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ATP Hydrolysis and DNA Binding by the *Escherichia coli* RecF Protein*

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The *Escherichia coli* RecF protein possesses a weak ATP hydrolytic activity. ATP hydrolysis leads to RecF dissociation from double-stranded (ds)DNA. The RecF protein is subject to precipitation and an accompanying inactivation *in vitro* when not bound to DNA. A mutant RecF protein that can bind but cannot hydrolyze ATP (RecF K36R) does not readily dissociate from dsDNA in the presence of ATP. This is in contrast to the limited dsDNA binding observed for wild-type RecF protein in the presence of ATP but is similar to dsDNA binding by wild-type RecF binding in the presence of the nonhydrolyzable ATP analog, adenosine 5′-*O*-(3-thio)triphosphate (ATPγS). In addition, wild-type RecF protein binds tightly to dsDNA in the presence of ATP at low pH where its ATPase activity is blocked. A transfer of RecF protein from labeled to unlabeled dsDNA is observed in the presence of ATP but not ATPγS. The transfer is slowed considerably when the RecR protein is also present. In competition experiments, RecF protein appears to bind at random locations on dsDNA and exhibits no special affinity for single strand/double strand junctions when bound to gapped DNA. Possible roles for the ATPase activity of RecF in the regulation of recombinational DNA repair are discussed.

Escherichia coli RecF is a multifunctional protein, with identified roles in recombinational DNA repair, homologous genetic recombination, and DNA replication. The phenotypes of recF mutants include decreased plasmid recombination (1, 2), moderate UV sensitivity (3), attenuated SOS response (4, 5), and deficiency in the repair of daughter strand gaps (6, 7). A role for RecF protein in replication was suggested by the requirement for recF (and recR) in the resumption of replication after the collapse of DNA replication forks (8) and by the requirement of recF for cell viability in a priA mutant background (9). A link between recombination and replication is implied in one model (10) which postulates that the *in vivo* role of RecF, in conjunction with RecO and RecR, is to divert damaged ssDNA[1] from the replication process so that recombinational DNA repair can take place. *In vitro*, RecF protein binds to both ssDNA and dsDNA and hydrolyzes ATP (11–14). The RecF and RecR proteins, acting together, limit the extension of RecA filaments beyond DNA gaps (15). All of the functions of RecF may come together in recombinational DNA repair, providing a useful paradigm within which to examine RecF protein activities (16, 17).

A detailed *in vitro* characterization of the RecF protein can help in elucidating function. The DNA binding properties of RecF protein have been described in several reports. The binding to ssDNA binding is ATP-independent (11, 12), although binding to dsDNA is ATP-dependent (13, 14). RecF protein binds to dsDNA weakly in the presence of ATP but binds much more stably when RecR is included or in the presence of the nonhydrolyzable ATP analog, ATPγS (14). The role of the ATP hydrolytic activity of RecF in dsDNA binding has not been analyzed. The RecF protein contains a consensus nucleotide binding fold (G*XX*G*X*GKT) in the N-terminal portion, which is highly conserved among the known recF gene sequences. The relevance of this nucleotide binding fold to the *in vivo* function of RecF was suggested by the observation that a single amino acid substitution within it (Lys to Arg at position 36) appears to be a null allele (recF4101) (18, 19). Comparable mutations in the conserved ATP binding sites of RecA (20), UvrB (21), and RAD3 (22) also produce mutant phenotypes *in vivo* and result in mutant proteins that bind but do not hydrolyze ATP. A further indication that the capacity to hydrolyze ATP plays a role in RecF function is the recF4101 (also RecF K36R, with a mutation in the ATP binding site) overexpression phenotype. Overexpression of wild-type RecF renders cells inviable at 42 °C. However, cells overexpressing the RecF4101 protein are viable (23). In light of these findings, we have studied the role of ATP hydrolysis in RecF protein function, focusing on DNA binding. We have also examined the effects of RecR protein and the binding of RecF protein to gap junctions in DNA.

MATERIALS AND METHODS

Enzymes and Reagents—E. coli RecF and RecR proteins were purified as described (14). The mutant RecF protein, RecF K36R, was purified according to the procedure used for wild-type RecF protein. All protein preparations were free of detectable endo- or exonuclease activities. Protein concentration was determined by absorbance at 280 nm using the extinction coefficients: $\epsilon_{280} = 3.87 \times 10^4\ M^{-1}\ cm^{-1}$ for RecF protein and RecF K36R protein (this work) and $\epsilon_{280} = 5.60 \times 10^3\ M^{-1}\ cm^{-1}$ for RecR protein (24). At no time during purification or storage was the RecF protein subjected to conditions, as defined in this work, under which the protein would aggregate and would be inactivated. The purified RecF and RecF K36R proteins are shown in Fig. 1.

Restriction endonucleases were purchased from New England Biolabs. MES and EPPS buffers, bovine serum albumin, ATP, glycerol, sodium chloride, phosphocreatine, creatine phosphokinase, NADH, and phosphoenolpyruvate were from Sigma. Oligonucleotides were synthesized by the University of Wisconsin Biochemistry Department Synthesis Facility. [α-^{32}P]dideoxyATP (3000 Ci/mmol) was from Amersham Pharmacia Biotech. ATPγS was purchased from Boehringer-Mannheim Biochemicals.

The extinction coefficient for native RecF protein was determined by a published procedure (25), modified as described (26). The results of four determinations were averaged to give an extinction coefficient of

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[1] The abbreviations used are: ss, single-stranded; ds, double-stranded; gDNA, gapped DNA; ATPγS, adenosine 5′-*O*-(3-thio)triphosphate; BSA, bovine serum albumin; MES, 2-(*N*-morpholino)ethanesulfonic acid; EPPS, *N*-2-hydroxyethylpiperazine-*N*′-3-propanesulfonic acid.

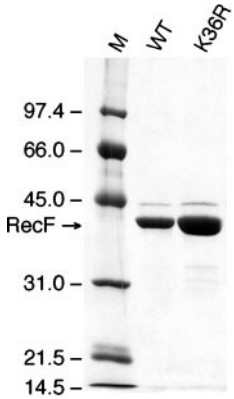

FIG. 1. **Purified RecF protein.** RecF and RecF K36R proteins were purified using the RecF protocol described previously (14). *Numbers* shown at the *left* of the gel represent the molecular mass of the marker proteins (M) in kDa.

native RecF as $\epsilon_{280,\,M,\,native} = 3.87 \pm 0.12 \times 10^4\ M^{-1}\ cm^{-1}$. The RecF concentrations determined using this method were in good agreement with the concentration determined by the Bradford method. This same extinction coefficient was used for the RecF K36R mutant.

DNA—Supercoiled circular duplex ϕX174 RFI DNA was purchased from Life Technologies, Inc. Linear ϕX174 dsDNA was prepared by complete digestion by *Pst*I. Circular ssDNA from M13mp8.1037 was prepared as described (24). Bacteriophage M13mp8.1037 was derived from M13mp8, having a 1037-base pair insertion (*Eco*RV-*Eco*RV fragment from the *E. coli* galT gene) in the *Sma*I site. Linear dsDNA radiolabeled at the 3′-end was generated using terminal transferase and [α-^{32}P]dideoxyATP. The concentrations of dsDNA and ssDNA stock solutions were measured by absorbance at 260 nm, using 50 and 36 μg ml^{-1} $A_{260}{}^{-1}$, respectively, as the conversion factors. DNA concentrations are expressed in terms of total nucleotides. DNA was stored in TE (10 mM Tris-HCl, pH 7.5, plus 1 mM EDTA)

Gapped DNA with a precisely defined gap length was prepared essentially as described (27). Gapped DNA having a 1037 base single-stranded gap (GD_{1037}) was prepared by annealing NaOH-denatured M13mp8 linearized by *Sma*I digestion to M13mp8.1037 (28) circular ssDNA. GD_{3329} was prepared by annealing the 4937-base pair fragment of a *Sma*I-*Bsp*HI digest of M13mp8 to M13mp8.1037 circular ssDNA. Thus GD_{1037} and GD_{3329} have a common 8266 base M13mp8.1037 inner circle and have different length linear DNAs annealed to it (7229 and 4937 bp, respectively). As an additional purification step, to remove contaminating M13mp8.1037 circular ssDNA and linear M13mp8 dsDNA after hydroxylapatite chromatography, the gapped DNA was electrophoresed on a 0.8% SeaPlaque GTG-agarose gel, and the DNA band corresponding to GD_{1037} or GD_{3329} was excised. The gel slice was melted at 65 °C and extracted 1:1 with phenol/chloroform/isoamyl alcohol (25:24:1) and chloroform/isoamyl alcohol (24:1). The final preparation of DNA was precipitated twice with ethanol.

Construction of RecF Mutant RecF K36R—To generate a plasmid containing the mutant recF allele, recF4101, and thus permitting overexpression of the RecF K36R protein, site-directed mutagenesis was performed using an oligonucleotide similar to that used by Sandler *et al.* (19) (5′-CAGCACGCTGGTACGGCCGCTGCCGTTGGCA-3′) and using pBLW20 as the template (14). The resulting plasmid encoded a mutant recF gene with the lysine at position 36 changed to arginine. This change was verified by sequencing, and the *Xba*I-*Nco*I fragment containing this mutation was then ligated back into pBLW20 to produce pBLW23. Overexpression of RecF K36R protein was performed exactly as for wild-type RecF protein, by transforming pBLW23 into the *E. coli* strain K38/pGP1–2 (29).

Reaction Conditions—Unless stated otherwise, reactions were performed in buffer A: 25 mM buffer (see below), 10 mM $MgCl_2$, 50 mM sodium chloride, 5% (w/v) glycerol, 0.1 mM EDTA, and 0.1 mM dithiothreitol. MES buffer was used for reactions at pH 5.7, 6.1, and 6.5. EPPS buffer was used for reactions at pH 6.7, 7.2, 7.6, 8.1, and 8.5. The reported pH of reaction mixtures reflect pH measurements (at 25 °C) of solutions containing all reaction components (except that storage buffers were substituted for proteins).

RecF Protein Aggregation Studies—We observed that RecF protein aggregates under certain conditions, causing an increase in turbidity at 340 nm when introduced into reactions monitored spectrophotometrically. Therefore, we used this signal as a measure of RecF protein aggregation to determine reaction components that prevent this effect. A Perkin-Elmer Lambda 7 double-beam recording spectrophotometer equipped with two thermojacketed cuvette holders, each capable of holding six cuvettes, was used for absorbance measurements. The cell length and the band pass were 0.5 cm and 2 nm, respectively. All components indicated except RecF and RecR were added to a 400-μl reaction mixture containing buffer A (EPPS, pH 7.6, except as stated otherwise). This was preincubated in the spectrophotometer at 37 C until the absorbance reading at 340 nm was stable. Then RecF protein (0.5 μM) and, as indicated, RecR protein (1.0 μM) were added, and the apparent absorbance (signal increase reflecting scattered light) was automatically measured every 30 s. Relative RecF protein aggregation was measured as an increase in the absorbance at 340 nm because of turbidity. The average of duplicate experiments is reported. The data is used comparatively, and no absolute measure of the degree of RecF protein aggregation is either known or implied.

Electron Microscopy—Reactions for observing RecF K36R protein binding to linear dsDNA were performed in buffer A (EPPS, pH 7.2) including 3 μM ϕX174 linear dsDNA, 1 mM ATP, and 0.6 μM RecF K36R protein. The reactions for observing RecF protein binding to dsDNA in the presence of ATPγS were performed in buffer A (pH 8.1) including 1 μM ϕX174 linear dsDNA, 1 mM ATPγS, and 0.2 μM RecF protein. The pH studies of wild-type RecF protein were performed in buffer A at the indicated pH including 1 μM ϕX174 linear dsDNA, 1 mM ATP or ATPγS, and 0.2 μM RecF protein. In all experiments, after the addition of RecF protein (or RecF K36R protein), the reactions were incubated at 37 °C for 10 min. The samples were then spread undiluted and without cross-linking for microscopy, as described previously (14). The grids were washed in reaction buffer containing 15% glycerol and of the appropriate pH before staining and shadowing.

RecF ATPase Assays—ATP hydrolysis by RecF protein was measured by a coupled enzyme assay. A Perkin-Elmer Lambda 7 was used for absorbance measurements in this assay. The regeneration of ATP from ADP and phosphoenolpyruvate coupled to the oxidation of NADH can be followed by the decrease in absorbance at 340 nm (the absorption maximum, which provides a level of sensitivity sufficient for the measurement of the relatively weak ATPase activity of RecF protein). Rates of ATP hydrolysis were measured at 37 °C at the following pH values: 5.7, 6.1, 6.5, 6.7, 7.2, 7.6, 8.1, and 8.5. Reaction mixtures (400 ml) contained buffer A of the indicated pH, 20 μM ϕX174 linear dsDNA, and 3 mM ATP. An ATP regenerating system (1.5 mM phosphoenolpyruvate, 4.5 units ml^{-1}, and 3 mM potassium glutamate) and a coupling system (0.6 mM NADH and 4.5 units ml^{-1} lactate dehydrogenase) were also included. The basal level of the decrease in absorbance at 340 nm was measured for 45 min prior to the addition of RecF protein. The rate of ATP hydrolysis catalyzed by RecF protein was calculated by subtracting this basal level from the decrease in absorbance at 340 nm measured for 45 min after the addition of RecF protein (2.5 μM). At each pH, the average of duplicate experiments is reported. Duplicate measurements of rates greater than 0.4 μM min^{-1} varied by no more than 7%, and those below 0.4 μM min^{-1} varied by no more than 15%.

Gel Shift Experiments—Agarose gel assays were carried out to detect complexes between RecF protein and linear dsDNA, as described previously (14). Challenge reactions were performed by preincubating RecF protein with ^{32}P-labeled dsDNA in the presence of either ATP or ATPγS prior to challenging with unlabeled dsDNA to determine how the stability of RecF·dsDNA complexes was affected by nucleotide cofactor and other parameters. RecF protein (1.33 μM) was preincubated with ^{32}P-labeled linear dsDNA (20 μM, as indicated in appropriate figure legends) in a 25-μl reaction mixture containing either ATP or ATPγS in buffer A (EPPS, pH 8.1) at 37 °C. After 10 min, unlabeled linear dsDNA (10 or 100 μM, as indicated) was added to the reaction (or TE for the control reaction). Portions of the reaction were stopped at the indicated times by the addition of EDTA (10 mM). A gel loading buffer (25 mM Tris-HCl, pH 7.5, 50% glycerol, and 0.02% bromphenol blue) was then added, and samples were electrophoresed on a 0.7% agarose gel at 3 V/cm with recirculation of the running buffer (20 mM Tris-HCl, pH 7.5, 4 mM sodium acetate, 0.1 mM EDTA) at room temperature. The gel was then dried, and the DNA was detected by autoradiography.

A modified assay patterned after a published procedure (30) was used to detect complexes between RecF and linear dsDNA or gapped DNA. Competition reactions in which one DNA substrate was preincubated with RecF prior to challenging with the second DNA substrate were used to detect any preferential binding. The reaction buffer contained 20 mM Tris-HCl (61% cation), 50 mM NaCl, 10 mM $MgCl_2$, 0.1 mM EDTA, and 5% (w/v) glycerol. Reactions containing 15 μM DNA (GD_{1037}, M13mp8.1037 linear dsDNA, or both as indicated), 0.3 μM RecF (giving one RecF per 50 nucleotides), and 1 mM ATPγS were incubated at 37 °C

for 30 min. The final pH of the mixture was 7.59 at 25 °C. For the challenge reactions, RecF was preincubated with one of the DNA cofactors for 10 min at 37 °C, followed by addition of the second DNA with the incubation continued for 30 min. A gel loading buffer (25 mM Tris-HCl, pH 7.5, 50% (w/v) glycerol, and 0.025% bromphenol blue) was added to each reaction, and the samples were immediately electrophoresed on a 0.8% agarose gel at 2.5 V/cm in 25 mM Tris-HCl, pH 7.5, 4 mM sodium acetate, and 1 mM EDTA with recirculation of the buffer. The DNA was then detected by ethidium bromide staining.

RESULTS

RecF Protein Undergoes Aggregation in Vitro—In the presence of ATP, the RecF protein appears to exist as a monomer in solution. In gel filtration trials carried out on columns equilibrated with buffer containing 1 mM ATP and 100 mM NaCl, RecF protein (40.5 kDa) consistently migrates slightly slower than the 22-kDa RecR protein (14).[2] Under these conditions, Umezu and Kolodner (31) reported that RecR existed as a dimer, a result we have confirmed,[2] so that the slower migration of RecF protein is most consistent with a monomeric species.

Our own observations and hints in some published reports suggested that the activity of RecF proteins may be affected by instability and/or insolubility *in vitro*. Madiraju and Clark (12) noted that incubation of prediluted RecF protein for 30 min prior to its addition to a reaction mixture resulted in total loss of DNA binding activity. We observed that RecF protein loaded onto a gel filtration column does not elute unless the column is equilibrated and developed in buffer containing either ATP or high salt (1 M NaCl) (14). Subsequently, we noticed that solutions containing RecF protein became visibly cloudy with what appeared to be precipitated or aggregated protein under a variety of conditions. We do not know whether the cloudy appearance of the solutions is because of precipitated protein or simply large aggregates. We generally employ the term "aggregation" throughout this paper, without implying any particular physical effect.

To follow up on these observations, we have used changes in turbidity as a measure of RecF protein aggregation or precipitation *in vitro*. As applied, this method is not intended to represent a quantitative measurement of the fraction of RecF protein aggregated. Instead, it was used to compare relative levels of RecF protein aggregation under different conditions and to identify solution conditions where this could be avoided.

RecF protein added to a reaction mixture containing no ATP or DNA at 37 °C aggregated almost immediately, as indicated by a large increase in turbidity (Fig. 2*A*). With RecF protein concentrations above 1 μM, the aggregation was manifested by a visible cloudy precipitate in the solution. The effect was temperature-dependent, as RecF protein was completely stable at 4 °C but aggregated shortly after a shift to 37 C (Fig. 2*A*). Exclusion of Mg^{2+} from the buffer delayed but did not eliminate this phenomenon (data not shown). To determine whether RecF protein was irreversibly inactivated by aggregation, the resulting white precipitate was collected by centrifugation, resuspended in storage buffer, and assayed for DNA binding activity. No activity was detected (data not shown). We do not know whether the protein is partially denatured and thereby inactive, or if it is simply present as large aggregates that are inaccessible to substrates. In either case, to date we have been unable to restore RecF protein to a form capable of ATP hydrolysis or DNA binding once the protein has come out of solution.

Because diluted RecF protein has been shown to be active for DNA binding and ATP hydrolysis under a variety of solution conditions, the reaction components that prevent or delay the aggregation of RecF protein were determined. As shown in Fig. 2*B*, aggregation is not affected by the addition of dsDNA but is decreased by ATP. Increasing the ATP concentration from 1 to 3 mM resulted in markedly less aggregation (data not shown). When present together, dsDNA and ATP slow the precipitation even more, suggesting a synergistic effect. Like ATP, ATPγS had only a small effect on aggregation. However, ATPγS in combination with dsDNA greatly reduced the observed turbidity change. These are conditions under which RecF protein is stably bound to dsDNA (14). Even though RecF protein remains in solution much longer when bound to DNA, it does slowly precipitate even under these conditions (Fig. 2*B*).

In the absence of ATP, RecR protein had no effect on RecF protein aggregation either in the presence or absence of dsDNA. RecR protein also had no effect in the presence of ATP when dsDNA was not also present (data not shown). However, the combination of the RecR protein, ATP, and dsDNA greatly reduced the turbidity increase (Fig. 2*B*). These are again conditions under which RecF protein (and RecR) are stably bound

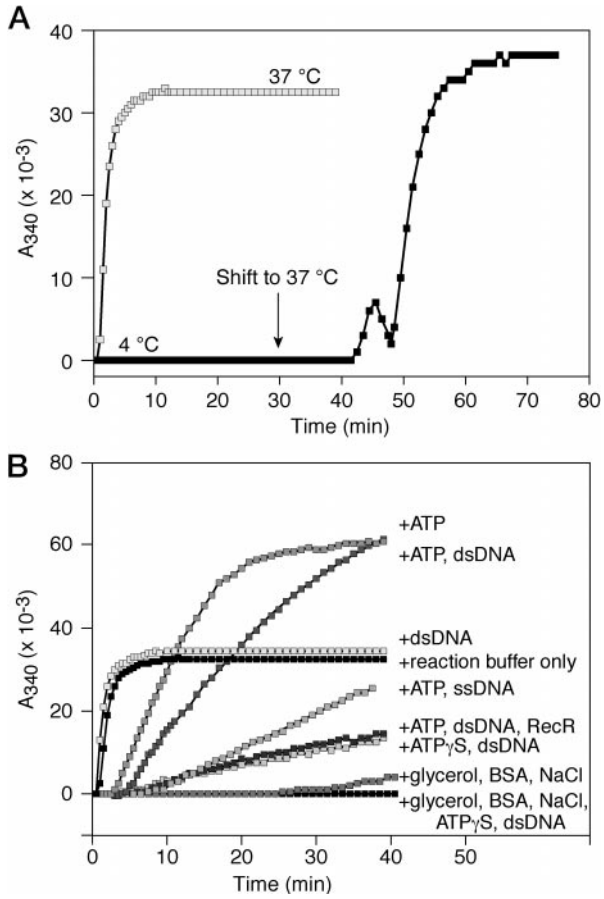

FIG. 2. **Aggregation of RecF protein *in vitro*.** The aggregation of RecF following its addition to a reaction mixture was monitored by increases in apparent absorption caused by turbidity at 340 nm. Reactions were carried out as described under "Materials and Methods" and contained buffer A (EPPS, pH 7.6) and the indicated reaction components. After the addition of RecF (0.5 μM) and, as indicated, RecR (1.0 μM), absorbance readings were automatically taken every 30 s. *A*, RecF aggregation occurred rapidly when added to buffer A at 37 °C. RecF added to buffer A equilibrated at 4 °C remained in solution until the temperature was shifted to 37 °C. The temperature shift required about 15 min to complete in this experiment. Aggregation detectable by turbidity increases is not evident until the temperature approaches 37 °C. *B*, aggregation of RecF protein in buffer A at 37 °C in the presence of the indicated reaction components. The concentration of ATP and ATPγS was 1 mM. Linear φX174 dsDNA and M13mp8.1037 circular ssDNA were both used at 10 μM. Where indicated, 30% (v/v) glycerol, 2 mg/ml BSA, and 150 mM NaCl were also added.

[2] B. L. Webb, unpublished results.

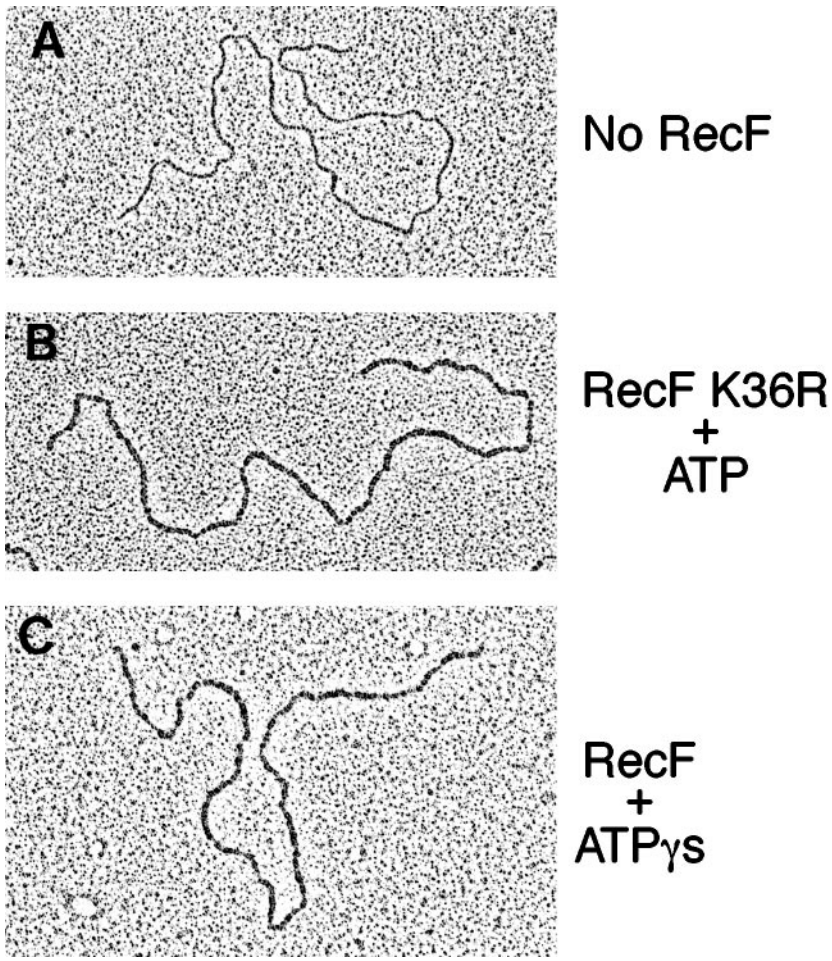

FIG. 3. **Comparison of linear dsDNA bound with RecF K36R in the presence of ATP and with wild-type RecF in the presence of ATPγS.** Binding reactions were carried out as described under "Materials and Methods." After a 10-min incubation at 37 °C, the samples were spread for microscopy without cross-linking or dilution. *A*, linear ϕX174 dsDNA without RecF added. *B*, linear ϕX174 dsDNA (3 μM) was incubated with RecF K36R (0.6 μM) in the presence of ATP. *C*, linear ϕX174 dsDNA (1 μM) was incubated with wild-type RecF (0.2 μM) in the presence of ATPγS.

to the DNA (14, 15). Interestingly, although the ssDNA binding activity of RecF has been reported to be ATP-independent, aggregation of RecF protein as measured by changes in turbidity was reduced by ssDNA in the presence of ATP (Fig. 2*B*) but not in the absence of ATP (data not shown).

The above studies indicate that RecF protein tends to aggregate and becomes inactive when it is not bound to DNA. In an attempt to find solution conditions which might stabilize RecF protein in the absence of DNA, we tested a variety of salts, detergents, and stabilizing agents for their ability to prevent RecF protein aggregation. We found that a combination of 30% glycerol, 2 mg/ml BSA, and 150 mM NaCl significantly reduced RecF protein precipitation in the absence of ATP or DNA. As indicated by a lack of significant increases in turbidity, RecF protein was completely soluble and unaggregated for at least 2 h when these stabilization components were combined with ATPγS and dsDNA (Fig. 2*B*). Although we have not fully characterized the effect of these conditions on RecF activities *in vitro*, they do not appear to significantly affect the dsDNA binding activity of RecF as measured by an agarose gel shift assay or its ATPase activity (data not shown).

The Role of ATP Hydrolysis in dsDNA Binding, a Mutant RecF Protein (RecF K36R) That Cannot Hydrolyze ATP Binds Tightly to dsDNA—Using electron microscopy, two previous observations inferred that ATP hydrolysis by RecF protein leads to its dissociation from dsDNA (14). First, RecF protein binds tightly to dsDNA in the presence of a nonhydrolyzable ATP analog, ATPγS. Second, much less RecF protein is bound in the presence of ATP, which is hydrolyzed by RecF protein. To verify that an inability to hydrolyze ATP blocks the dissociation of RecF protein from dsDNA, we generated and purified a RecF mutant protein that alters the ATP binding site (RecF K36R). The mutation is identical to the recF4101 mutant described by Sandler *et al.* (19). RecF K36R does not hydrolyze ATP (14).

RecF K36R protein was assayed for ATPase activity in six separate trials carried out over a period of 5 months. Four of these utilized the spectrophotometric assay described under "Materials and Methods," while the other two used the thin layer chromatography method (14). In two of the trials, multiple reactions were run side by side with and without the RecR protein, which stimulates ATP hydrolysis by the wild-type RecF protein (14). Reactions were carried out at pH 7.2 under standard reaction conditions, except that four of the trials included the optimal stabilizing conditions of 30% glycerol, 100 mM NaCl, 2 mg/ml BSA, and 3 mM ATP. ATP hydrolysis was not detected at any level in any of these trials (data not shown). We conclude that at least under these conditions, the RecF K36R mutant protein does not hydrolyze ATP.

As shown in Fig. 3, RecF K36R protein binds to and coats linear dsDNA in the presence of ATP. In a count of 98 molecules selected at random from two separate experiments, 97 or 99% were coated with protein. The molecule in Fig. 3*B* is typical of this population, with molecules varying somewhat in the number and lengths of small discontinuities in the protein coat. The DNA bound with mutant protein appears identical to dsDNA coated with wild-type RecF protein in the presence of ATPγS. In the presence of ATP, RecF K36R protein also retards the migration of dsDNA in an agarose gel to the same extent as wild-type RecF protein in the presence of ATPγS (data not shown; see Fig. 5 for an example of the result obtained with wild-type protein and ATPγS). Unlike the wild-type protein, the binding of the mutant RecF K36R protein to dsDNA was

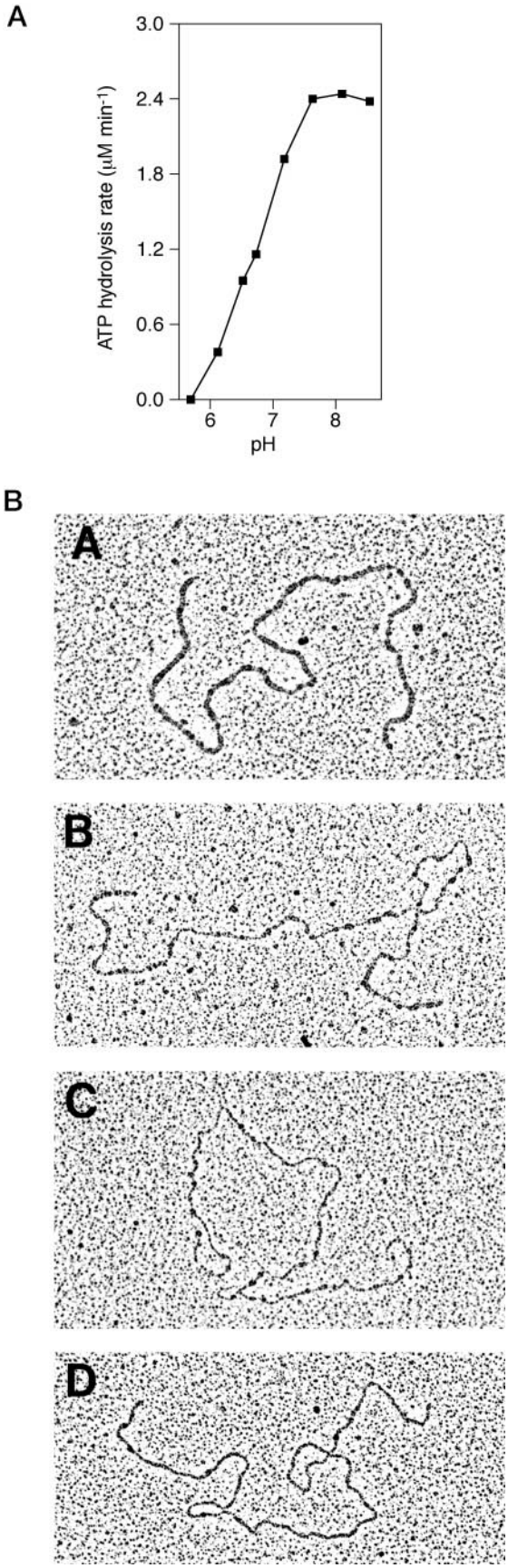

FIG. 4. **Dependence of RecF-mediated ATP hydrolysis and dsDNA binding in the presence of ATP on pH.** Reactions were performed at 37 °C in buffer A at the indicated pH, as described under "Materials and Methods." *A*, RecF ATP hydrolysis rates as a function of pH. Reactions contained 20 μM ϕX174 linear dsDNA, 3 mM ATP, and 2.5 μM RecF. The pH values of the individual reactions were 5.7, 6.1, 6.5, 6.7, 7.2, 7.6, 8.1, and 8.5. *B*, effect of pH on RecF binding to dsDNA in the presence of ATP. Reactions contained 1 μM ϕX174 linear dsDNA, 1 mM ATP, and 0.2 μM RecF and were carried out at pH 5.6 (*A*), 6.5 (*B*), 7.2 (*C*), and 8.1 (*D*). The molecules shown were chosen to be representative of their respective samples. At pH 5.7, the DNA is essentially fully coated with protein. The complexes have a much thicker appearance than free dsDNA. The protein coat has no visible fine structure under these preparative conditions. At pH 6.5, there is visibly less bound protein. Short portions of the DNA appear to be fully coated, but more usually there are a high diversity of individual protein blobs. At pH 7.2, there are no fully coated regions, and only individual protein blobs are observed. At pH 8.1, very few protein blobs are observed. Many DNA molecules have no detectable bound protein. There was no indication of cooperative protein binding in any of the samples.

reduced in the presence of ATPγS. This was true regardless of whether the stabilizing reaction conditions were employed. The reduced binding was manifested by protein-free or nearly protein-free DNA observed by electron microscopy in three separate trials, in contrast to the coated DNA seen when ATP was used in Fig. 3*B*. In a count of 182 individual DNA molecules (linear ϕX174 dsDNA) from three separate experiments, 158 or 87% had no bound protein. Small dots or short tracts appearing to be bound RecF K36R protein were seen on 24 molecules, all of these from one of the three samples. No molecules were completely coated with protein. In three gel retardation trials (data not shown), the reactions with ATPγS produced a variable smearing of the DNA band (data not shown) as opposed to the sharp retarded band seen with ATP. In the complete absence of nucleotide cofactor, little or no binding to dsDNA was observed with the wild-type RecF protein (12, 14). This also proved to be the case for the RecF K36R mutant protein, as measured by either electron microscopy or gel retardation (two trials each; data not shown). In the electron microscope, 107 of 114 molecules counted in two separate experiments, or 94%, had no bound RecF K36R protein in the absence of nucleotide cofactors. A few DNA molecules in both experiments (7 total) had small tracts that appeared to be bound protein. Inasmuch as the presence of ATP clearly facilitates the binding of RecF K36R protein to dsDNA, we inferred that the mutant protein was able to bind ATP as well as DNA.

Wild-type RecF protein binds stably to dsDNA in the presence of ATP at low pH—To further correlate the relationship between RecF ATP hydrolysis and dissociation from dsDNA, we studied the effect of pH on these two processes using wild-type RecF protein. The pH profile of the ATPase activity of RecF in the presence of linear dsDNA is shown in Fig. 4*A*. At pH 5.7, no ATP hydrolysis by RecF protein was detected. With increasing pH, the ATPase activity increased, reaching a maximum around pH 8.1.

The rates of ATP hydrolysis were monitored using a coupled spectrophotometric assay as a decrease in absorbance (because of NADH oxidation) with time at 340 nm. These measurements could be affected by RecF aggregation, which would cause a slower decrease in measured absorption because of increases in turbidity (which we measured at the same wavelength, albeit the reactions did not contain NADH), as well as the accompanying loss of RecF activity. For the reactions performed below pH 7.5, no significant change in rate was detected over the 45-min time course used to calculate the steady state ATP hydrolytic rate, suggesting that no measurable aggregation of RecF protein occurred. However, there was an apparent decline in the rate with time in the reactions performed at the higher pHs. In agreement with the precipitation studies above, this most likely reflects an increase in absorbance because of precipitation, which nullifies some of the decrease in absorbance brought about by NADH oxidation in the coupled reaction system. This became more noticeable with increasing pH. As a

result, the rates for reactions above pH 7.5 were calculated from the first 30 min of time points after the addition of RecF protein to minimize any effects of aggregation.

We previously reported a turnover number of 0.2 min^{-1} at pH 7.0, a result obtained with a different ATPase assay based on thin-layer chromatography (14). The rate for pH 7 derived from the data in Fig. 4*A* was 0.64 min^{-1}. We attribute much of the increase to the effects of the 5% glycerol present in the new experiments, which greatly reduces aggregation and its accompanying loss of activity. The highest rate observed, at pH 8.1, was 0.97 min^{-1}. Three other trials were carried out over a period of 4 months under conditions determined to stabilize the RecF protein (5% or more glycerol plus high concentrations of ATP), two using the thin layer chromatography assay (14) and the other using the spectrophotometric assay. All were carried out at or near pH 7 with 1 mM or greater ATP. The k_{cat} derived from these trials varied from 0.5 to 0.58 min^{-1}.

A pH titration of the dsDNA binding activity of RecF was carried out using electron microscopy. The amount of RecF protein bound to linear dsDNA in the presence of ATPγS was not affected by pH, within the range of 5.7–8.1; incubation of dsDNA with saturating amounts of RecF protein resulted in fully coated DNA at all pHs (data not shown). Fig. 4*B* shows the effect of pH on the binding of RecF protein to dsDNA in the presence of ATP. In agreement with our earlier report (14), very little RecF protein was bound to the dsDNA above neutral pH, where the ATPase activity is greatest. However, at pH 5.7, where the ATPase activity is suppressed, RecF protein bound and coated the dsDNA. Thus, the extent of the binding of RecF protein to dsDNA is correlated inversely with its ATP hydrolytic activity. Unlike at pH 7.6 (Fig. 2*B*), RecF protein exhibited little aggregation as reflected by measured turbidity in the presence of ATP and dsDNA at pH 5.7 (data not shown), presumably because it remains bound to the dsDNA.

Though the ATPase activity of RecF protein is relatively weak and accumulation of ADP in a 10-min incubation is unlikely, we tested the effect of including an ATP regeneration system on RecF protein binding to dsDNA in the presence of ATP at pH 8.1. As determined by electron microscopy, the extent of RecF binding to dsDNA was unchanged (data not shown).

RecF Protein Binds Stably to dsDNA in the Presence of ATPγS—The migration of linear dsDNA through an agarose gel is retarded by RecF protein to a greater extent in the presence of ATPγS than with ATP (14). To determine whether less RecF protein is bound to the dsDNA in the presence of ATP as a result of ATP hydrolysis-induced dissociation, we examined the stability of these RecF·dsDNA complexes over time using a challenge experiment. Radiolabeled linear dsDNA (20 μM) was preincubated with RecF protein (1.33 μM) in the presence of either ATP or ATPγS for 10 min prior to challenging with excess unlabeled linear dsDNA (100 μM). Aliquots of the reaction were stopped with EDTA at 5 and 10 min after the challenge and run on an agarose gel. RecF protein dissociated rapidly from the labeled dsDNA when bound in the presence of ATP. Within 5 min after the challenge DNA was added, the retardation in the migration of the labeled DNA was reduced substantially, almost to the point of running even with the unbound DNA marker (Fig. 5, *lane 1 versus lanes 2* and *3*). In contrast, the migration of dsDNA bound by RecF protein in the presence of ATPγS was retarded almost to the same extent before and after the challenge (Fig. 5, *lane 4 versus lanes 5* and *6*), indicating little dissociation of RecF protein. Thus, ATP hydrolysis correlates with the dissociation of RecF from the DNA.

The RecR Protein Slows the Dissociation of RecF Protein from dsDNA—Addition of the RecR protein improves the binding of RecF protein to dsDNA in the presence of ATP (14). To explore the molecular basis of this phenomenon, we examined the effect of RecR protein on RecF dissociation from dsDNA (Fig. 5, *lanes 7–9*). The presence of RecR protein does not prevent dissociation of RecF protein from the DNA, but the dissociation of the putative RecFR complexes are markedly slowed relative to the dissociation of RecF alone (compare *lanes 7–9* to *lanes 2* and *3*).

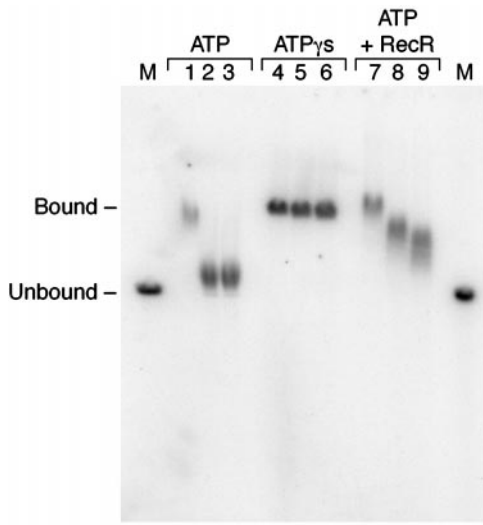

FIG. 5. **Dissociation of RecF from dsDNA in presence of ATP *versus* ATPγS.** A challenge experiment was used to measure dissociation of RecF bound to dsDNA. The reactions were carried out as described under "Materials and Methods." RecF (1.33 μM) was preincubated with ^{32}P-labeled ϕX174 linear dsDNA (20 μM) in the presence of either ATP or ATPγS, as indicated, 10 min prior to the addition of excess unlabeled ϕX174 linear dsDNA (100 μM). RecR protein (2.66 μM) was included in the reactions shown in *lanes 7–9*. Reactions were stopped by the addition of EDTA after 5 and 10 min, and the extent of RecF binding to the labeled dsDNA was determined using an agarose gel retardation assay. *M*, ϕX174 linear dsDNA markers.

RecF Protein or RecFR Complexes Do Not Exhibit a Preferential Association with ssDNA-dsDNA Junctions in Gapped DNA—A recent report (30) indicates that in the presence of ATPγS, RecF protein has a higher affinity for gapped DNA (gDNA) than for either dsDNA or ssDNA, implying it might bind tightly to the ss-ds junction of the gDNA. In contrast, a study of the effects of RecF and RecR proteins on the assembly of RecA filaments on gapped DNA provided no indication that RecFR complexes bind to the ss-ds junction (15). We wished to determine whether RecF protein alone exhibits an enhanced affinity for gap junctions that might be abolished by dATP/ATP hydrolysis and/or an interaction with RecR protein.

As a direct assessment of the relative affinity of RecF protein for gDNA *versus* dsDNA, gel shift DNA binding assays were carried out, patterned after the work by Hedge *et al.* (30) and done under essentially the same reaction conditions except for the inclusion of 5% glycerol and the use of somewhat different DNA substrates. Subsaturating amounts of RecF protein were incubated with either linear dsDNA (end-labeled with ^{32}P), GD_{1037}, or both in the presence of ATPγS (Fig. 6). To visualize the location of both DNAs simultaneously, we stained the agarose gel with ethidium bromide (as shown). The identity of each band was verified separately by exposing the dried gel to x-ray film (data not shown). With either DNA substrate alone, increasing the RecF protein concentration from 180 nM (1 RecF per 83 nucleotides) to 300 nM (1 RecF per 50 nucleotides) resulted in a readily discerned decline in the mobility of the DNA in the gel (Fig. 6, *lanes 3–6*). When both DNAs were

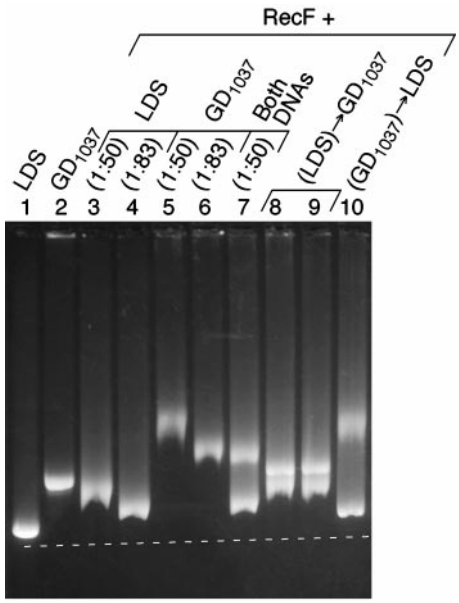

FIG. 6. **RecF exhibits no preferential binding to gapped DNA over dsDNA.** Reactions were carried out as described under "Materials and Methods," with 15 μM DNA (GD_{1037}, M13mp8.1037 linear dsDNA, or both as indicated), RecF (at the indicated concentration), and 1 mM ATPγS. At the end of the incubation, samples were electrophoresed on a 0.8% agarose gel. The gel was loaded as it was running such that the *lanes* at *right* have migrated for a somewhat shorter time than the *lanes* at *left*. The *dashed line* is drawn between points 1 cm below the linear dsDNA marker in *lane 1* and a similar marker in a *lane* to the *right* of *lane 10* (not shown), to provide a measure of the degree of retardation caused from left to right as a result of this loading effect. The DNA was detected by ethidium bromide staining. Controls: *lane 1*, linear dsDNA marker; *lane 2*, GD_{1037} marker; *lane 3*, 0.3 μM RecF with linear dsDNA (1 RecF:50 nucleotides total DNA); *lane 4*, 0.18 μM RecF with linear dsDNA (1:83); *lane 5*, 0.3 μM RecF with GD_{1037} (1:50); *lane 6*, 0.18 μM RecF with GD_{1037} (1:83). To determine whether RecF preferentially binds to gapped DNA, RecF (at 300 nM or 1:50 nucleotides of total DNA) was incubated with both DNAs for 30 min at 37 °C (lane 7), or was preincubated with one of the DNAs 10 min prior to challenging with the other DNA for an additional 30 min (linear dsDNA before GD_{1037} in *lanes 8* and 9; GD_{1037} before linear dsDNA in *lane 10*).

included in the experiment at equal concentrations, RecF protein (1 RecF per 50 total nucleotides) impeded the migration of each of them to a similar extent (*lane 7*), reflecting the binding of RecF protein to both DNAs. In this experiment, the amount of available dsDNA in the two types of DNA molecules is quite similar, differing only by the absence of one strand over about 15% of the length of the gDNA.

Challenge experiments were also carried out. Linear dsDNA was preincubated with RecF protein (300 nM, or 1 RecF per 50 nucleotides) for 10 min prior to challenging with an equal amount of GD_{1037}, followed by incubation for an additional 30 min (*lanes 8* and *9*). The linear dsDNA was shifted almost to the full extent of the control (*lane 3*) with linear dsDNA alone, while the GD_{1037} was shifted very little. In addition, the GD_{1037} band (the *upper band* in *lanes 8* and *9*) did not have the somewhat smeared appearance evident in the bound DNA bands in *lanes 3–6*. Thus, once bound to the linear dsDNA, very little RecF protein was seen to move to the GD_{1037}. Similarly, most of the RecF protein initially bound to the GD_{1037} remained there when challenged by linear dsDNA (*lane 10*). These data indicate that RecF protein binds with apparently equal facility to the dsDNA or to the dsDNA portion of gDNA. The experiments were carried out 5 times with consistent results. Elimination of the glycerol (5%) did not affect the result (data not shown). We found no evidence for a preferential binding of RecF protein to gap junctions.

DISCUSSION

Our primary conclusion is that ATP hydrolysis by RecF protein leads to its dissociation from dsDNA. Stable binding is observed in the presence of ATPγS, which is not hydrolyzed by the RecF protein, and at low pHs where ATP hydrolysis is minimal. The RecF K36R mutant, which does not hydrolyze ATP, also binds stably to dsDNA when ATP is present. The dissociation of RecF protein from dsDNA is slowed by the RecR protein.

We also show that RecF protein aggregates *in vitro* at 37 °C, becoming both insoluble and inactive under conditions in which it is not bound to DNA. The aggregation is slowed by the addition of glycerol, NaCl, and BSA to the reaction, or by incubation at relatively low temperatures. If DNA and an appropriate nucleoside triphosphate are present, aggregation is minimized. The aggregation of RecF at 37 °C may contribute to the formation of inclusion bodies in cells in which RecF is highly expressed (14). Soluble RecF protein is obtained in greater yield when the expressing cells are grown at reduced temperatures. The aggregation and accompanying inactivation of RecF protein *in vitro* may complicate the interpretation of any experiments involving RecF protein done under conditions where it is not bound to DNA.

We previously showed that RecF binding to dsDNA in the presence of ATP is limited (14). Here we show that inhibiting RecF ATP hydrolysis, either by replacing ATP with ATPγS, by using a RecF mutant that is unable to hydrolyze ATP, or by lowering the pH such that the hydrolytic activity of wild-type RecF is blocked, leads to stable binding of RecF protein to dsDNA and prevents its dissociation. In addition, RecF protein dissociation from dsDNA in the presence of ATP but not ATPγS was observed directly using a DNA challenge experiment.

The precedents for such a role of ATP hydrolysis in DNA binding proteins that bind ATP are numerous. The ADP-bound forms of the RecA protein (24, 32), Rep helicase (33), and UvrA (34) protein all have lower affinity for their DNA substrates than ATP-bound forms, resulting in an increased tendency to dissociate following ATP hydrolysis. The functional significance of dissociation from DNA in each of these cases is specific to the individual protein and the role they play in DNA metabolism.

The RecF K36R mutant protein binds to dsDNA in the presence of ATP, but the binding is much reduced when ATPγS is substituted for ATP. We do not have an explanation for the effects of ATPγS. The analogous mutation in the ATP binding site of the RecA protein, RecA K72R, results in a protein that does not hydrolyze NTPs and functions in certain RecA activities. However, this mutant RecA functions with either dATP or ATPγS, but not with ATP (20, 35).

The binding of RecF protein to both ssDNA and dsDNA is readily demonstrated (11–15). However, we have been unable to confirm a report that RecF protein binds preferentially to gap junctions (30). Complexes of the RecF and RecR proteins appear to bind randomly to the dsDNA portion of gapped DNA molecules, where they halt RecA filament extension (15). In the present study, direct comparisons of the binding of RecF protein alone to dsDNA or gapped DNA failed to reveal significant differences between the two.

The molecular function of ATP hydrolysis and subsequent dissociation from dsDNA by RecF protein *in vivo* is not entirely clear. One obvious function could be the recycling of the protein. However, there are many other possibilities. RecF may interact with a number of recombination and replication proteins *in vivo*. The RecF and RecR proteins interact *in vitro* (14), and together they limit the extension of RecA protein filaments beyond single-stranded DNA gaps (15). Therefore, one function

of RecF protein may be to modulate RecA filament assembly. The weak ATPase activity of RecF protein could also play some role in regulating the interface between recombination and replication systems during recombinational DNA repair (17). A complete understanding of the role of RecF-mediated ATP hydrolysis must await analysis of the effects of additional recombination and replication proteins on RecF and of the effects of RecF on these proteins.

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