Biochemical Basis of the Constitutive Coprotease Activity of RecA P67W Protein†

Julie K. Mirshad‡,§ and Stephen C. Kowalczykowski*,‖

Department of Chemistry, University of California, Davis, California 95616, and Sections of Microbiology and of Molecular and Cellular Biology, Center for Genetics and Development, University of California, Davis, California 95616

Received November 25, 2002; Revised Manuscript Received March 25, 2003

ABSTRACT: The mutation of Pro67 to Trp (P67W) in the *Escherichia coli* RecA protein results in reduced recombination and constitutive coprotease phenotypes. We examined the biochemical properties of this mutant in an effort to understand these altered behaviors. We find that RecA P67W protein can access single-stranded DNA (ssDNA) binding sites within regions of secondary structure more effectively than wild-type protein, and binding to duplex DNA is both faster and more extensive as well. This mutant is also more effective than wild-type RecA protein in displacing SSB protein from ssDNA. An enhancement in SSB protein displacement has been shown previously for RecA441, RecA730, and RecA803 proteins, and similarly, this improved ability to displace SSB protein for RecA P67W protein correlates with an increased rate of association with ssDNA. As for the aforementioned mutant RecA proteins, we expect that this enhanced activity will allow RecA P67W protein to bind ssDNA naturally occurring in undamaged cells and to constitutively induce the SOS response. The DNA strand exchange activity of RecA P67W protein is also altered. Although the rate of duplex DNA uptake into joint molecules is increased compared to that of wild-type RecA protein, the resolution to the nicked circular dsDNA product is reduced. We suggest that either a limited amount of DNA strand reinvasion or a defect in DNA heteroduplex extension is responsible for the impaired recombination ability of this mutant protein.

The *Escherichia coli* RecA protein is an essential component for both homologous recombination and induction of the SOS response (*1*−*3*). Central to these processes, which are important for the generation of genetic diversity, maintenance of genetic integrity, and the proper segregation of chromosomes, are the pairing and exchange of homologous DNA strands and the catalysis of LexA repressor autocleavage.

The formation of a ternary complex, consisting of RecA protein, ssDNA,[1] and a nucleotide cofactor, is vital to the function of the enzyme. The binding of ATP to RecA protein results in a conformational change, which increases the affinity of RecA protein for ssDNA (*4*, *5*). Once ATP binds, a high-affinity binding state is assumed by the RecA protein in which the nucleoprotein filament is extended (*6*−*8*). ATP hydrolysis regulates RecA protein binding and dissociation from DNA; the resultant ADP induces a low-affinity, inactive form of the enzyme, characterized by a compact filament. The high-affinity conformation is necessary for all RecA protein enzymatic activities (*9*).

The site for ATP binding in the RecA protein, the phosphate binding loop (P-loop), is highly conserved among many nucleotide binding proteins. It is defined by the Walker A sequence, G/AXXXXGKT/S, where X is a nonconsensus residue (*10*). This sequence in the RecA protein has been identified as residues 66−73, which corresponds to the sequence GPESSGKT. An alignment of 64 full-length bacterial RecA protein homologues confirmed that the Walker A sequence was highly conserved with no variance among six of the residues (*3*).

The study of RecA protein function and how it is related to ATP binding and hydrolysis was undertaken by Konola and Logan by developing mutants with single, double, and multiple amino acid substitutions in the P-loop motif (*11*−*13*). The mutants were examined for recombination proficiency and coprotease activity *in vivo*, in the presence and absence of DNA-damaging agents. These studies showed that Pro67 was able to tolerate mutation, even though it is highly conserved among eubacterial proteins.

Of particular interest is the mutant Pro67 substituted with Trp (RecA P67W). This mutant is constitutive for coprotease activity and moderately inhibited for recombination (a 15−50% reduction in UV survival and an 88% reduction in λ phage plating efficiency) (*12*, *13*). The length of the oligonucleotide cofactor that can support coprotease function was investigated by Konola et al. (*13*) since it was shown that RecA441 protein, which is also constitutive for coprotease activity, can use shorter oligonucleotides for the cleavage of λ cI repressor than wild-type RecA protein (*14*). In the case of LexA repressor cleavage, both wild-type RecA protein and RecA P67W protein exhibited the same minimum effective length of oligonucleotide cofactor, although RecA P67W protein produced more extensive cleavage for all the lengths that were examined. The extent of cleavage of λ

† This work was supported by National Institutes of Health Grant GM-62653.

* To whom correspondence should be addressed: Sections of Microbiology and of Molecular and Cellular Biology, Center for Genetics and Development, One Shields Ave., Briggs Hall, University of California, Davis, CA 95616. Telephone: (530) 752-5938. Fax: (530) 752-5939. E-mail: sckowalczykowski@ucdavis.edu.

‡ Department of Chemistry.

§ Present address: LumiCyte, Inc., 48480 Lakeview Blvd., Fremont, CA 94538.

‖ Center for Genetics and Development.

[1] Abbreviations: ssDNA, single-stranded DNA; dsDNA, double-stranded DNA.

10.1021/bi027232q CCC: $25.00
Published on Web 04/24/2003

cI repressor was also measured, and a length dependence was found. Using dT oligomers, it was established that RecA P67W protein can use shorter polynucleotide cofactors for the cleavage of λ cI repressor than wild-type RecA protein, like RecA441 protein (*14*). This suggests that RecA P67W protein may be activated by DNA oligomers that do not support enzymatic activity by wild-type RecA protein (*13*).

RecA mutant proteins, RecA1202 and RecA1211, are able to utilize rRNA and tRNA as polynucleotide cofactors for LexA repressor cleavage (*15*). It was possible that these alternate polynucleotide cofactors could induce the high-affinity conformation in RecA P67W protein which would enable coprotease function in the absence of DNA damage. However, no coprotease activity was measured *in vitro* with tRNA for RecA P67W protein (*13*).

RecA P67W protein, as well as other mutant RecA proteins such as RecA1202 and RecA1211 (*16*), has a relaxed specificity for the NTP cofactor for coprotease activity. RecA P67W protein was activated for coprotease activity *in vitro* with the ribo, deoxy, and dideoxy forms of ATP, CTP, TTP, UTP, and GTP. As compared to wild-type RecA protein, which showed a greater specificity for the cofactor and an inability to utilize GTP, dGTP, ddGTP, TTP, and ddTTP, RecA P67W protein was able to function with any of these cofactors, although with reduced activity in some cases (see Table 3 of ref *13*).

Konola et al. (*13*) suggested that mutations at residue 67 may exert their effect through interactions with the primary DNA binding site and LexA repressor binding site. Substitutions at position 67 may interact with Gln194 or residues within loop 2, which were proposed to be part of the primary DNA binding site (*17*), and therefore affect recombination and coprotease activity. Additionally, a substitution with Trp may alter coprotease activity by reaching further than Pro into the nucleoprotein filament helical groove, which is proposed to be the site of LexA repressor binding (*13*, *18*, *19*). Another alternative is that this effect could be mediated by other residues in contact with residue 67 and the LexA repressor binding site.

In this study, the biochemical properties of RecA P67W protein are characterized *in vitro* in an attempt to account for the reduced level of recombination and the constitutive coprotease phenotype. We show that RecA P67W protein exhibits an enhanced ability to displace SSB protein from ssDNA which alters the competition between these two proteins. The displacement of SSB protein by RecA P67W protein is the result of an increased rate of association with ssDNA (*20*−*22*). By gaining access to the ssDNA that occurs naturally in the cell through the displacement of SSB protein, RecA P67W protein becomes activated for coprotease activity in the absence of DNA damage. We find that RecA P67W protein is capable of carrying out DNA strand exchange with an increase in the level of pairing of dsDNA; this finding is seemingly inconsistent with the *in vivo* data showing an increased UV sensitivity and an impaired ability to support plaque formation as a measurement of recombination activity (*12*, *13*). However, we also observed that RecA P67W protein appears to have an impaired ability to resolve joint molecules in a DNA strand exchange assay; therefore, the recombination defect may be a result of an increased level of reinvasion by the displaced DNA strand or a defect in branch migration.

EXPERIMENTAL PROCEDURES

Chemicals and Buffers. All chemicals were reagent grade, and buffers were prepared with Barnstead Nanopure water.

BCA assay reagents were purchased from Pierce and used according to recommended standard reaction conditions.

DNA. Circular M13 ssDNA was prepared from bacteriophage M13 mp7 as described previously (*23*). Circular pBS ssDNA was prepared using a modification of the M13 procedure. Duplex pBS DNA was purified by alkaline lysis followed by a cesium chloride gradient (*24*, *25*) and digested with *Bam*HI restriction endonuclease to form linear dsDNA. Poly(dT) was from Amersham Pharmacia Biotech and, as provided by the manufacturer, was approximately 200−250 nucleotides in length. Extinction coefficients at 260 nm of 8250, 8784, and 6500 M^{-1} cm^{-1} for poly(dT), ssDNA, and dsDNA, respectively, were used to determine concentrations. Concentrations are reported in nucleotides. Etheno M13 ssDNA was prepared according to the method described in ref *4*.

Proteins. Wild-type RecA protein was purified from strain GE1710 following a protocol based on spermidine acetate precipitation (*26*). All purification steps were performed at 4 °C. Cells were suspended in 4 mL/g of a buffer containing 50 mM Tris-HCl (pH 7.5), 25% (w/v) sucrose, 25 mM NaCl, 5 mM EDTA, and 1 mM DTT and then lysed using a French press. Phenylmethanesulfonyl fluoride (PMSF) was added before and during lysis to a final concentration of 10 μL/mL. The protein in the cleared lysate was precipitated by the addition of a 10% solution of polyethyleneimine in water to a final concentration of 0.5%. The resulting pellet was extracted several times with R buffer [20 mM Tris-HCl (pH 7.5), 10% (v/v) glycerol, 0.1 mM dithiothreitol, and 0.1 mM EDTA] containing 150 mM ammonium sulfate and then with R buffer containing 300 mM ammonium sulfate. The protein in the supernatant from the latter set of extractions was precipitated by the addition of ammonium sulfate to 0.3 g/mL. The protein pellet was dissolved in phosphate buffer [20 mM KH_2PO_4/K_2HPO_4 (pH 6.5), 10% glycerol, 0.1 mM DTT, and 0.1 mM EDTA] with 200 mM NaCl, dialyzed into the same buffer, and then passed over a Whatman P-11 phosphocellulose column. The pooled flow-through fractions were precipitated again by the addition of ammonium sulfate, as before. The resulting pellet was resuspended in spermidine acetate buffer [20 mM spermidine acetate (pH 7.5), 7 mM spermidine (pH 7.5), and 0.1 mM DTT] and dialyzed extensively into several changes of the same buffer. The resulting precipitate was pelletted, then dissolved in phosphate buffer containing 25 mM EDTA, and dialyzed into the same buffer and then into phosphate buffer containing 50 mM NaCl. The solution was applied to a DNA cellulose column equilibrated with phosphate buffer containing 50 mM NaCl, and the RecA protein was eluted with phosphate buffer containing 250 mM NaCl and 1 mM ATP. The protein was again precipitated by the addition of ammonium sulfate, then dissolved in Tris buffer [20 mM Tris-HCl (pH 7.5), 0.1 mM EDTA, and 0.1 mM DTT] containing 100 mM NaCl, and dialyzed into the same buffer. The protein solution was then applied to a Mono-Q column equilibrated in the same buffer and eluted with a NaCl gradient in Tris buffer. The protein-containing fractions were checked for nuclease activity, pooled, and then precipitated with the addition of ammonium

sulfate. The final pellet was dissolved in R buffer containing 1 mM DTT and dialyzed into the same buffer. The concentration of the protein was determined using an extinction coefficient at 280 nm of 2.7×10^4 M^{-1} cm^{-1}. This procedure was used to purify wild-type RecA protein, a variety of mutant RecA proteins (*20*, *22*, *27*, *28*), and a similar procedure was used to purify human Rad51 protein (*29*).

RecA P67W protein (*12*, *13*) was purified from strain DE 1663′ harboring a pTRecA103 construct using a variation of the wild-type RecA protein preparation. RecA P67W protein could not be eluted from a ssDNA−cellulose column with ATP, as is wild-type RecA protein; instead, 400 mM NaCl was used. The concentration of the protein was determined using the BCA protein concentration assay with wild-type RecA protein as a standard. The extinction coefficient determined from this assay at 280 nm was 9.77×10^4 M^{-1} cm^{-1}.

SSB protein was purified from strain RLM727 following the method described in ref *30*. An extinction coefficient at 280 nm of 3.0×10^4 M^{-1} cm^{-1} was used to determine the protein concentration (*31*).

Lactate dehydrogenase and pyruvate kinase were purchased as ammonium sulfate suspensions from Sigma. For use in the assays, a sample of the suspension was centrifuged and the resulting protein pellet was dissolved in reaction buffer.

ATPase Assay. A spectrophotometric assay that couples the production of ADP to the oxidation of NADH was used to monitor the hydrolysis of ATP (*32*, *33*). Reactions were carried out at 37 °C in a buffer containing 25 mM Tris-acetate (pH 7.5), 0.1 mM dithiothreitol, 0.2 or 0.4 mg/mL NADH, 1 mM ATP, 1.5 mM phosphoenolpyruvate, 30 units/mL pyruvate kinase, 30 units/mL lactate dehydrogenase, and varying concentrations of magnesium acetate, as noted. All reaction mixtures contained the indicated nucleic acid at 3 μM and varying amounts of RecA protein, as noted. For reactions with RecA protein added first, a steady-state rate of ATP hydrolysis was attained before the addition of 0.6 μM SSB protein.

SSB Displacement Assays. The displacement of SSB protein from ssDNA by RecA protein was followed by monitoring the appearance of ATPase activity, as mentioned above, with 10 mM magnesium acetate. M13 ssDNA (3 μM) was incubated at 37 °C with 0.3 μM SSB protein for 5 min, after which time 1.5 μM RecA protein was added. The intrinsic fluorescence of the SSB protein was also used to measure the displacement of SSB protein from ssDNA (*34*). In a buffer containing 25 mM Tris-acetate (pH 7.5), 0.1 mM dithiothreitol, 10 mM magnesium acetate, 1 mM ATP, 1.5 mM phosphoenolpyruvate, and 30 units/mL pyruvate kinase, 3 μM M13 ssDNA was added to 0.3 μM SSB protein; after binding was observed, 1.5 μM RecA protein was added and the change in the fluorescence signal was measured.

Association Assay. The association of RecA protein with ssDNA was examined by monitoring the change in intrinsic fluorescence of etheno M13 DNA upon RecA binding (*20*). In a buffer consisting of 25 mM Tris-HCl (pH 7.5), 0.1 mM dithiothreitol, 150 mM NaCl, 1 mM ATP, and 1 mM magnesium acetate, at 20 °C, the association of 0.1 μM RecA protein with 6 μM etheno M13 DNA was followed. All of the RecA protein is assumed to be bound at the fluorescence signal plateau since the DNA is present in a 20-fold excess.

DNA Strand Exchange Assay. Reactions for examining DNA strand exchange activity were performed at 37 °C in a buffer containing 25 mM Tris-acetate (pH 7.5), 1 mM dithiothreitol, 6 mM magnesium acetate, 1 mM ATP, 3 mM phosphoenolpyruvate, and 10 units/mL pyruvate kinase (*35*). First, pBS ssDNA (5 μM) was incubated with either 3 μM RecA protein or 0.45 μM SSB protein for 1 min, and then whichever protein had not been added first was added next. After a further 1 min incubation, the reaction was started with the addition of 10 μM pBS linear dsDNA. Aliquots of the reaction mixture were removed at the specified time points, and the reaction was stopped by addition to a stop buffer at final concentrations of 4% Ficoll, 0.02 mM EDTA, 2% SDS, 0.05 mg/mL bromophenol blue, and 0.05 mg/mL xylene cyanol FF. Time points were run on a 1% agarose gel for 600 Vh, stained with ethidium bromide, and quantified using Gel Pro Analyzer (Media Cybernetics, L.P.) software.

RESULTS

The ssDNA-Dependent ATPase Activity Reveals that RecA P67W Protein Can Access ssDNA More Effectively than Wild-Type RecA Protein. Konola et al. (*12*, *13*) reported that RecA P67W protein is constitutive for LexA coprotease activity and moderately inhibited for homologous genetic recombination and recombinational DNA repair. ATP hydrolysis activity was measured to examine the formation and activity of RecA protein−ATP−ssDNA ternary complexes (*33*, *36*). The ATPase activity of wild-type RecA protein and RecA P67W protein, using M13 ssDNA as the nucleic acid cofactor in the absence and presence of SSB protein, is shown in Figure 1a. In the absence of SSB protein, RecA P67W protein is able to attain a higher rate of ATP hydrolysis than is the wild-type RecA protein. Additionally, wild-type RecA protein shows apparent saturation of the ssDNA at a protein concentration lower than that of RecA P67W protein. This indicates that RecA P67W protein is capable of binding more fully to M13 ssDNA, in the absence of SSB protein, than is wild-type RecA protein, which is partially inhibited because of the secondary structure present in the DNA.

The binding of SSB protein removes the secondary structure, making more of the M13 ssDNA available for wild-type RecA protein binding (*33*). The increase in the rate of hydrolysis upon addition of SSB protein is 45% for wild-type RecA protein; however, for RecA P67W protein, the effect is only 15% (Figure 1a). Both wild-type RecA protein and RecA P67W protein show a binding stoichiometry in the presence of SSB of approximately three nucleotides per RecA protein monomer (*33*, *37*). The k_{cat} values obtained from these data are 29 min^{-1} for wild-type RecA protein and 34 min^{-1} for RecA P67W protein. The finding that both enzymes have comparable k_{cat} values is also consistent with previous findings (*12*, *13*); however, as has been extensively documented for RecA protein (*1*, *9*, *33*, *34*, *37*), the observed k_{cat} values are approximately twice as great when the RecA protein concentration is greater than the ssDNA concentration (as in our experiments here), compared to the opposite experimental situation, which was used previously for RecA P67W protein (*13*).

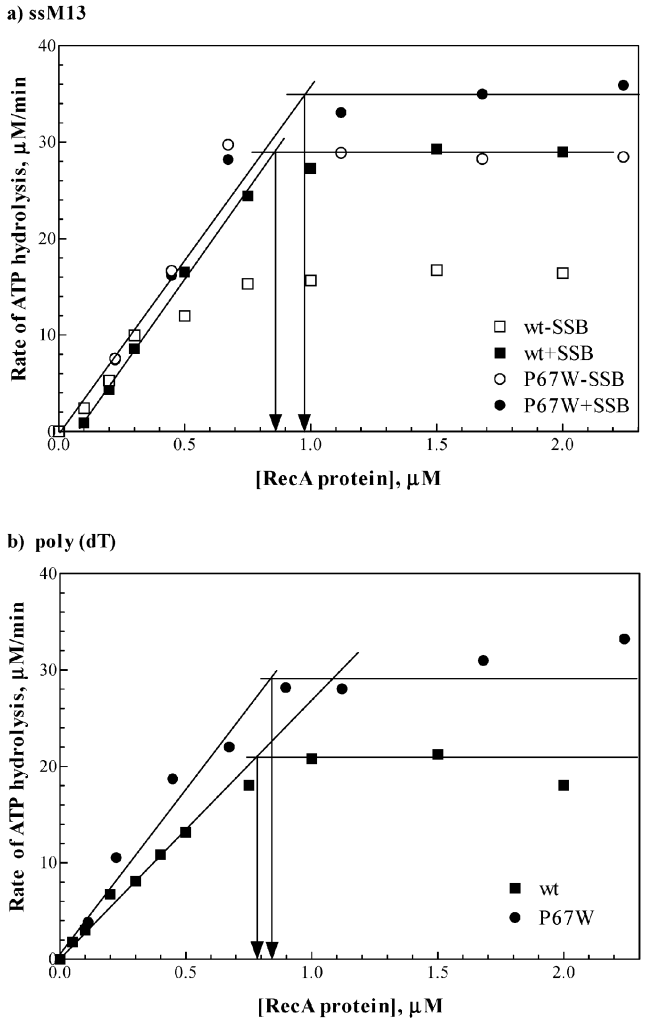

FIGURE 1: Dependence of ssDNA-dependent ATP hydrolysis activity on RecA protein concentration. RecA P67W protein can more efficiently use ssDNA within secondary structure. Varying concentrations of RecA protein, as indicated, were incubated with 3 μM ssDNA in a buffer containing 10 mM magnesium acetate, as described in Experimental Procedures. After a steady state of hydrolysis had been attained, 0.6 μM SSB protein was added, where noted. Empty symbols are for reaction mixtures without SSB protein, and filled symbols are for reaction mixtures containing SSB protein. The lines indicate the initial straight part of the rate vs concentration curve and the final (maximum) rate obtained; their point of intersection indicates the site size: (a) reactions that included M13 ssDNA and (b) reactions that included poly(dT).

The ATP hydrolysis activity was also assayed with poly(dT) as the nucleic acid cofactor (Figure 1b), since this DNA contains no secondary structure and the site size could be determined without addition of SSB protein. The binding site size is approximately 3.5 nucleotides per RecA protein monomer for both wild-type RecA protein and RecA P67W protein. Consistent with the results obtained with M13 ssDNA in the presence of SSB protein, RecA P67W protein shows a higher rate of hydrolysis as indicated by the corresponding k_{cat} values of 21 min^{-1} for wild-type RecA protein and 29 min^{-1} for RecA P67W protein [the lower rate of ATP hydrolysis seen with poly(dT) and wild-type RecA protein was observed previously (*28*), and likely results from the shorter DNA length (*33*)].

RecA P67W Protein Can Displace SSB Protein More Effectively than Wild-Type RecA Protein. The ability of RecA protein to displace SSB protein is dependent on the magnesium ion concentration; at 1 mM magnesium ion, wild-type RecA protein cannot displace SSB protein (*34*). However, the ATPase activity of RecA441, RecA803, and RecA730 proteins is not inhibited by SSB protein at 1 mM magnesium ion, revealing that these RecA proteins are able to compete with SSB protein for ssDNA binding under these conditions (*21*, *22*, *38*). The ATPase activity of RecA P67W protein was measured at various magnesium ion concentrations to investigate its ability to compete with SSB protein (added after RecA protein addition). In the absence of SSB protein, RecA P67W protein is able to attain a higher level of ATP hydrolysis activity than wild-type RecA protein at all magnesium acetate concentrations that were examined, as shown in Figure 2. The trend in activity without SSB protein present is similar for both RecA proteins, with a decrease in activity seen at 4 mM magnesium acetate, although this effect is more pronounced for wild-type RecA protein. In the presence of SSB protein, the trend changes dramatically only for wild-type RecA protein, but not significantly for RecA P67W protein. Wild-type RecA protein is inhibited by SSB protein at 1 mM magnesium acetate (*34*), whereas RecA P67W protein is able to maintain its high level of activity in the presence of SSB protein under all magnesium acetate conditions that were assayed, demonstrating its improved ability to displace SSB protein from ssDNA.

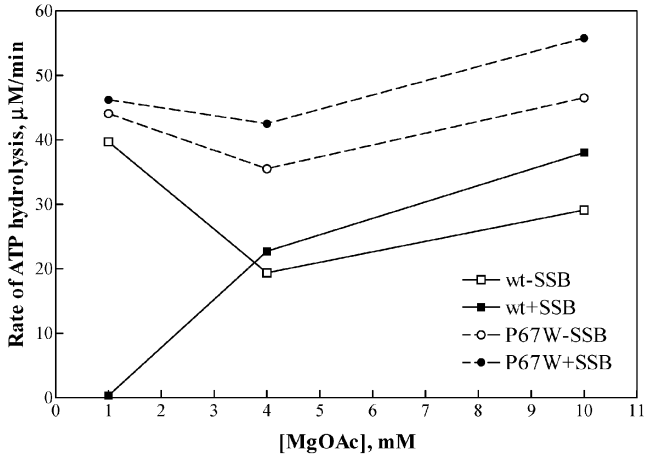

FIGURE 2: RecA P67W protein both utilizes ssDNA as a substrate for ssDNA-dependent ATP hydrolysis activity and resists inhibition by SSB protein better than wild-type RecA protein at all magnesium acetate concentrations. RecA protein (1.5 μM) was incubated with 3 μM ssDNA, and after a steady state of hydrolysis had been attained, 0.6 μM SSB protein was added, where noted. Empty symbols are for reaction mixtures without SSB protein, and filled symbols are for reaction mixtures containing SSB protein.

RecA P67W Protein Has Enhanced Duplex DNA-Dependent ATPase Activity. Duplex DNA is able to act as a polynucleotide cofactor for the hydrolysis of ATP by wild-type RecA protein (*36*, *39*−*41*). Hydrolysis is characterized by a lag phase, during which the DNA is proposed to be "opened" to permit binding to a strand of DNA, followed by a steady-state rate of ATP hydrolysis (*39*, *41*). RecA P67W protein was assayed for activity with dsDNA as the nucleic acid cofactor, as shown in Figure 3, and found to have a shorter lag time for the appearance of a steady state of hydrolysis than wild-type RecA protein. The lag times measured were 1150 and 380 s for wild-type RecA protein and RecA P67W protein, respectively. Additionally, RecA

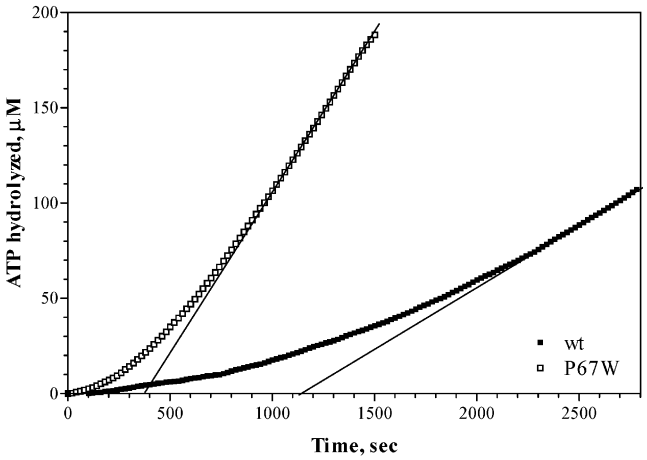

FIGURE 3: RecA P67W protein can use dsDNA as a substrate for DNA-dependent ATP hydrolysis activity better than wild-type RecA protein. pBS linear dsDNA (3 μM) was incubated with 1.5 μM RecA protein. The lag times were measured by extending the linear portion of the trace, after a steady state had been obtained, back to zero and determining their point of intersection.

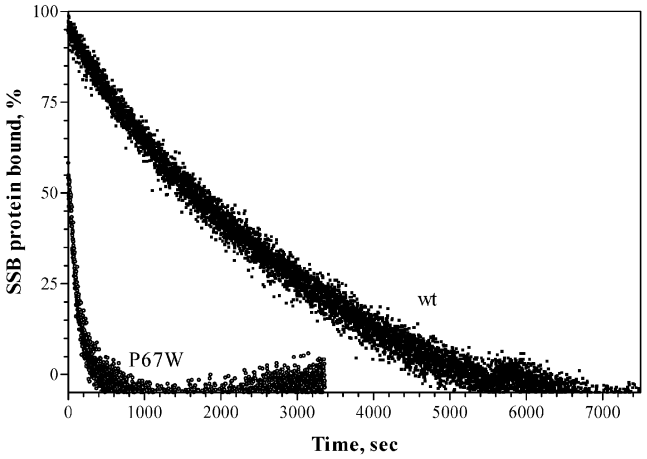

FIGURE 4: Direct measurement reveals that RecA P67W protein displaces SSB protein from M13 ssDNA more quickly than wild-type RecA protein. The intrinsic fluorescence of SSB protein was used to measure the displacement of SSB protein from ssDNA. SSB protein (0.3 μM) was added to 3 μM M13 ssDNA in a standard buffer containing 10 mM magnesium acetate. After binding was observed, 1.5 μM RecA protein was added and the change in fluorescence signal was measured. The percentage of SSB protein bound was calculated by comparing the unquenched SSB protein fluorescence measurement with the fluorescence measurement after the addition of M13 ssDNA and RecA protein (correcting for RecA protein fluorescence).

P67W protein was able to attain a higher final observed rate of hydrolysis, 10.2 μM/min, as compared to the wild-type RecA protein rate of 4.3 μM/min, at the same concentration of protein. These data suggest that RecA P67W protein has the ability to bind dsDNA and form an active nucleoprotein filament both faster and more extensively than wild-type RecA protein (*21*, *42*).

RecA P67W Protein Can Displace SSB Protein from M13 ssDNA Faster than Wild-Type RecA Protein. The binding of SSB protein to ssDNA prior to the addition of RecA protein acts as an impediment to RecA protein binding (*33*). To assess the ability of RecA P67W protein to compete for ssDNA binding sites, the displacement of SSB protein from M13 ssDNA by RecA protein was examined by monitoring the intrinsic fluorescence of SSB protein as shown in Figure 4. The rate of SSB protein displacement from the ssDNA is dramatically different for these two proteins; RecA P67W protein clearly displaces SSB protein considerably faster than does wild-type RecA protein. The time for half of the SSB protein to be displaced is approximately 5 and 1675 s for RecA P67W protein and wild-type RecA protein, respectively.

If ATP hydrolysis by RecA protein is monitored instead of SSB protein displacement, a lag is observed when SSB protein-coated ssDNA is used. This lag varies with experimental conditions, and corresponds to the kinetics of SSB protein displacement (*21*). Figure 5 shows the amount of ATP hydrolyzed in this experiment comparing wild-type RecA and RecA P67W proteins. Precoating ssDNA with SSB protein before the addition of RecA protein results in a lag of 600 and 150 s for wild-type and mutant RecA protein, respectively. The final observed rates of ATP hydrolysis, expressed as a percentage of the maximum rate (which is the rate measured with the subsequent addition of SSB protein), are different for each protein. RecA P67W protein can attain a higher rate of ATP hydrolysis, 91% of its maximum rate, indicating more complete displacement of SSB protein. Wild-type RecA protein reaches only 39% of its maximum activity, indicating that it is more inhibited by SSB protein. The displacement of SSB protein by RecA P67W protein is both faster and more complete than for wild-type RecA protein. The onset of ATP hydrolysis is delayed relative to the direct displacement fluorescence measurements as was seen previously (*22*); this difference might suggest the need for a requisite amount of SSB protein displacement prior to formation of an active nucleoprotein filament before observation of ATPase activity (*22*).

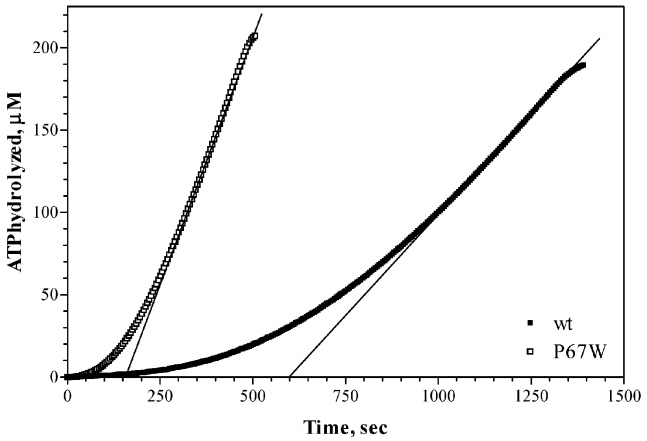

FIGURE 5: Displacement of SSB protein measured as the appearance of ATP hydrolysis by RecA protein concurs with the direct displacement measurements. M13 ssDNA (3 μM) was incubated at 37 °C with 0.3 μM SSB protein in a standard buffer containing 10 mM magnesium acetate for 5 min, after which time 1.5 μM RecA protein was added. The lag times were measured by extending the linear portion of the trace, after a steady state had been obtained, back to zero and determining their point of intersection.

Association of RecA Protein with Etheno M13 DNA. The enhanced ability to displace SSB protein observed for RecA441, RecA730, and RecA803 proteins was shown to be due to an increased rate of association with ssDNA, as compared to that of wild-type RecA protein (*21*, *22*, *35*, *38*). The kinetics of RecA protein association were examined using a fluorescence assay which measures the increase in the intrinsic fluorescence of etheno M13 DNA that ac-

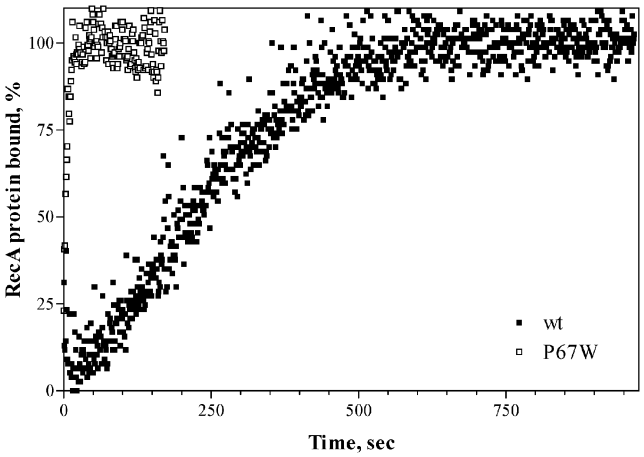

FIGURE 6: RecA P67W protein associates with etheno M13 ssDNA more rapidly than wild-type RecA protein. The association of 0.1 μM RecA protein with 6 μM etheno M13 ssDNA was followed by monitoring the change in intrinsic fluorescence of the DNA over time.

companies the binding of RecA protein. The association of wild-type RecA protein and RecA P67W protein was assessed at 25 °C and 1 mM $MgCl_2$ to slow the binding enough to make it observable with this assay (*20*); the results are shown in Figure 6. Even with the suboptimal conditions for ssDNA binding, the association of RecA P67W protein with etheno M13 DNA is too fast to monitor accurately. The difference between the rates of association of RecA P67W protein and wild-type RecA protein is clear; RecA P67W protein associates with the DNA considerably faster. The half-time for RecA protein binding is approximately 230 and 3 s for wild-type and RecA P67W proteins, respectively. RecA P67W protein has a greatly increased rate of association with ssDNA, as compared to wild-type RecA protein, which correlates with the enhanced ability to displace SSB protein for binding to ssDNA.

RecA P67W Protein Can Catalyze DNA Strand Exchange. The order of RecA protein and SSB protein addition affects DNA strand exchange; the prior addition of SSB protein to ssDNA inhibits the activity of wild-type RecA protein (*37*, *43*). As shown by the aforementioned SSB protein displacement assays, RecA P67W protein is able to bind ssDNA coated with SSB protein more efficiently than is wild-type RecA protein. To determine the effect of SSB protein on the DNA strand exchange activity of RecA P67W protein, the activity was examined with either prior or subsequent addition of SSB protein; the results are shown in Figure 7. The addition of RecA protein prior to the addition of SSB protein resulted in approximately 90% of the dsDNA being utilized by both RecA proteins after 60 min (Figure 7a, lanes 4 and 7). However, wild-type RecA protein forms more nicked circular heteroduplex product under these conditions than does RecA P67W protein, 84 versus <1%, respectively. The majority of the dsDNA is contained in joint molecules in the RecA P67W reaction, perhaps the result of a decrease in the efficiency of branch migration or a reinvasion of the displaced DNA strand.

The effect of SSB protein binding to ssDNA prior to RecA protein on DNA strand exchange is shown in Figure 7b. The rate of uptake of dsDNA is decreased for both RecA proteins compared to that in reactions in which SSB protein was added second: 24% dsDNA usage for wild-type RecA

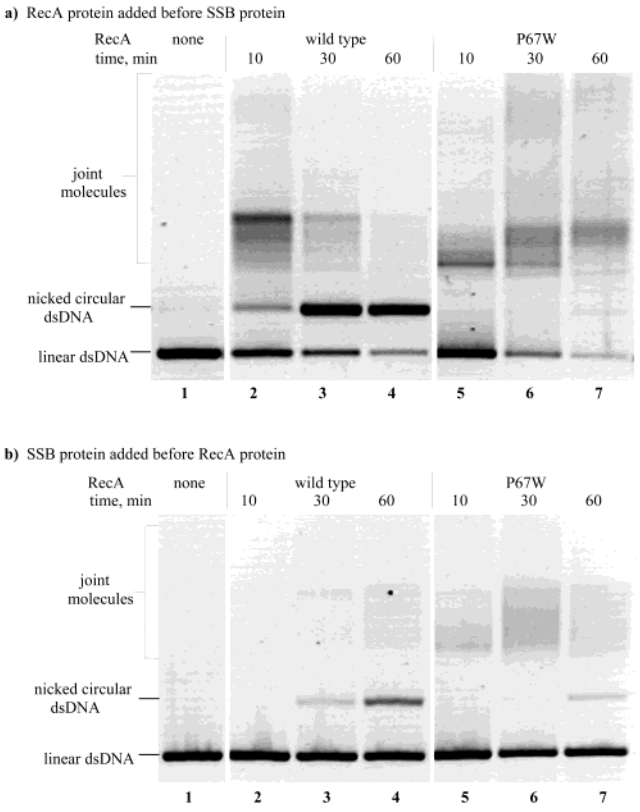

FIGURE 7: DNA strand exchange promoted by wild-type and mutant RecA proteins. First, pBS ssDNA (5 μM) was incubated with either 3 μM RecA protein or 0.45 μM SSB protein for 1 min, and then whichever protein had not been added first was added second. After a further 1 min incubation, the reaction was started with the addition of 10 μM linear pBS dsDNA: (a) the addition of RecA protein before SSB protein and (b) the addition of SSB protein before RecA protein. Lane 1 in both panels shows a control reaction lacking proteins; lanes 2−4 show wild-type RecA protein reactions, and lanes 5−7 show RecA P67W protein reactions. The figure was created using Adobe Photoshop 5.0 and Micrografx Designer to select the lanes of interest and to adjust the contrast for publication; quantification was performed using Image-QuaNT software and the original image.

protein and 45% for RecA P67W protein. This result is similar to the DNA strand exchange activity observed for RecA441 protein (*35*); the addition of SSB protein prior to RecA protein diminishes the amount of dsDNA utilization relative to the subsequent addition of SSB protein. This reduction in the level of dsDNA usage is smaller in magnitude for RecA P67W protein than wild-type RecA protein due to its enhanced ability to displace SSB protein from ssDNA. However, as before, wild-type RecA protein forms more nicked circular heteroduplex product than RecA P67W protein, 25 versus 8%, respectively. Although the amount of dsDNA uptake by RecA P67W protein is decreased in comparison to that found in the experiments that had subsequent addition of SSB protein, the amount of nicked circular product that is formed is increased. Again, however, the majority of the substrate dsDNA that participated in DNA heteroduplex formation in the RecA P67W protein reaction was converted to joint molecules.

DISCUSSION

The mutation of residue 67 from Pro to Trp in the RecA protein results in constitutive coprotease and reduced re-

combinational DNA repair activity (*12*, *13*). In this study, we examined the biochemical properties of RecA P67W protein *in vitro* in an attempt to explain the altered phenotype. RecA P67W protein was found to be enhanced in several activities, compared to wild-type RecA protein. This mutant protein exhibited an improved ability to bind ssDNA within secondary structure, a faster displacement of SSB protein, an enhanced ability to bind dsDNA, and a more rapid association with ssDNA. In a manner similar to that proposed for RecA441 and RecA730 proteins (*21*, *22*), the enhanced ability to displace SSB protein gives RecA P67W protein the ability to access ssDNA that occurs naturally in the cell in the absence of DNA-damaging agents.

It is possible that the ability to bind duplex DNA more effectively could lead to a constitutive coprotease phenotype. RecA1202 and RecA1211 proteins have more coprotease activity with dsDNA than wild-type RecA protein (*15*). An increase in the extent and rate of RecA P67W protein binding to dsDNA was observed (Figure 3). However, the use of dsDNA as a nucleic acid cofactor for induction of the SOS response by RecA P67W protein is unlikely since dsDNA was found to be ineffective in promoting coprotease activity *in vitro* (*13*).

RecA441, RecA730, and RecA803 proteins have been shown to possess an improved ability to compete with SSB protein for the binding of ssDNA (*22*, *35*, *38*, *44*). The competition of RecA protein with SSB protein is known to parallel the rate of association; hence, the improvement in the competitive ability of these mutant proteins is the result of a faster rate of association with ssDNA (*21*, *22*, *35*, *38*). The enhanced ability to compete with SSB protein for ssDNA may allow more ssDNA to be available for RecA protein binding, such as that naturally existing in the cell, without the addition of DNA-damaging agents (*22*). *RecA441* and *RecA730* strains are constitutive for SOS induction, although a *RecA803* strain is not, which suggests a minimum level of SSB protein displacement may be required (*22*). These proteins may be able to bind the transient ssDNA in an undamaged cell, such as at replication forks. In support of this, the coprotease activity of RecA441 protein can be suppressed by the *dnaB252* mutation, in which initiation of replication is inhibited, thereby eliminating replication forks and hence the available ssDNA (*45*). As is seen with RecA441 and RecA730 proteins, the data in this study suggest that the constitutive coprotease activity of RecA P67W protein can be credited to an enhanced ability to displace SSB protein. This enhanced competitiveness of RecA P67W protein is the result of an increased rate of association with ssDNA, as is also the case with RecA441 and RecA730 proteins (*22*).

Although RecA P67W protein shows an improved uptake of dsDNA in forming joint molecules, the yield of nicked circular dsDNA product is less than that of wild-type RecA protein. The formation of joint molecules by RecA P67W protein without their resolution to the final products of DNA strand exchange is possibly the result of either a slower rate of DNA heteroduplex extension, perhaps by the enhanced rate of dsDNA binding, or reinvasion by the displaced DNA strand. The branch migration process might also be hampered by a limited amount of reinvasion by the displaced DNA strand, which would result from the enhanced ability of RecA P67W protein to displace SSB protein, as is seen for RecA441 protein (*35*). However, if reinvasion by the displaced strand were occurring, an increase in the extent of homology-dependent network formation would be expected (*35*), yet this is not the case for RecA P67W protein; therefore, the increased level of dsDNA binding may be the contributing factor. *In vivo*, the joint molecules formed may not mature into stable recombination intermediates due to the instability of the complexes at this slower rate of DNA heteroduplex extension, and they may also be subjected to local reinvasion by the displaced DNA strand. The decreased level of conversion of joint molecules to fully exchanged product may result in the defective recombination phenotype.

In summary, the constitutive coprotease activity of RecA P67W protein can be explained by its enhanced ability to displace SSB protein from ssDNA. In the absence of DNA damage, RecA P67W protein may be able to displace SSB protein from replication forks and become activated for LexA repressor cleavage. The faster and more extensive displacement of SSB protein by RecA P67W protein, as compared to that of wild-type RecA protein, is the result of its increased rate of association with ssDNA. The observed reduction in the extent of recombinational repair may result from an impaired ability of RecA P67W protein to produce heteroduplex DNA that is sufficiently extended to be stable for subsequent maturation into recombinant products (*12*, *13*).

ACKNOWLEDGMENT

We thank the members of our laboratory, Piero Bianco, Mark Dillingham, Naofumi Handa, Cynthia Hazeltine, Noriko Kantake, Alex Mazin, Olga Mazin, Jim New, and Yun Wu for their helpful comments and critical reading of the manuscript. We are grateful to Kendall Knight for sending the strains used in the isolation of RecA P67W protein.

REFERENCES

1. Bianco, P. R., and Kowalczykowski, S. C. (1999) in *Encyclopedia of Life Sciences*, Nature Publishing Group, London.
2. Eggleston, A. K., and Kowalczykowski, S. C. (1991) *Biochimie 73*, 163−176.
3. Roca, A. I., and Cox, M. M. (1997) *Prog. Nucleic Acid Res. Mol. Biol. 56*, 129−223.
4. Menetski, J. P., and Kowalczykowski, S. C. (1985) *J. Mol. Biol. 181*, 281−295.
5. Menetski, J. P., Varghese, A., and Kowalczykowski, S. C. (1988) *Biochemistry 27*, 1205−1212.
6. Stasiak, A., and Egelman, E. H. (1988) in *Genetic Recombination* (Kucherlapati, R., and Smith, G. R., Eds.) pp 265−308, American Society for Microbiology, Washington, DC.
7. Heuser, J., and Griffith, J. (1989) *J. Mol. Biol. 210*, 473−484.
8. DiCapua, E., Schnarr, M., Ruigrok, R. W., Lindner, P., and Timmins, P. A. (1990) *J. Mol. Biol. 214*, 557−570.
9. Kowalczykowski, S. C. (1991) *Annu. Rev. Biophys. Biophys. Chem. 20*, 539−575.
10. Walker, J. E., Saraste, M., Runswick, M. J., and Gay, N. J. (1982) *EMBO J. 1*, 945−951.
11. Logan, K. M., and Knight, K. L. (1993) *J. Mol. Biol. 232*, 1048−1059.
12. Konola, J. T., Logan, K. M., and Knight, K. L. (1994) *J. Mol. Biol. 237*, 20−34.
13. Konola, J. T., Nastri, H. G., Logan, K. M., and Knight, K. L. (1995) *J. Biol. Chem. 270*, 8411−8419.
14. McEntee, K., and Weinstock, G. M. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 6061−6065.
15. Wang, W.-B., Tessman, E. S., and Tessman, I. (1988) *J. Bacteriol. 170*, 4823−4827.
16. Wang, W.-B., Sassanfar, M., Tessman, I., Roberts, J. W., and Tessman, E. S. (1988) *J. Bacteriol. 170*, 4816−4822.

17. Story, R. M., Weber, I. T., and Steitz, T. A. (1992) *Nature 355*, 318−325.
18. Story, R. M., and Steitz, T. A. (1992) *Nature 355*, 374−376.
19. Yu, X., and Egelman, E. H. (1993) *J. Mol. Biol. 231*, 29−40.
20. Menetski, J. P., and Kowalczykowski, S. C. (1990) *J. Mol. Biol. 211*, 845−855.
21. Lavery, P. E., and Kowalczykowski, S. C. (1988) *J. Mol. Biol. 203*, 861−874.
22. Lavery, P. E., and Kowalczykowski, S. C. (1992) *J. Biol. Chem. 267*, 20648−20658.
23. Messing, J. (1983) *Methods Enzymol. 101*, 20−78.
24. Birnboim, H. C., and Doly, J. (1979) *Nucleic Acids Res. 7*, 1513−1523.
25. Vinograd, J., and Leibowitz, J. (1966) *J. Gen. Physiol. 49*, 103−125.
26. Griffith, J., and Shores, C. G. (1985) *Biochemistry 24*, 158−162.
27. Kowalczykowski, S. C., Burk, D. L., and Krupp, R. A. (1989) *J. Mol. Biol. 207*, 719−733.
28. Kowalczykowski, S. C., and Krupp, R. A. (1989) *J. Mol. Biol. 207*, 735−747.
29. Baumann, P., Benson, F. E., Hajibagheri, N., and West, S. C. (1997) *Mutat. Res. 384*, 65−72.
30. LeBowitz, J. (1985) Ph.D. Thesis, Johns Hopkins University, Baltimore, MD.
31. Ruyechan, W. T., and Wetmur, J. G. (1975) *Biochemistry 14*, 5529−5534.
32. Kreuzer, K. N., and Jongeneel, C. V. (1983) *Methods Enzymol. 100*, 144−160.
33. Kowalczykowski, S. C., and Krupp, R. A. (1987) *J. Mol. Biol. 193*, 97−113.
34. Kowalczykowski, S. C., Clow, J. C., Somani, R., and Varghese, A. (1987) *J. Mol. Biol. 193*, 81−95.
35. Lavery, P. E., and Kowalczykowski, S. C. (1990) *J. Biol. Chem. 265*, 4004−4010.
36. Roman, L. J., and Kowalczykowski, S. C. (1986) *Biochemistry 25*, 7375−7385.
37. Menetski, J. P., and Kowalczykowski, S. C. (1989) *Biochemistry 28*, 5871−5881.
38. Madiraju, M. V. V. S., Lavery, P. E., Kowalczykowski, S. C., and Clark, A. J. (1992) *Biochemistry 31*, 10529−10535.
39. Kowalczykowski, S. C., Clow, J., and Krupp, R. A. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 3127−3131.
40. Pugh, B. F., and Cox, M. M. (1987) *J. Biol. Chem. 262*, 1337−1343.
41. Pugh, B. F., and Cox, M. M. (1988) *J. Mol. Biol. 203*, 479−493.
42. Lu, C., and Echols, H. (1987) *J. Mol. Biol. 196*, 497−504.
43. Cox, M. M., and Lehman, I. R. (1982) *J. Biol. Chem. 257*, 8523−8532.
44. Madiraju, M. V. V. S., Templin, A., and Clark, A. J. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 6592−6596.
45. D'Ari, R., George, J., and Huisman, O. (1979) *J. Bacteriol. 140*, 381−387.

BI027232Q