexes and allow the two-stranded intermediate to form. Since this analog cannot be hydrolyzed to ADP, the complex cannot break down, resulting in the formation of networks of interconnected complexes. The implications of this model with respect to the role of ATP hydrolysis in recA protein-promoted DNA-pairing reactions will be elaborated elsewhere.

Acknowledgments—We thank the Department of Biochemistry, University of California, Berkeley, for the use of its electron microscope and facilities.

REFERENCES