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Biochemistry

Homologous pairing in genetic recombination: Complexes of recA protein and DNA*

[stoichiometric requirement for recA protein/adenosine 5′-*O*-(3-thiotriphosphate)/ATPase/D loops]

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ABSTRACT recA protein, which is essential for general genetic recombination in *Escherichia coli*, promotes the homologous pairing of single-stranded DNA with double-stranded DNA to form a D loop. The amount of recA protein required for the reaction was directly proportional to the amount of single-stranded DNA and was unaffected by similar variations in the amount of double-stranded DNA. The ATP analog, adenosine 5′-*O*-(3-thiotriphosphate) (ATPγS), which was not rapidly hydrolyzed by recA protein, blocked the formation of D loops but promoted the formation of stable complexes of recA protein and single-stranded DNA. These complexes, in turn, bound homologous or heterologous double-stranded DNA and partially unwound it. Because ATPγS competitively inhibited the ATPase activity of recA protein ($K_m/K_i \approx 300$), we infer that ATPγS binds at a site that overlaps the site for ATP and that the functional complexes formed in the presence of the analog probably represent partial steps in the overall reaction. If the complexes formed in the presence of ATPγS reflect natural intermediates in the formation of D loops, recA protein must promote homologous pairing either by moving juxtaposed single-stranded and double-stranded DNA relative to one another or by forming and dissociating complexes reiteratively until a homologous match occurs.

Nothing is more intriguing about homologous recombination than its beginning. How, for example, do homologous double-stranded molecules recognize each other, and what enzymic events, overcoming the energetic barrier posed by the stability of duplex DNA, begin an exchange of parts? Current ideas on these questions are based, on the one hand, on the structure and enzymology of DNA (2) and, on the other hand, on biological observations, particularly those on transformation in bacteria and on meiotic recombination in fungi (3–5). Until recently, however, we had little direct information about the initiation of homologous recombination. A start has been made as the result of a series of experiments that began with the discovery of the *recA* gene, which is essential for recombination in *Escherichia coli* (6, 7). In recent years, the cloning of the *recA* gene (8–10) facilitated the purification of its protein product (9, 11, 12). Following the inference drawn by Holloman and Radding (13) that one of the functions controlled by *recA* might be the formation of D loops and encouraged by evidence that the product of the *recA* gene is directly involved in recombination (14), Shibata *et al.* (12) discovered that purified recA protein catalyzed the homologous pairing of superhelical DNA and single-stranded fragments (Fig. 1). McEntee *et al.* (15) and Cunningham *et al.* (1) have since reported that relaxed DNA also serves as a substrate in the formation of D loops by recA protein. The reaction requires ATP and Mg^{2+}. Consistent with the observation that recA protein lacks topoisomerase activity (1), the formation of a D loop by recA protein appears to require that the single-stranded DNA have a free end (12). We can

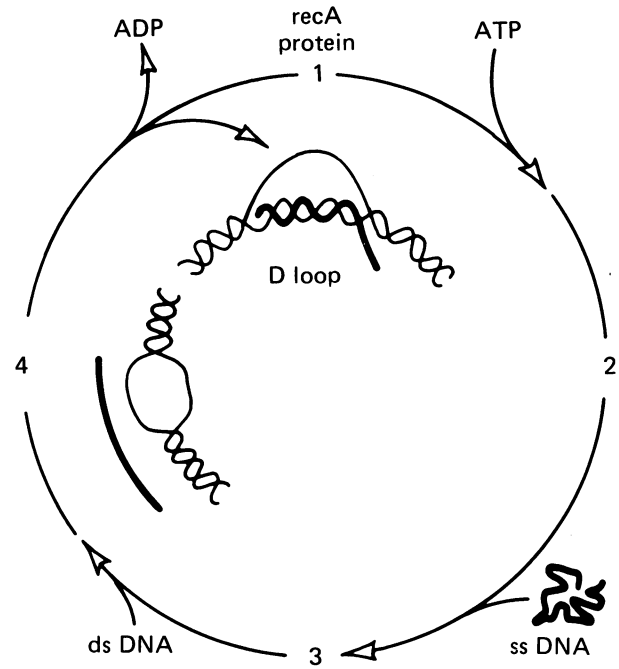

FIG. 1. Model for the promotion of homologous pairing by recA protein. The numbers represent states of the recA protein, possibly different conformations, as inferred from the effects of the indicated ligands (see *Discussion*). Single-stranded and double-stranded DNA are abbreviated ss DNA and ds DNA, respectively.

imagine at least four components of this reaction: (*i*) unfolding of single-stranded DNA to make its bases available for pairing, (*ii*) unwinding of the duplex recipient, (*iii*) homologous base-pairing, and (*iv*) formation of a double helix containing the new strand, rather than rewinding of the original helix (Fig. 1). Astonishingly, a protein constructed from a single polypeptide chain of molecular weight 40,000 suffices *in vitro* to catalyze this complex reaction.

The DNA-dependent ATPase activity of recA protein (9, 16) provided a strong clue to the interaction of recA protein with DNA as well as an assay for purification of the protein (12). Analogs of ATP (17), a number of which are readily available, have proven useful in studying the action of ATPases. Studies of DNA gyrase provided an immediate precedent for the current work. Sugino *et al.* (18) showed that in the presence of the nonhydrolyzable analog adenyl-5′-yl imidodiphosphate, stoichiometric amounts of DNA gyrase would partially unwind DNA. Elsewhere, we have reported that recA protein partially

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Abbreviations: We use the following designations for the different forms of double-stranded DNA from phages φX174 and fd: form I, superhelical DNA; form II, nicked circular DNA; form III, linear DNA. ATPγS, adenosine 5′-*O*-(3-thiotriphosphate).

* This is paper no. 3 in a series. Paper no. 2 is ref. 1.

unwinds DNA in the presence of adenosine 5′-*O*-(3-thiotriphosphate) (ATPγS) and either single strands or oligodeoxynucleotides (1). The experiments reported here relate to the mechanisms by which recA protein forms D loops, mechanisms suggested largely by observations on the effects of ATPγS.

METHODS

Formation of D Loops or Complexes of recA Protein and DNA. The standard reaction mixture for making D loops contained, in 20.5 μl, 31 mM Tris·HCl (pH 7.5), 1.8 mM dithiothreitol, 88 μg of bovine serum albumin per ml, 1.3 mM ATP, 25 mM $MgCl_2$, 8.8 μM double-stranded fd [^{3}H]DNA, 12 μM fragments of single-stranded fd [^{32}P]DNA, and 2.4 μM recA protein (1). Samples were incubated at 37°C for 30 min. In some experiments, we used 6.7 mM $MgCl_2$, which supports D-loop formation by superhelical (form I) DNA but not by nicked circular (form II) DNA. Amounts of DNA are expressed in moles of nucleotide.

To study the formation of complexes of recA protein with DNA, we usually replaced ATP by 0.5 or 1.3 mM ATPγS. For assay C, we also reduced the $MgCl_2$ to 6.7 mM in order to avoid the trapping of single-stranded DNA by nitrocellulose, which occurs at higher concentrations of Mg^{2+}.

Assay D. This is the method that we have used to detect the formation of D loops (12, 19). It measures the retention by nitrocellulose of DNA with single-stranded regions or of complexes of protein and DNA that are not dissociated by heating at 50°C for 4 min in 1.5 M NaCl/0.15 M Na citrate at pH 7. We used the Sartorius Membranfilter type SM 11306, which has a pore size of 0.45 μm.

Assay C. This assay measures the retention by nitrocellulose of complexes of protein and DNA. Less than 1% of either single-stranded or double-stranded DNA is retained in the absence of protein. For this assay, we diluted the reaction mixture 1:10 in a buffer containing 31 mM Tris·HCl (pH 7.5), 6.7 mM $MgCl_2$, 1.8 mM dithiothreitol, and 88 μg of bovine serum albumin per ml. The filters (Millipore DAWP, pore size 0.65 μm) were soaked in the same buffer prior to use and washed with 1 ml of buffer after filtration of 0.2 ml of sample. In some experiments the above buffer contained 0.2 M NaCl.

Oligodeoxynucleotides. A boiled digest of double-stranded DNA, prepared as described (1), was the source of oligodeoxynucleotides.

Other Methods. These were as described (1, 12).

RESULTS

Stoichiometric Relationship Between recA Protein and Single-Stranded DNA. The formation of D loops is a nonlinear function of the concentration of recA protein. No D loops are formed until a certain concentration of recA protein is present, after which the yield of D loops rises sharply (refs. 12 and 15 and Fig. 2). We varied, one at a time, the concentration of single-stranded DNA (Fig. 2*A*) or double-stranded DNA (Fig. 2*B*) and measured the D loops formed in 30 min at 37°C as a function of the concentration of recA protein added. The experiments showed that the amount of single-stranded DNA determines the amount of recA protein that is required to form D loops. Similar variations in the concentration of form I DNA had no detectable effect on the amount of recA protein needed. The minimal amount of recA protein was 1 molecule per 10–18 bases of single-stranded DNA. Less than a doubling in the concentration of recA protein produced the optimal formation of D loops, consistent with our previous estimate (12), of 1 molecule of recA protein per 5–10 nucleotide residues of single-stranded DNA.

Competitive Inhibition of recA ATPase by ATPγS. recA protein hydrolyzed 27 moles of ATP per mole of enzyme per min when both ATP and single-stranded DNA were present in excess (Fig. 3). The K_m for ATP, estimated from the data presented in Fig. 3, is 0.77 mM. The analog, ATPγS, inhibited the ATPase activity: double-reciprocal plots, which relate the rates of hydrolysis to the concentrations of ATP and ATPγS, show that the inhibition is competitive and that the K_i for the inhibitor is 2.9 μM (Fig. 3).

To determine whether recA protein hydrolyzes ATPγS, we examined the products of the standard reaction by thin-layer chromatography (12). Under ultraviolet illumination, absorbent material appeared only at positions corresponding to ATP, ATPγS, and ADP. Lacking radioactive ATPγS, we scraped the material from the thin-layer strips at the position corresponding to ADP, extracted it with 10 mM HCl, and examined it spec-

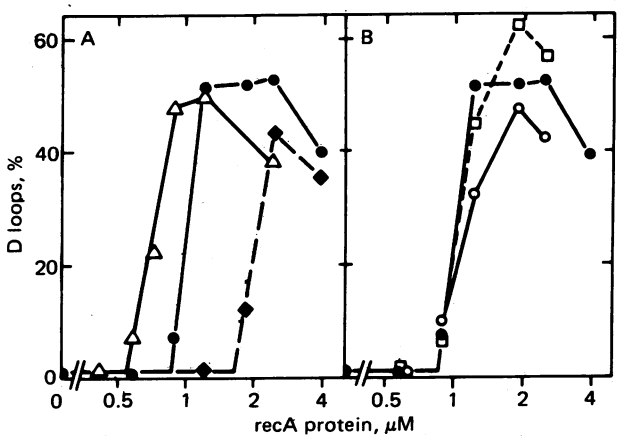

FIG. 2. Stoichiometric requirement for recA protein. Reaction mixtures were as described in *Methods* except for the indicated variations in the concentrations of DNA and recA protein. (*A*) Concentration of fd form I DNA was held constant at 8.8 μM. Concentration of fragments of single-stranded fd DNA was varied: △, 5.9 μM; ●, 12 μM; ◆, 29 μM. (*B*) Concentration of single-stranded fragments was held constant at 12 μM. Concentration of form I DNA was varied: □, 4.4 μM; ●, 8.8 μM; ○, 22 μM.

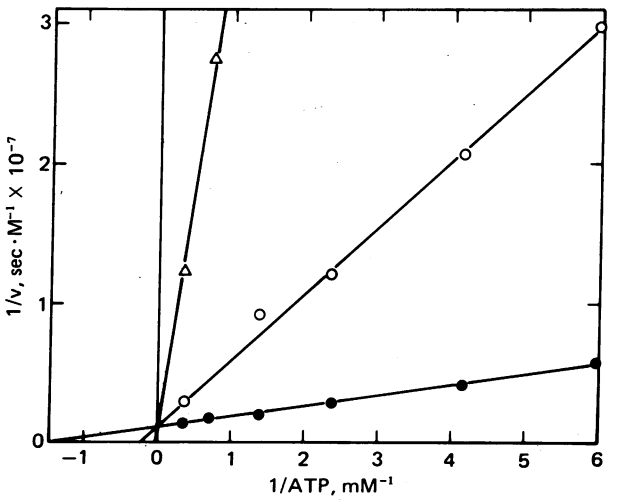

FIG. 3. Competitive inhibition of the ATPase activity of recA protein by ATPγS. The ATPase activity was assayed as described (12) except that the incubation was stopped at 12 min. Reaction mixtures, 18 μl in volume, contained 50 μM single-stranded DNA of phage φX174, 6.7 mM $MgCl_2$, 35 mM Tris·HCl (pH 7.5), 2 mM dithiothreitol, 100 μg of bovine serum albumin per ml, 1.4 mM [^{3}H]ATP, 2.2 μM recA protein, and ATPγS as indicated. ●, No ATPγS; ○, 15 μM ATPγS; △, 100 μM ATPγS.

trophotometrically. Reaction mixtures containing ATPγS included controls lacking either recA protein or single-stranded DNA. None of these differed significantly in the amount of ADP produced, as measured by the ultraviolet absorption spectrum of the extracted material. From the chromatogram of the reaction mixture containing ATP, we recovered a sample of 13 μM ADP. In the reaction mixtures containing ATPγS, we would have detected an increase of ADP relative to controls of amounts in excess of 1/10th of that produced from ATP. We conclude that recA protein hydrolyzed ATPγS at a rate that was less than 1/10th of that for ATP.

Binary Complexes of recA Protein and DNA. To detect complexes of recA protein and DNA, we used nitrocellulose filters under two conditions as described in *Methods*. When recA protein was incubated with double-stranded DNA and ATP in the absence of homologous single-stranded fragments, only a few percent of double-stranded DNA was retained by nitrocellulose filters in assay D (ref. 12 and Table 1, lines 1 and 7). However, when ATPγS replaced ATP, recA protein caused the retention of a large fraction of either form I or form II DNA by the filters (Table 1, lines 2, 5, 6, and 8). These complexes resisted heating at 50°C for 4 min in 1.5 M NaCl/0.15 M Na citrate, a treatment that is part of standard assay D, but complexes were dissociated by alkaline pH (Fig. 4) or by Sarkosyl (Table 2).

These observations show that ATPγS causes recA protein to form very stable complexes with either form I or form II DNA. When such complexes were made with form I DNA, there was no change in the fraction of closed circular DNA, as measured by alkaline denaturation and renaturation (Fig. 4, open circles). In addition, Cunningham *et al.* (1) have shown that recA protein lacks any topoisomerase activity under the same conditions. We found no evidence even for transient covalent linkage of recA protein and double-stranded DNA; thus, the stable complexes described here probably involve noncovalent bonds.

To detect the binding of recA protein to single-stranded DNA, we looked for conditions under which nitrocellulose filters would not trap single-stranded DNA alone. In assay C, nitrocellulose filters retained neither single-stranded DNA nor double-stranded DNA of phages fd and φX174 (Fig. 5 a and b). Single-stranded DNA passed through these filters even when the concentration of NaCl was as high as 1 M. However, when incubated with recA protein and ATPγS, 100% of single-stranded DNA was trapped by the filter (Fig. 5e). These complexes were stable in 0.2 M NaCl (Fig. 5f). Some 30% of single-stranded DNA was retained when it was incubated with recA protein in the absence of ATP or ATPγS (Fig. 5c). These complexes, by contrast with those formed in the presence of ATPγS, were 80–90% dissociated by 0.2 M NaCl (Fig. 5d).

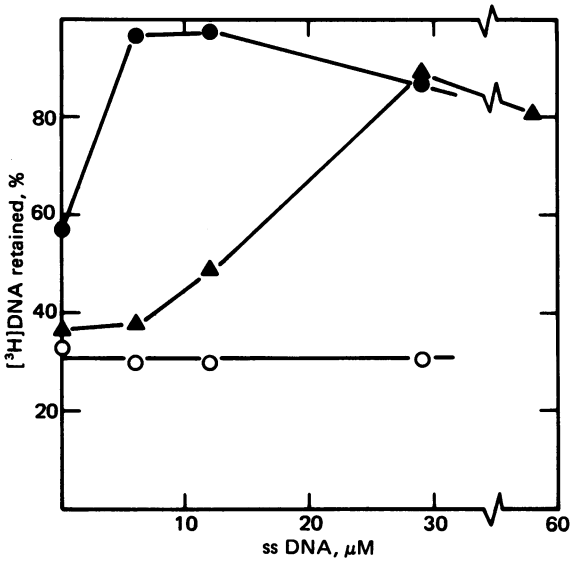

FIG. 4. Formation of complexes of recA protein and DNA. In the standard reaction mixture, ATP was replaced by 0.5 or 1.3 mM ATPγS. ●, 8.8 μM double-stranded fd [³H]DNA (70% form I and 30% form II DNA) and fragments of single-stranded fd DNA at the concentrations indicated; complexes were detected by assay D. ○, Samples from the above experiment were assayed for nicked circular DNA by the method of Kuhnlein *et al.* (20). ▲, 8.8 μM fd form II [³H]DNA and oligodeoxynucleotides at the concentrations indicated; complexes were detected by assay D except that the nitrocellulose filter was a Millipore filter with a pore size of 0.65 μm.

Ternary Complexes of recA Protein and DNA. By both assays D and C, we observed the formation, in the presence of ATPγS, of ternary complexes of recA protein, double-stranded DNA, and single-stranded DNA. Under the conditions of assay D, fragments of single-stranded DNA increased the retention of double-stranded DNA (Fig. 4 and Table 1). The effectiveness of heterologous single-stranded fragments (Table 1, line 4) indicated that most of the complexes formed in the presence of ATPγS were not D loops, an inference that we confirmed by experiments described below (Table 2).

The binary complexes of recA protein and double-stranded DNA (Fig. 4 and Table 1) were not efficiently trapped under the conditions of assay C (Fig. 5h). However, all of the double-stranded DNA was retained when the reaction mixture contained single-stranded fragments in addition to recA protein

Table 1. Complexes of recA protein and DNA formed in the presence of ATPγS

ss DNA	Triphosphate	recA protein	% fd ds DNA trapped
fd form I			
1. None	ATP	+	2
2. None	ATPγS	+	53
3. fd	ATPγS	+	77
4. φX	ATPγS	+	92
5. fd	ATPγS	None	1
6. fd	None	+	1
fd form II			
7. None	ATP	+	2
8. None	ATPγS	+	33
9. fd	ATPγS	+	94

The concentration of $MgCl_2$ was 6.7 mM. Complexes were detected by assay D. ss DNA, single-stranded DNA; ds DNA, double-stranded DNA.

Table 2. ATPγS inhibits formation of D loops*

Treatment	ATP +fd (a)	ATP +φX (b)	ATPγS +fd (c)	ATPγS +None (d)
None	29	2.3	87	76
Sarkosyl (0.5%)	29	2.0	2.4	0.9

The concentration of $MgCl_2$ was 25 mM. To eliminate complexes of recA protein and DNA, we diluted a sample of the mixture, after incubation, in an equal volume of 25 mM EDTA, added 1/20 vol of 10% Sarkosyl, and incubated the sample at 37°C for 5 min more. The rest of the procedure was that of the standard assay D.

* Values are the percent of fd form I [³H]DNA trapped in the presence of ATP or ATPγS and the indicated single-stranded DNAs.

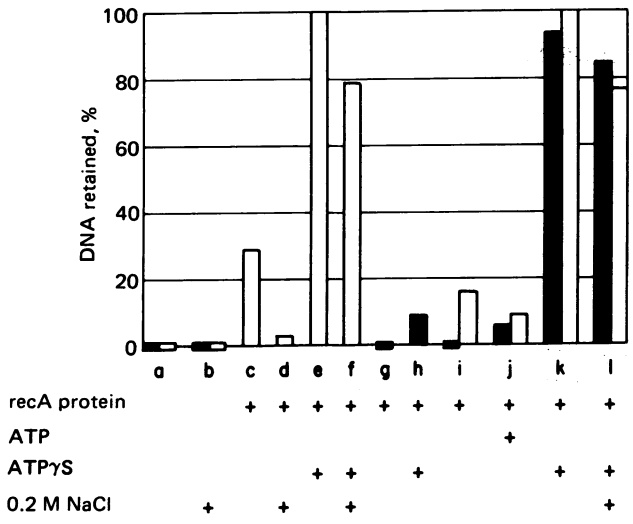

FIG. 5. Formation of complexes of recA protein and DNA. Reaction mixtures contained, in 20.5 μl, 31 mM Tris·HCl (pH 7.5), 6.7 mM $MgCl_2$, 1.8 mM dithiothreitol, 88 μg of bovine serum albumin per ml, and the following ingredients as indicated: 2.4 μM recA protein, 8.8 μM fd form I [^{3}H]DNA, 12 μM fragments of fd single-stranded [^{32}P]DNA, 1.3 mM ATP, and 0.5 mM ATPγS. Incubation was at 37°C for 30 min. Complexes were trapped on nitrocellulose filters under the conditions of assay C. Open bars represent single-stranded DNA; closed bars represent double-stranded DNA. A pair of bars with no space between them indicates that both kinds of DNA were in the reaction mixture.

and ATPγS (Fig. 5k). Again, heterologous fragments were as effective as homologous fragments in causing the trapping of double-stranded DNA: 74% of fd form I DNA was retained in the presence of recA protein, ATPγS, and fragments of single-stranded φX174 DNA.

Using a modification of assay D (namely, nitrocellulose filters with a pore size of 0.65 μm), we observed that oligodeoxynucleotides also stimulated the trapping of double-stranded DNA by nitrocellulose filters in the presence of recA protein and ATPγS (Fig. 4). Under these conditions, less than 1% of the oligodeoxynucleotides themselves were retained when they were incubated with only recA protein and ATPγS. The optimal concentration of oligodeoxynucleotides was similar to that required for the stimulation of partial unwinding of duplex DNA by recA protein (ref. 1 and unpublished observations).

When ATP was the cofactor in the reaction of recA protein with fd form I DNA and fd single-stranded fragments, neither the D loops that were presumably formed nor single-stranded DNA was trapped by nitrocellulose filters in assay C (Fig. 5j; see *Discussion*).

ATPγS Inhibits Formation of D Loops. D loops formed by recA protein were not dissociated by treatment with concentrations of sodium dodecyl sulfate or Sarkosyl that did not interfere with the assay (ref. 12 and Table 2, column a). When we mixed form I DNA and homologous single-stranded fragments with ATPγS in 25 mM $MgCl_2$ (Table 2, column c), all of the complexes formed were eliminated by treatment in 0.5% Sarkosyl, which shows that the complexes made in the presence of ATPγS were not D loops.

When we added both ATP and ATPγS to the reaction mixture containing form II DNA, and treated with Sarkosyl after incubation, we found little or no D-loop formation, which shows that ATPγS inhibits the formation of D loops when the analog competes with ATP.

DISCUSSION

Observations on partial reactions that occur in the presence of the ATP analog, ATPγS, as well as observations on the complete reaction that occurs in the presence of ATP, help us to picture how recA protein catalyzes the homologous pairing of single-stranded and double-stranded DNA to form a D loop (Fig. 1). ATPγS, which is not hydrolyzed at an appreciable rate, competitively inhibits the ATPase activity of recA protein. The latter observation suggests that ATP and ATPγS bind to overlapping sites. Therefore, we start from the premise that the partial reactions seen in the presence of the analog reflect the normal mechanism of recA protein up to the hydrolysis of ATP. We can organize our observations and interpretations under three rubrics:

***(i)* The Binding of ATP Activates recA Protein.** (See 1 → 2, Fig. 1.) In the presence of ATPγS, recA protein binds single-stranded DNA, forming a stable complex that is efficiently retained by a nitrocellulose filter. Because the filter is only an empirical tool, increased trapping by the filter may mean that ATPγS changed the affinity of recA protein for single-stranded DNA or that ATPγS changed the size or shape of the complex. The increased stability of the complexes in 0.2 M salt, which is independent of the properties of the filter, suggests that ATPγS changed the affinity of recA protein for single strands. Moreover, any of these explanations implies that the binding of ATP changes the conformation of recA protein.

***(ii)* The Binding of Single-Stranded DNA to Activated recA Protein Governs Its Interaction with Double-Stranded DNA.** (See 2 → 3 → 4, Fig. 1.) In the presence of ATPγS, either single strands or oligonucleotides caused recA protein to bind all of the double-stranded DNA in stable complexes that were trapped by nitrocellulose filters (Fig. 5 k and l). In experiments described elsewhere (1), we found that DNA ligase could seal more than half of form II DNA in such complexes and that when protein was removed all of the sealed DNA was negatively superhelical, which indicated that recA protein had partially unwound the duplex DNA. Both the binding of double-stranded DNA (Fig. 5) and its partial unwinding by recA protein (1) are strongly stimulated by either homologous or heterologous single strands. The dissociation of these complexes by detergent (Table 2), including those formed by homologous DNA, shows that single-stranded and double-stranded DNA are not extensively base-paired to one another. Thus, if we accept the premise stated at the beginning of this discussion, the complex that contains single-stranded DNA and partially unwound double-stranded DNA represents an intermediate that is formed prior to homologous pairing. The accumulation of these complexes may be attributable to the ability of recA protein to bind ATPγS without hydrolyzing it. We have interpreted the formation of these complexes as evidence that recA protein, stimulated by single strands, unwinds duplex DNA to enable a search for homology (1).

There is also evidence that single-stranded DNA governs the interaction of recA protein with double-stranded DNA in the presence of ATP, which supports the complete reaction. The amount of recA protein required to form D loops was directly proportional to the amount of single-stranded DNA present, but was unaffected by similar variations in the concentration of double-stranded DNA (Fig. 2). The stoichiometry, one molecule of recA monomer per 5–10 nucleotide residues of single-stranded DNA, and the failure to form any D loops until there is nearly that optimal amount of recA protein suggest that the single-stranded DNA must be completely covered with protein. The stoichiometric relationship between the amount of recA protein and the amount of single-stranded DNA is a sufficient explanation for the nonlinear effect of recA protein.

***(iii)* Homologous Pairing of Single-Stranded DNA with Double-Stranded DNA and Detachment of recA Protein Entail Hydrolysis of ATP.** (See Fig. 1.) When ATP is present, recA protein forms D loops, but neither these nor single-stranded DNA was retained by nitrocellulose under conditions that trapped complexes made in the presence of ATPγS (Fig. 5j). Thus, the association of recA protein with DNA is either weakened or qualitatively altered when ATP is hydrolyzed. More interesting is the possible role of ATP hydrolysis in the promotion of base-pairing. As described above, when hydrolysis is blocked by the use of an ATP analog, the single-stranded DNA need not be homologous to promote the binding and unwinding of double-stranded DNA by recA protein, and bound homologous DNA molecules are not extensively base-paired to each other. If these complexes reflect a normal intermediate in the formation of D loops, then single-stranded and double-stranded DNA must move relative to each other or the complexes must be formed and dissociated reiteratively until a homologous match occurs. The hydrolysis of ATP might drive either kind of motion.

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