Kinetic Mechanism for the Formation of the Presynaptic Complex of the Bacterial Recombinase RecA*

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RecA protein from Escherichia coli catalyzes DNA strand exchange during homologous recombination in a reaction that requires nucleoside triphosphate cofactor. In the first step of this reaction RecA protein polymerizes on single-stranded DNA to form a filament with a stoichiometry of three nucleotides/RecA monomer called the presynaptic complex. We have used fluorescence anisotropy of a fluorescein-labeled oligonucleotide to investigate presynaptic complex formation. RecA-ATPγS bound to oligonucleotide by a two-step process. Kinetic studies revealed an intermediate in the polymerization reaction that had greater mobility than the final product filament. The intermediate was transformed into the final product by a process that was independent of filament concentration and temperature at 25 °C. In whole cells, the intermediate is a transient dissociation of the filament (17–19). Furthermore the use of oligonucleotide substrates can destabilize nucleoprotein filaments by reducing the number of protein-protein interactions. Here we use the dissociation-reassociation reaction and oligonucleotide nucleoprotein filaments to investigate the mechanism of presynaptic complex formation.

Homologous recombination of DNA is a universal biochemical process required for chromosomal segregation and certain mechanisms of DNA replication and repair (1). Recombinases act in nucleoprotein filaments whose essential features have been conserved from phage to man (2, 3). The most thoroughly studied recombinase, Escherichia coli RecA protein, carries out the principle reactions of homologous recombination in vitro (2, 4). RecA protein has two functional DNA binding sites (5). In the first step of the reaction, multiple RecA monomers bind ssDNA at one site of the protein to form a right-handed helical polymer, 3 nt per RecA monomer, known as a presynaptic complex. In the presence of ATP, this filament binds dsDNA, and if the DNA sequences are complementary, strand exchange occurs.

RecA protein catalyzes DNA-dependent ATP hydrolysis uniformly throughout the nucleoprotein filament (6). The presynaptic complex is necessary and sufficient for ATPase activity, even though this filament can bind additional ssDNA or dsDNA (7). The effect of ATP hydrolysis on the DNA strand exchange reaction carried out by RecA protein has been investigated using stable ATP analogs such as ATPγS (8, 9) or the mutant RecA protein K72R (10, 11). In these conditions the presynaptic filament binds but does not hydrolyze cofactor. This filament nevertheless carries out limited DNA strand exchange and is considered to reproduce some of the early steps of homologous recombination. In particular, binding of ATP or ATPγS produces a filament in which DNA is extended (5.1 Å per base pair) and underwound (18 base pair/turn) (12–14). Concomitantly ATP or ATPγS increase the stability of the nucleoprotein filament, while ADP destabilizes the filament relative to no cofactor (15).

The mechanism by which RecA protein forms the presynaptic complex is poorly understood. Equilibrium binding studies have been carried out for the reaction of RecA with ssDNA in the absence of cofactor (15, 16). However, equilibrium constants and rate constants for formation of the presynaptic complex with ATP or ATPγS have not been investigated in part because of the high stability of the filament in the presence of cofactor. It has been reported that addition of nucleoside triphosphate cofactor to a 3/1 nt/RecA filament may induce transient dissociation of the filament (17–19). Furthermore the use of oligonucleotide substrates can destabilize nucleoprotein filaments by reducing the number of protein-protein interactions. Here we use the dissociation-reassociation reaction and oligonucleotide nucleoprotein filaments to investigate the mechanism of presynaptic complex formation.

We have recently developed fluorescence anisotropy methods to study the binding of oligonucleotide to the presynaptic filament in the presence of ATPγS (20). The anisotropy of a 5′-fluorescein-labeled oligonucleotide increases as protein binds to DNA. Control experiments show that the 5′-fluorescein label does not affect the DNA strand exchange reaction and that the spectroscopic signal reflects the properties of the nucleoprotein filament rather than the local behavior of the fluorophore. We apply this method to study the presynaptic complex.

EXPERIMENTAL PROCEDURES

Fluorescein-labeled oligonucleotide, 5′-F CCA TCC GCA AAA ATG ACC TCT TAT CAA AAG GA where F is fluorescein, was synthesized by Genosys. RecA protein and M13 DNA were purified, and concentrations were determined as described (20, 21). RecA concentration units are M protein monomer; oligonucleotide concentrations are M nt. TMDG buffer is 20 mM Tris acetate, 2 mM Mg(CH3COO)2, 1 mM dithiothreitol, and 5% glycerol, pH 7.5.

Unless otherwise stated, nucleotide filaments were prepared with stoichiometry of 3/1 nt/RecA protein in TMDG buffer at 25 °C ± 1 °C; concentration units of the filament are M nt. In a typical experiment, nucleoprotein complex in the absence of cofactor was prepared and equilibrated in the cuvette, cofactor was added at the beginning of the reaction, t = 0 s, and fluorescence signal followed for 1000 s. Manual mixing time for addition of cofactor was 15 s.
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**Fig. 1.** Spectroscopic changes caused by addition of nucleotide cofactor to the nucleoprotein filament formed by RecA protein and fluorescein-labeled oligonucleotide. 1.0 μM nt and 0.33 μM RecA protein were incubated at 25 °C in TMDG buffer. 100 μM nucleotide cofactor or buffer was added immediately before the time designated as t = 0 s, and the signal followed as a function of time. a and b, upper curve, buffer; lower curve, ADP. c and d, upper curve, ATP+S; lower curve, ATP added to 1.0 μM nt and 0.33 μM RecA protein; middle curve, ATP added to 4.8 μM nt and 1.6 μM RecA protein. a and c, fluorescence anisotropy; b and d, fluorescence quenching.

Fluorescence experiments were performed using a model MOS-400 spectrophotometer from Bio-Logic (Claix, France). Measurements were taken of samples in a 1.0 × 0.4-cm cuvette stirred continuously at the indicated temperature ± 1 °C. To maximize sensitivity, a single polarizer configuration was used to determine fluorescence anisotropy, A, and fluorescence intensity, Fl (V) (22). Fluorescence is reported as quenching, Q = (Fl/Flo) where Flo is the signal from oligonucleotide alone. Samples were excited at 490 nm, and total emission intensity was measured at 1-s intervals using a 515-nm cutoff filter.

**RESULTS**

**Dissociation of Nucleoprotein Filament by ATPγS—**We first wished to show that dissociation and reassociation of the RecA nucleoprotein filament can be studied by fluorescence anisotropy. The fluorescence signals of various complexes of 1.0 μM 5'-fluorescein 32-mer oligonucleotide and 0.33 μM RecA protein are shown in Fig. 1. Fluorescence anisotropy of free oligonucleotide in the absence of RecA was A = 0.05 ± 0.005. Anisotropy of the 3/1 nt/RecA complex without cofactor was A = 0.19 ± 0.005 and fluorescence quenching was 0.3 ± 0.05. Addition of ADP to this complex decreased the anisotropy to 0.05, the same value as free oligonucleotide; fluorescence quenching decreased to 0.025, and these spectroscopic signals did not change with time. NaCl decreased the anisotropy and fluorescence quenching of the 3/1 complex of oligonucleotide and RecA protein in absence of cofactor, while the signal of the corresponding filament in the presence of ATPγS was stable in 800 mM salt (not shown). ADP is known to destabilize the RecA protein-DNA complex, while ATPγS stabilizes it with respect to salt denaturation (15). Hence these fluorescence signals measure known effects of cofactor and salt on the formation and stability of the 3/1 nt/RecA nucleoprotein filament.

When ATPγS was added to the filament without cofactor, the anisotropy signal decreased. In this experiment, the intensity at t = 0 s, which is defined as the beginning of measurement after mixing, was A = 0.12 and signal gradually increased over 15 min to A = 0.19 (Fig. 1c). In the same experiment quenching decreased immediately after addition of ATPγS to 0.2; signal then increased for about 50 s to Q = 0.26 and thereafter slowly decreased to Q = 0.22. After addition of ATP, the anisotropy and fluorescence signals initially decreased; signals then increased for about 50 s in these experiments and subsequently decreased. The time-dependent spectroscopic signals observed for the reaction with ATP are consistent with a combination of cofactor binding followed by hydrolysis to ADP.

To further understand the kinetic effects brought about by nucleoside triphosphate binding (separate from its hydrolysis), we investigated the reaction with ATPγS. If the decrease in fluorescence anisotropy produced by the addition of ATPγS (Fig. 1c) was caused by cofactor-induced dissociation, the resulting free RecA protein should react with competitive nonfluorescent DNA. To test this hypothesis, various concentrations of M13 phage ssDNA were added with ATPγS at the beginning of the reaction (Fig. 2). The decrease in the anisotropy and fluorescence quenching signals at t = 0 s caused by addition of ATPγS was not influenced by 0–4 μM competitor DNA. However, the recovery of fluorescence signal was inhibited in a concentration-dependent manner. Adding 10 μM nonfluorescent ssDNA at the end of the reaction caused a small decrease in the anisotropy and no change in the quenching (not shown), indicating that once it is formed the ATPγS-RecA nucleoprotein filament was not significantly perturbed by competitor DNA. These results support the hypothesis that addition of ATPγS dissociated protein molecules from the 3/1 oligonucleotide/RecA complex.

The anisotropy increase following this dissociation (designated t > 0 s, Fig. 1c) could be caused by reassociation of RecA protein with DNA. To test this possibility, we compared two reactions (Fig. 3). First, addition of cofactor to a 3/1 nt/RecA complex led to a decrease in the anisotropy followed by recovery of signal as in Fig. 1c. Second, for the same concentrations of reactants, RecA protein and ATPγS were added to fluorescent oligonucleotide. For this reaction, the anisotropy of the oligonucleotide alone was 0.05 and signal increased after addition of
RecA as protein bound to oligonucleotide. The increased anisotropy and fluorescence quenching changes were the same for the two reactions. These results were confirmed in eight independent experiments using 3/1 nt/RecA complexes made with 0.5–10 μM nt. Hence the increase in fluorescence anisotropy observed after addition of cofactor corresponds to the reaction of RecA-ATPγS with fluorescent oligonucleotide.

**Dependence of the Reaction on ATPγS Concentration**—The kinetics of the dissociation-reassociation reaction were unchanged for ATPγS concentrations above 15 μM. To study the requirement of the reaction for ATPγS, 0–10 μM ATPγS were added to a 3/1-nt/RecA complex, 1 μM nt (Fig 4). The anisotropy at t = 0 s decreased from A = 0.2 to A = 0.15 as ATPγS concentrations increased from 0 to 2 μM. Binding of RecA protein to oligonucleotide was greater without ATPγS or for ATPγS concentrations > 10 μM than for intermediate concentrations.

Low concentrations of ATPγS would be expected to produce a mixture of RecA molecules with and without ATPγS. These results show that binding of such a mixed population of RecA molecules to single-stranded DNA was qualitatively different than for either species by itself (see “Discussion”).

**Dependence of the Reaction on Filament Concentration**—Complexes of 3/1 nt/RecA containing from 0.5 μM to 10 μM oligonucleotide were prepared, ATPγS was added, and the anisotropy and fluorescence quenching were observed as a function of time (Fig 5). The effect of filament concentration on several features of the anisotropy kinetic curves is shown in Fig 6a. In absence of ATPγS, 3/1 RecA nt formed a complex at high filament concentrations with A = 0.21 ± 0.005 and Q = 0.35 ± 0.05 (designated t < 0 s, Fig 5; circles, Fig 6a). These spectroscopic values correspond to a complex in which fluorescent oligonucleotide is saturated by RecA protein (20). Spectroscopic signals of this complex decreased for oligonucleotide concentrations below 2.0 μM. The simplest explanation of this observation would be that oligonucleotide and protein dissociate at low concentrations of filament.

The intensity of the signals immediately after addition of ATPγS, designated t = 0 s in Fig 5, increased with filament concentration and remained constant above 2.5 μM complex: A = 0.17 ± 0.01, Q = 0.30 ± 0.05 (Fig 6a, squares). At all concentrations the value of anisotropy was significantly less than the signal observed for the reactant or the product complexes. These results show that reassociation after addition of ATPγS occurred in two kinetically distinct steps. A rapid reac-

![Fig. 3: Reassociation of RecA protein and oligonucleotide. Dark curve, ATPγS was added to a mixture of 0.5 μM oligonucleotide 0.167 μM RecA at t = 0 s. Light curve, a mixture of 0.167 μM RecA and ATPγS was added to a 0.5 μM oligonucleotide at t = 0 s. Reaction conditions are as in Fig 1.](image1)

![Fig. 4: Effect of ATPγS concentration on reassociation kinetics. 1 μM oligonucleotide and 0.33 μM RecA protein were incubated at 25 °C in TMG buffer. The indicated concentrations of ATPγS were added at 0 s, and anisotropy followed as a function of time.](image2)

![Fig. 5: Effect of filament concentration on fluorescence anisotropy. Complexes of (i) 0.5 μM, (ii) 1 μM, (iii) 2 μM (light curve), or (iv) 3 μM fluorescent oligonucleotide were equilibrated with RecA protein, 3 nt/protein molecule; 100 μM ATPγS was added at t = 0 s. Reaction conditions as in Fig 1. a, fluorescence anisotropy; b, fluorescence quenching.](image3)
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Fig. 6. a, anisotropy values of different parts of the kinetic curves in Fig. 5a. Circles, prior to addition of ATP·S, t < 0 s; triangles, t = 1000 s, b, dissociation of the presynaptic filament in presence of ATP·S. •, dilution of 4.8 μM nt 3/1 nt/RecA complex, 100 μM ATP·S, into TMDG buffer with cofactor; ■, titration of buffer and cofactor with 3/1 nt/RecA complex, 4.8 μM nt.

Table I

| Timea | Speciesb | Fluorescence quenching | Anisotropy |
|-------|----------|------------------------|------------|
| t < 0 s | RF       | 0.35 ± 0.05            | 0.21 ± 0.005 |
| t = 0 s | RGF      | 0.30 ± 0.05            | 0.17 ± 0.01 |
| t = 1000 s | RGF     | 0.23 ± 0.05            | 0.21 ± 0.005 |

a Time at which spectroscopic parameters were determined from kinetic curves (Fig. 5a) for filament concentration > 2.5 μM nt.
b Abbreviations for various RecA oligonucleotide complexes (Fig. 8).  

The nucleoprotein complex formed in the absence of ATP·S appeared to be unstable at low MgCl₂ concentrations. Addition of 0.167 μM RecA protein to 0.5 μM fluorescent oligonucleotide at 25 °C in 20 mM Tris buffer, pH 7.4, and no MgCl₂ initially produced a filament with A = 0.16 and Q = 0.3. The anisotropy and fluorescence quenching subsequently decreased during 15 min to A = 0.07, Q = 0.12. The decrease was less apparent at higher filament concentration; e.g. for a 2 μM complex, spectroscopy signals decreased less than 10%. Similar instability was also apparent under certain conditions in standard buffer. For example, a slow decrease of anisotropy from A = 0.18 to

A = 0.1 was observed in TMDG buffer for 3/1 nt/RecA complexes with 1 μM nt at 37 °C (Fig. 7a) but not at 25 °C (Fig. 1a).

The effect of low MgCl₂ concentration was reversible. Addition of 15 mM Mg(CH₃COO)₂ 20 min after incubation of the complex at 25 °C in the absence of MgCl₂ restored the 3/1 complex as well as the ATP·S-induced dissociation and reassociation reactions. The instability of the 3/1 complex in the absence of cofactor and MgCl₂ may be due to slow aggregation of free protein into a nonproductive complex as previously reported (24). It is worth noting that high MgCl₂ concentrations dissociate the presynaptic complex (20).

Addition of NaCl favored the ATP·S-induced dissociation reaction (Fig. 7b, t = 0 s) as expected from known salt effects on filament stability (15, 20). NaCl also inhibited the reassociation reaction with 50% inhibition observed for 250 mM NaCl. In contrast, addition of these concentrations of NaCl at the end of the reaction did not greatly decrease the anisotropy of product filament, as previously reported (15); for example, addition of 1 μM NaCl to the product filament with 1 μM nt decreased the anisotropy from 0.18 to 0.14. Hence salt concentrations that inhibited formation of the ATP·S-RecA-oligonucleotide complex did not destabilize the complex once it was formed. Similar results were observed using etheno-modified M13 DNA (not shown).

Kinetic Analysis—The preceding results indicate that a mechanism for the reaction of ATP·S with the 3/1 nt/RecA filament would include at least the following elements (Fig. 8). i) Prior to addition of ATP·S, free RecA protein (R) and fluorescent oligonucleotide (F) are in equilibrium with the nucleoprotein complex (RF) (reaction 1). ii) The slow dissociation of RF, which was observed in the absence of MgCl₂, indicates a competitive reaction in which free RecA formed aggregates (24) that can not bind to the oligonucleotide (reaction 2). iii) Reaction of ATP·S with free RecA dissociates the 3/1 nt/RecA filament (reaction 3, see "Discussion"). iv) Subsequently RecA-ATP·S (RG) binds to the oligonucleotide to form a stable reaction intermediate RG·F (reaction 4). v) The product of reaction 4 is slowly transformed into the final product RG·F (reaction 5). At low filament concentrations, an equilibrium was also observed between free RecA protein and product filament in the presence of ATP·S (Fig. 6b).

Time-dependent spectroscopic signals were analyzed in an
attempt to determine the rate constants for the association reactions 4 and 5. Kinetics were studied for addition of ATPγS to 1 μM filament. Reaction 5 was approximated as a first order reaction. Rate constants were determined for the mechanism shown in Fig. 9a, which further assumes that the binding of RecA is a two-step reaction between free protein, Rg, and oligonucleotide, F. Kinetic equations were solved numerically (25)

\[
F(t) = \sum c_i(t)q_i \\
A(t) = \frac{\sum c_i(t)A_i}{\sum c_i(t)q_i} \\
(Eq. 2)
\]

where \(A(t)\) and \(F(t)\) are the time-dependent anisotropy and fluorescence. \(c_i(t)\) are the concentrations of fluorescent species \(i\) (μM nt) calculated from the equation in Fig. 9A. \(A_i\) and \(q_i\) are the anisotropy and effective quantum yields of each species; the latter was approximated by fluorescence intensity (25). Calculations were carried out as follows. i) Initial values of \(A_i\) and \(q_i\) were determined from steady state parameters, Table I; the spectroscopic signals of free oligonucleotide were kept constant, \(A = 0.05, F = 0.375 V\). ii) Rate constants for the forward and reverse steps of reaction 4, \(k_{4f}\) and \(k_{4r}\), and for the first order reaction 5 were adjusted to visually fit the experimental data (Fig. 9B, Table II).

Good agreement between experimental and theoretical curves was observed for data \(t > 150 s, k_0 = 0.36 \text{ min}^{-1}\). The rate constant for the second step of the reaction was confirmed by nine additional experiments in which ATPγS was added to 3/1 nt/RecA complexes containing 2–8 μM filament, conditions where only this reaction was observed (Fig. 6a); anisotropy kinetic curves in these experiments were identical, indicating a reaction mechanism that was independent of filament concentration. These curves could be fit by a single exponential with average rate ± S.D., \(k_5 = 0.4 \pm 0.1 \text{ min}^{-1}\). A concentration-independent rate constant indicates that neither protein binding nor aggregation contributes significantly to the time-dependent spectroscopic signals. Hence the increased anisotropy and fluorescence that occur during the second step of the reaction (Fig. 9) appear to be caused by a process other than protein binding, such as a conformational change (see “Discussion”). The rate constant for the second step of the reaction was independent of temperature between 25 °C and 37 °C (Table III).

Fit of the early part of the reaction was poor, and rate constants were not consistent with \(K_0\) obtained from the dissociation of the filament at low concentrations (Fig. 6b). The DNA binding reaction 4 probably needs to be represented by another mechanism than a simple two-state process. For example, the reaction of RecA protein with ssDNA can be analyzed in the general context of cooperative binding between a ligand with a finite binding site size and a one-dimensional lattice (26, 27). This formalism shows that binding is a function of the size of the binding site, cooperative interactions, and effects of oligonucleotide extremities as well as the association

**Discussion**

*E. coli* RecA protein forms a nucleoprotein filament with ssDNA in the presence of ATPγS, 3 nt/RecA monomer, which is able to carry out the initial steps of homologous recombination in vitro and is considered to be a good model for the presynaptic complex (8, 9). We have investigated this RecA nucleoprotein filament using the fluorescence anisotropy of a 5′-fluorescein-labeled 32-mer oligonucleotide. This method is sensitive to hydrodynamic properties of the fluorophore (28), and consequently the fluorescence anisotropy of the oligonucleotide increases with RecA binding (20). Here we demonstrate that addition of nucleoside triphosphate to the nucleoprotein filament can cause its dissociation under some conditions as previously suggested from biochemical experiments (18, 19). Furthermore, the spectroscopic measurements showed a two-step

**TABLE II**

| Species | \(q_i(V)\) | Fluorescence quenching | Anisotropy \(A_i\) | Rate constant |
|---------|-------------|------------------------|-------------------|--------------|
| F       | 0.375       | 0.0                    | 0.05              |              |
| RgF     | 0.266       | 0.29                   | 0.165             |              |
| RgF'    | 0.285       | 0.25                   | 0.191             |              |
| \(k_{4f}\) |            |                        | \(21 \text{ (μM nt min)}^{-1}\) |              |
| \(k_{4r}\) |            |                        | 0.18 min^{-1}     |              |
| \(k_5\)  |            |                        | 0.36 min^{-1}     |              |

*Abbreviations for various RecA oligonucleotide complexes (see Fig. 8).*

**FIG. 9.** Kinetic analysis. A, two-state mechanism for the association reaction. Abbreviations are as in Fig. 8. B, kinetic analysis of the association reaction. Anisotropy (a) and fluorescence (b) changes after addition of ATPγS to 0.33 μM RecA protein, reaction conditions are as in Fig. 1; symbols, average values of from 11 independent experiments; lines, theoretical curves for parameters Table II (see “Results”). Residuals for the fit of the anisotropy (c) and fluorescence data (d).

**FIG. 8.** Proposed reaction mechanism for the cofactor-induced dissociation and reassociation of the 3/1 nt/RecA filament. R, RecA; F, fluorescent oligonucleotide; RF, 3/1 nt/RecA complex in absence of cofactor; Rg, RecA-ATPγS complex; RgF and RgF', 3/1 nt/RecA complexes in presence of cofactor.
association reaction between RecA and ssDNA. The most important result of this study is the observation of a previously unknown intermediate in the polymerization of RecA protein on ssDNA that we will attempt to define in this Discussion. Polymerization of RecA protein on the oligonucleotide in the presence of ATP·S took place in two steps. In the first, fluorescence was quenched by 0.30 ± 0.05 and fluorescence anisotropy increased from 0.05 to 0.17 ± 0.005 (Fig. 6a, Table I). Kinetics of this reaction could be observed below filament concentrations of 2 μM nt; at higher concentrations the reaction was complete in the mixing time of the experiments, 15 s (Figs. 5, 6a). In the second step of the reaction the anisotropy increased to 0.21 ± 0.005 and fluorescence quenching decreased to 0.23 ± 0.05. The rate of this reaction was independent of filament concentration (Fig. 5) and temperature (Table III), $k_3 = 0.3 ± 0.1$ min$^{-1}$. Concentration-independent kinetics indicate that neither aggregation nor protein binding contribute significantly to the reaction.

The fluorescence measurements give some information about the relative physical properties of the reaction intermediate and the product. Both filaments appear to have quantitatively bound RecA protein at filament concentrations above 2 μM (Fig. 6a). However, the intermediate is more hydrodynamically mobile judging from its lower anisotropy (28). RecA protein is known to extend the nucleoprotein filament (12-14). An increased mobility of the intermediate could be explained if it had a smaller persistence length than the product filament.

Low concentrations of ATP·S decreased the anisotropy of the nucleoprotein filament, while higher concentrations of ATP·S restored anisotropy (Fig. 4). The maximum decrease was observed for 2 μM ATP·S. In these conditions the anisotropy of the complex was reduced to $A = 0.15 ± 0.01$, which corresponds to the anisotropy of the polymerization intermediate described above (Fig. 6a). This filament was stable for at least 15 min. Although the equilibrium constants for the reaction between RecA protein and nucleotide cofactors are not known, sufficiently low concentrations of the nucleoside triphosphate should give two populations of RecA protein, with and without ATP·S. Hence, these results suggest that the intermediate filament formed during the polymerization reaction may have a conformation similar to a mixed filament containing RecA molecules with and without ATP·S. The low anisotropy of this latter filament could be explained if relatively rigid segments of polymerized RecA (R) and RecA-ATP·S (Rg) were separated by flexible “hinges” caused by the incompatible protein-protein interactions between R and Rg monomers (29, 30). At high ATP·S concentrations, cofactor would bind to all protein and the more rigid product filament would form via reaction 5 (Figs. 4 and 8). This reaction is apparently inhibited in a filament containing a mixed population of RecA monomers with and without cofactor.

These considerations suggest that a rearrangement takes place in the slow step of the polymerization reaction that increases the persistence length of the filament (reaction 5 in Fig. 8). Reported kinetics of ATP hydrolysis suggest a possible mechanism for this process. Using a combination of intrinsic protein fluorescence and ATP hydrolysis measurements, Paulus and Bryant demonstrated a two step mechanism for ATP hydrolysis in similar reaction conditions to those used in our experiments (31). Rapid binding of ATP is followed by rate-limiting “isomerization” of the filament. When ATP·S is used in the reaction, isomerization is observed without hydrolysis. ATP·S hydrolyzes in these conditions but the rate, $k = 0.01$ min$^{-1}$, is too slow to contribute to our reaction 5. Pre-steady state kinetic analysis of ATP hydrolysis and its inhibition by ATP·S showed that the forward rate constant for the isomerization reaction is the same as that for ATP hydrolysis, $k = 20$ min$^{-1}$ at 37°C. Hence in our reaction, this isomerization has likely occurred in the intermediate complex. The reverse rate for the isomerization, $k' = 0.1-0.2$ min$^{-1}$ corresponds well to the rate constant for the slow reaction observed in our experiments, $k_3$ (Tables II and III). These results suggest that reversal of isomerization is required for the transition from the flexible intermediate to the more rigid product.

To summarize, we present spectroscopic evidence for a previously unknown intermediate in the reaction that forms the RecA nucleoprotein filament in the presence of ATP·S. Fluorescent anisotropy measurements indicate that the intermediate is less rigid than the final product. It has anisotropy similar to mixed filaments of extended and condensed RecA monomers observed at low ATP·S concentrations. However, the intermediate is observed in the presence of a large excess of nucleotide cofactor, and presumably all RecA monomers are in the extended conformation. The greater flexibility of the intermediate filament might result from some sort of incorrect binding between relatively rigid segments of polymerized RgF (Fig. 8). This defect could be due to faulty protein-protein or protein-DNA interactions at the site between the segments. Our preferred hypothesis is that polymerization may initiate from multiple sites on the oligonucleotide and leave gaps of 1 or 2 nt between adjacent polymerization events, which are too small to react with RecA, whose binding site size is 3 nt (2, 4). Whatever the mechanism responsible for this flexibility, it disappears by the slow second step of the reaction. Comparing the rate of this process with published data (31) suggests that reversal of the nucleoside triphosphate dependent isomerization of the RecA filament is required to reshuffle RecA monomers in the filament to form a more rigid structure.

Taken together these results also show that the 32-mer oligonucleotide-RecA complex studied here can exist in two forms with different hydrodynamic properties. The more rigid form was observed (i) in filaments without cofactor and (ii) for the product filament in presence of ATP·S. The more flexible structure was observed (iii) during the polymerization reaction in presence of ATP·S and (iv) in mixed filaments of RecA protein with and without ATP·S. We argue that flexibility is caused by a break between segments of polymerized RecA protein. In the reaction intermediate (iii), the break is probably due to a discontinuity in the polymer, perhaps the result of multiple polymerization events. In the mixed filament (iv), the break likely comes from the inability of extended and condensed RecA monomers to form adequate protein-protein interactions for polymerization (29).

A transition between filament conformations with different mobilities such these could be coupled to ATP hydrolysis. RecA monomers are released from the nucleoprotein filament during
ATP hydrolysis, albeit with a low efficiency compared with the hydrolysis rate (32). Furthermore, ATP hydrolysis can introduce conformational changes that alter protein-protein interactions between adjacent RecA monomers (29,30). Either of these effects could introduce a flexible hinge in the filament at the site of ATP hydrolysis thereby introducing local segmental mobility, which could play a role in homologous recombination.

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