Enzymatic Properties of the RecA803 Protein, a Partial Suppressor of *recF* Mutations†

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ABSTRACT: The RecA803 protein suppresses the recombinational repair defect of *recF* mutations and displays enhanced joint molecule formation in vitro (Madiraju et al., 1988). To understand the physical basis for these phenomena, the biochemical properties of RecA803 protein were compared with those of the wild-type protein. The RecA803 protein shows greater DNA-dependent ATPase activity than the wild-type protein with either M13 single-stranded (ss) DNA, which contains secondary structure, or double-stranded DNA. This increased activity reflects an enhanced ability of the mutant protein to form active complexes with these DNA molecules rather than an enhanced catalytic turnover activity, because identical k_{cat} values for ATP hydrolysis are obtained when DNA substrates lacking secondary structure are examined. In addition, the ssDNA-dependent ATPase activity of RecA803 protein displays greater resistance to inhibition by SSB (single-stranded DNA binding) protein. These properties of the RecA803 protein are not due to either an increased binding affinity for ssDNA or an increased kinetic lifetime of RecA803 protein–ssDNA complexes, demonstrating that altered protein–DNA stability is not the basis for the enhanced properties of RecA803 protein. However, the nucleation-limited rate of association with ssDNA is more rapid for the RecA803 protein than for wild-type RecA protein. Consequently, we suggest that altered protein–protein interactions may account for the differences between these two proteins. The implications of these results with regard to the partial suppression of *recF* mutations by *recA803* are discussed (Madiraju et al., 1988).

The protein encoded by the *recA* gene of *Escherichia coli* plays a vital role in homologous genetic recombination (Clark & Margulies, 1965). Extensive in vitro characterization of the RecA protein has identified a variety of biochemical properties that contribute to this recombinational role in vivo (Cox & Lehman, 1987; Radding, 1988; Kowalczykowski, 1991a,b). These properties include the ability to form a helical filament with single-stranded or double-stranded DNA (ssDNA or dsDNA)[1] (Dunn et al., 1982; Flory & Radding, 1982; Stasiak et al., 1981), the hydrolysis of ATP, the renaturation of denatured DNA, and the pairing and exchange of homologous DNA.

Although RecA protein, alone, can promote both homologous pairing and DNA strand exchange and the cleavage of cellular repressor proteins in vitro, genetic data implicate the involvement of other gene products, or accessory factors, in these in vivo processes [see Clark and Low (1988) for a review]. The RecF protein is one such accessory factor; mutations in *recF* cause defects in both DNA recombination and repair. However, these defects in *recF* can be partially suppressed by certain extragenic mutations that map to the *recA* gene. One of these mutations, *recA803*, results in cells that can overcome the need for RecF protein in recombinational repair, but not for induction of the *lexA* regulon, suggesting that the activities of the RecA803 protein have been altered to compensate partially for the loss of functions normally provided by the RecF protein in vivo.

In an effort to understand RecF protein function, the ability of the RecA803 protein to carry out joint molecule formation in vitro was examined (Madiraju et al., 1988). These initial studies demonstrated that, under certain conditions, RecA803 protein promotes joint molecule formation at a higher initial rate and to a greater final yield than wild-type RecA protein. These conditions involved the use of ssDNA substrates that are suboptimal for wild-type protein activity: (1) ssDNA containing secondary structure and (2) ssDNA precomplexed with SSB protein. Filaments of RecA protein and ssDNA are obligatory intermediates in the reaction between ssDNA and homologous dsDNA to form joint molecules (Cox & Lehman, 1987; Radding, 1988; Kowalczykowski, 1991a,b). Since the binding of RecA and SSB proteins to ssDNA is competitive (Kowalczykowski & Krupp, 1987), filament formation requires that RecA protein compete effectively with SSB protein. The outcome of this competition using mutant RecA proteins in vitro correlates with the phenotype of *recA* mutations, suggesting that SSB protein displacement is essential to proper RecA protein function both in vitro and in vivo (Kowalczykowski, 1991b). Initial observations with RecA803 protein suggested that it is capable of both the utilization of secondary structures present in ssDNA and the efficient displacement of SSB protein from ssDNA. Consequently, we hypothesized that the partial suppression by *recA803* of a *recF* mutant phenotype defect is due to its enhanced ability to carry out recombinational repair reactions

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[1] Abbreviations: ssDNA, single-stranded DNA; dsDNA, double-stranded DNA; SSB protein, *Escherichia coli* single-stranded DNA binding protein; RecA(wt), wild-type RecA protein; etheno M13 DNA, M13 ssDNA containing 1,N^6-ethenoadenine and 3,N^4-ethenocytidine residues; poly(dT), poly(thymidylic acid).

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in vivo with native ssDNA or with ssDNA complexed with SSB protein (Madiraju et al., 1988).

If the mutation at codon 37 that gives rise to the RecA803 mutation (Madiraju et al., 1988) resulted in a mutant protein with an enhanced ability to interact with DNA, this could explain the enhanced activity of RecA803 protein in joint molecule formation. This possible new property, resulting from an altered protein–DNA interaction, would contribute to the enhanced ability of RecA803 protein to compete effectivley with SSB protein for ssDNA binding. This hypothesis is consistent with results showing that the recombinational repair defect associated with the *ssb*-113 mutation is partially suppressed by *recA803* (Madiraju & Clark, 1990), since the SSB-113 protein binds ssDNA more tightly than wild-type SSB protein (Chase & Williams, 1986). Therefore, Madiraju and Clark (1990) suggested that RecF protein could assist RecA protein to overcome inhibition caused by SSB protein. Using a compelmentary approach, Moreau (1987, 1988) concluded that wild-type cells overproducing SSB protein show a *recF* mutant phenotype suggesting that RecF and SSB proteins compete for ssDNA binding in vivo.

In the present investigation, we test the hypothesis that RecA803 protein has an enhanced ability to interact with DNA. The binding of RecA protein to ssDNA in the presence of ATP results in the hydrolysis of ATP. Therefore, ATP hydrolysis is a measure of the formation of a ternary complex of RecA protein, ATP, and DNA, even though hydrolysis facilitates dissociation of the complex rather than the binding of RecA protein to DNA (Menetski & Kowalczykowski, 1985). Another method to measure both the formation and stability of ternary complexes is to use the chemically-modified fluorescent M13 ssDNA called etheno M13 DNA (Silver & Fersht, 1982; Menetski & Kowalczykowski, 1985). The binding of RecA protein to this DNA results in the formation of complexes whose characteristic fluorescence and stability depend on the nucleotide cofactor present (Menetski & Kowalczykowski, 1985). We find that, despite our initial expectation, the stability of the RecA803 protein–ssDNA complex is unaltered; however, the rate of RecA803 protein association with ssDNA is enhanced. The results obtained are discussed in terms of the ability of the *recA803* protein partially to suppress *recF* mutations (Madiraju et al., 1988).

EXPERIMENTAL PROCEDURES

Chemicals and Buffers. ATP and ADP were purchased from Pharmacia P-L Biochemicals and were dissolved in distilled H_2O as concentrated stock solutions at pH 7.5. NADH was obtained from Sigma Chemical Co. Unless otherwise mentioned, all reactions were carried out in buffer consisting of 25 mM Tris-HCl (pH 7.5) and 0.1 mM dithiothreitol (TD buffer); $MgCl_2$ at the indicated concentration was added. Whenever ATP was used, an ATP regenerating system consisting of 2 units of pyruvate kinase/mL and 1.5 mM phosphoenolpyruvate was included.

Proteins. Wild-type RecA protein was purified from *E. coli* strain JC12772 (Uhlin & Clark, 1981) using a procedure (S. Kowalczykowski, unpublished results) based on precipitation by spermidine acetate (Griffith & Shores, 1985). RecA803 protein was purified from *E. coli* strain JC15369 as described (Madiraju et al., 1988). SSB protein was purified from *E. coli* strain RLM727 as described by LeBowitz (1985). Protein concentrations were determined at 280 nm using molar extinction coefficients of 2.7×10^{-4} M^{-1} cm^{-1} for RecA protein and 3×10^4 M^{-1} cm^{-1} for SSB protein.

DNA. Both ssDNA and dsDNA were prepared from bacteriophage M13mp7 using the procedure described by Messing (1983). Nucleotide concentrations were determined at 260 nm using molar extinction coefficients of 6500 M^{-1} cm^{-1} for dsDNA and 8780 M^{-1} cm^{-1} for ssDNA. Linear dsDNA was obtained by digesting circular dsDNA with *Eco*RI restriction nuclease followed by phenol extraction. Etheno M13 DNA was obtained by treating M13mp7 circular ssDNA with chloroacetaldehyde as described (Menetski & Kowalczykowksi, 1985); its concentration was determined using an extinction coefficient of 7000 M^{-1} cm^{-1} at 260 nm (Menetski & Kowalczykowski, 1985). Poly(dT) was purchased from P-L Biochemicals, and its concentration was determined using an extinction coefficient of 8540 M^{-1} cm^{-1} (Menetski & Kowalczykowski, 1987).

ATPase Assay. The hydrolysis of ATP was monitored using a spectrophotometric assay described by Kreuzer and Jongeneel (1983) and as adapted for use with RecA protein by Kowalczykowski and Krupp (1987). This assay is based on the fact that upon hydrolysis of ATP to ADP, 1 equiv of phosphoenolpyruvate is converted to pyruvate by pyruvate kinase, resulting in the regeneration of ATP from ADP. In the presence of lactate dehydrogenase, pyruvate is reduced to lactate with the concurrent oxidation of 1 equiv of NADH to NAD^+. The resultant decrease in NADH absorbance at 340 nm is followed spectrophotometrically. The rate of change of the absorbance (decrease) at 340 nm is directly proportional to the steady-state rate of ATP hydrolysis; at 340 nm, a decrease of 1 OD unit is equivalent to the hydrolysis of 0.16 mM ATP (Kowalczykowski & Krupp, 1987). Phosphoenolpyruvate, at a final concentration of 1.5 mM, and pyruvate kinase and lactate dehydrogenase, at approximately 2.5 units/mL each, were used in all reactions. M13mp7 circular ssDNA, linear M13 dsDNA, and poly(dT) were used as indicated.

DNA Binding Assay. The binding of RecA protein to etheno M13 DNA was measured as described (Menetski & Kowalczykowski, 1985). Titration of the DNA with protein (referred to as a "protein titration") was carried out in TD buffer by adding aliquots of concentrated RecA protein to 3 μM etheno M13 DNA and measuring the increase in fluorescence upon complex formation. The stability of the RecA protein–DNA complexes to disruption by salt (referred to as a "salt titration") was measured by adding aliquots of concentrated NaCl to complexes of RecA protein and etheno M13; the salt titration midpoint is defined as the concentration of NaCl required to dissociate half of the protein–DNA complexes as measured by the accompanying fluorescence decrease (Menetski & Kowalczykowski, 1985).

Transfer Reactions. RecA protein–etheno M13 DNA complexes were formed in TD buffer containing the indicated concentration of $MgCl_2$, 4.2 μM etheno M13 DNA, 0.56 μM RecA protein, and nucleotide cofactors at the indicated concentration. The addition of poly(dT) results in a decrease in etheno M13 DNA fluorescence as RecA protein transfers to the poly(dT). At zero time, 25 μM poly(dT) was added, and the decrease in the fluorescence was monitored as described (Menetski & Kowalczykowski, 1987).

Kinetic Association Experiments. The kinetics of association of RecA protein with etheno M13 DNA were measured as described (Menetski & Kowalczykowski, 1990). Reactions were in TD buffer containing 4 mM $MgCl_2$, 150 mM NaCl, 500 μM ATP, and 6 μM etheno M13 DNA and were initiated by addition of 0.1 μM RecA protein.

RESULTS

ATP Hydrolysis by RecA803 Protein. The formation of a ternary nucleoprotein (presynaptic) complex containing

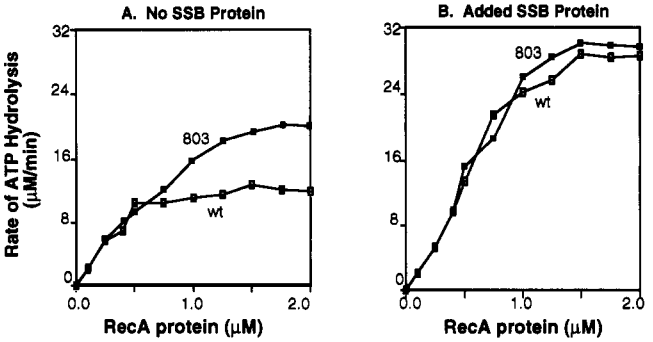

FIGURE 1: RecA protein concentration dependence of ssDNA-dependent ATPase activity. All reactions were performed at 37 °C in buffer containing 10 mM $MgCl_2$, 1 mM ATP, and 3 μM ssDNA. In panel A, SSB protein was omitted. In panel B, 0.6 μM SSB protein was added immediately following RecA protein addition. Open squares represent wild-type RecA protein, and closed squares represent RecA803 protein.

RecA protein, ssDNA, and ATP is an essential step in joint molecule formation. A consequence of complex formation is the hydrolysis of ATP by RecA protein (Cox & Lehman, 1987; Radding, 1988; Kowalczykowski, 1991a). The amount of complex formed by RecA protein depends on several factors and can be limited both by DNA secondary structure and by SSB protein bound to ssDNA. Since presynaptic complex formation is a prerequisite to joint molecule formation, we examined whether RecA803 protein was more proficient in forming this complex than wild-type protein. This was assessed by measuring the ssDNA-dependent ATPase activity at different RecA protein concentrations using M13 ssDNA. M13 ssDNA has regions of secondary structure that limit binding of RecA protein; consequently, the observed rate of ATP hydrolysis is reduced, relative to that observed with ssDNA that has no secondary structure, when the concentration of RecA protein is not limiting (Kowalczykowski & Krupp, 1987). Figure 1A shows that the steady-state rate of ATP hydrolysis is the same for both proteins up to about 0.5 μM RecA protein. At this protein concentration, the molar ratio of RecA protein to ssDNA (in nucleotide residues) is 1:6 and is below saturation of the ssDNA. This result demonstrates that when ssDNA is not limiting, the rate of ATP hydrolysis is essentially identical for both proteins. This, in turn, implies that the k_{cat} for ATP hydrolysis (i.e., moles of ATP hydrolyzed per mole of protein per minute) is identical for both proteins. Above 0.5 μM RecA protein, however, the rate of ATP hydrolysis by wild-type RecA protein saturates, while that of RecA803 protein continues to increase. This observation demonstrates that the ssDNA available for protein binding becomes limiting for wild-type protein but not for RecA803 protein and suggests that the RecA803 protein can use more of the ssDNA that is sequestered in secondary structure than wild-type protein. Consistent with these statements, the observed rates of ATP hydrolysis in the plateau region for each protein are approximately equal (i.e., per micromolar protein–DNA complex), when normalized to reflect the fact that, in the plateau region, nearly 2-fold more RecA803 protein is involved in formation of the complex with M13 ssDNA (as determined from the intersection of the initial linear portion of Figure 1A with a horizontal extension of the respective plateau region).

The inhibitory effects of DNA secondary structure on complex formation can be alleviated by the addition of SSB protein (Kowalczykowski & Krupp, 1987). To verify that the differences noted in Figure 1A are due solely to a greater ability of RecA803 protein to utilize secondary structure within M13 ssDNA for ATP hydrolysis, assays in the presence of SSB protein were conducted. Figure 1B shows that, in the presence of SSB protein, the steady-state rate of ATP hydrolysis for each protein is essentially the same at all protein concentrations, further demonstrating that the k_{cat} values for ATP hydrolysis are identical (Figure 1B). These data confirm that the differences observed between the two proteins in Figure 1A are due to their different abilities to invade secondary structures present in ssDNA, rather than to differences in their turnover numbers for ATP hydrolysis. However, since the maximal rate of ATP hydrolysis by RecA803 protein is greater in the presence of SSB protein than in its absence (compare Figure 1B with Figure 1A), there is some DNA secondary structure which RecA803 protein alone is incapable of invading. Finally, in complete agreement with the results obtained with the native M13 ssDNA, the steady-state rate of ATP hydrolysis is identical at all concentrations of wild-type and RecA803 proteins when poly(dT) (which is devoid of secondary structures) is used instead of M13 ssDNA (data not shown). Thus, RecA803 and wild-type RecA proteins do not differ in their intrinsic rate of ATP turnover, but rather in their ability to utilize regions of DNA secondary structure.

RecA803 Protein Is Less Sensitive to Competitive Effects of SSB Protein. SSB protein can either stimulate or inhibit RecA protein-dependent reactions depending on both the order in which RecA and SSB proteins are bound to ssDNA and the reaction conditions. When SSB is added to preformed complexes of RecA protein, ssDNA, and ATP, it disrupts the DNA secondary structures and permits RecA protein to form efficient presynaptic complexes; this results in stimulation of both ssDNA-dependent ATPase activity (Kowalczykowski & Krupp, 1987) and joint molecule formation (Cox & Lehman, 1981). In contrast, when SSB protein is complexed with ssDNA prior to RecA protein addition, it resists displacement by RecA protein, reducing both ssDNA-dependent ATPase activity (Kowalczykowski & Krupp, 1987) and joint molecule formation (Cox & Lehman, 1981). When such preformed complexes of SSB protein and ssDNA are used to assay ATP hydrolysis by RecA protein, the time course for ATP hydrolysis displays a pronounced pre-steady-state (lag) phase (Lavery & Kowalczykowski, 1988) that parallels the physical displacement of SSB protein from the DNA (Lavery & Kowalczykowski, 1992). Since joint molecule formation by RecA803 protein is less inhibited than wild-type RecA protein by the prior binding of SSB protein to ssDNA (Madiraju et al., 1988), we imagined that RecA803 protein could displace SSB protein from ssDNA more effectively than wild-type RecA protein. To test this prediction, the rate of ssDNA-dependent ATP hydrolysis was measured at various times after addition of RecA protein to previously formed complexes of SSB protein and ssDNA. The results indicate that RecA803 protein catalyzes more ATP hydrolysis than wild-type RecA protein at all times (Figure 2) and also show that there is a more rapid acceleration of ATP hydrolysis rates for RecA803 protein than for wild-type RecA protein. These data suggest that RecA803 protein replaces SSB protein on the ssDNA more rapidly and completely than wild-type RecA protein.

The ability of wild-type RecA protein to displace SSB protein from ssDNA is sensitive to magnesium ion concentration (Kowalczykowski & Krupp, 1987). At 10 mM $MgCl_2$, wild-type RecA protein can compete with SSB protein for binding to ssDNA, whereas at 1 mM $MgCl_2$ it cannot. We find that at 1 mM $MgCl_2$, RecA803 protein catalyzes a significant amount of ATP hydrolysis (Figure 2), whereas

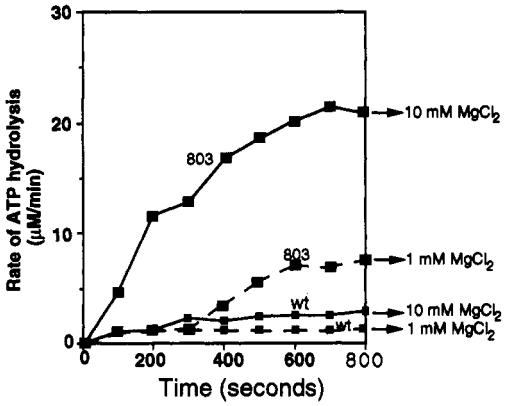

FIGURE 2: ATP hydrolysis by RecA protein using ssDNA that is complexed with SSB protein. Reaction mixtures consisting of 3 μM M13 ssDNA, 0.6 μM SSB protein, 1 mM ATP, and the indicated concentration of $MgCl_2$ were incubated in buffer at 37 °C for 2 min before RecA protein was added. The change in NADH concentration at 100-s intervals was converted to units of micromolar ATP hydrolyzed per minute. Open squares represent wild-type RecA protein, and closed squares represent RecA803 protein.

Table I: Inhibition of Poly(dT)-Dependent ATP Hydrolysis by SSB Protein[a]

	half-time for decay of ATP hydrolysis (min)	
[$MgCl_2$] (mM)	RecA(wt) protein	RecA803 protein
4	1.0	2.7
10	1.5	11

[a] All reactions were performed at 37 °C in buffer containing the indicated $MgCl_2$ concentration, 500 μM ATP, 3 μM poly(dT), and 0.75 μM recA protein. After recA protein had attained a steady-state rate of ATP hydrolysis, 0.6 μM SSB protein was added. Half-times for the inhibition of ATP hydrolysis were determined from a non-linear least-squares fit of the data to a single-exponential decay.

wild-type RecA protein displays no detectable activity (<0.1 μM/min). This result indicates that RecA803 protein, unlike wild-type RecA protein, effectively displaces SSB protein from ssDNA even at 1 mM $MgCl_2$.

The observation that RecA803 protein can effectively overcome inhibition of both ssDNA-dependent ATPase activity (Figure 2) and joint molecule formation by SSB protein (Madiraju et al., 1988) suggests not only that RecA803 protein is capable of SSB protein displacement but also that it could be more resistant than wild-type RecA protein to inhibition by SSB protein under conditions which favor net SSB protein binding to ssDNA. Due to differences in DNA binding affinity, SSB protein inhibits the poly(dT)-dependent ATPase activity of RecA protein under all conditions examined (Kowalczykowski & Krupp, 1987); consequently, we measured the rates of poly(dT)-dependent ATP hydrolysis by RecA803 protein after adding SSB protein. Table I shows that the half-time for decay of ATPase activity after SSB protein addition is greater for RecA803 protein than for wild-type RecA protein, demonstrating that RecA803 protein possesses a greater resistance to inhibition by SSB protein than the wild-type protein. These differences are more striking at 10 mM $MgCl_2$ than at 4 mM $MgCl_2$. These experiments were also performed using etheno M13 DNA in place of poly(dT), and similar results were obtained, except that the inhibition half-time for RecA803 protein is only 2 times greater than that for wild-type RecA protein (data not shown). These data show that the net steady-state amount of RecA803 protein–ssDNA complexes is greater after SSB protein addition, suggesting that RecA803 protein either dissociates more slowly from or associates more rapidly with ssDNA, or both, than does wild-type RecA protein.

Table II: ssDNA Binding Affinities of RecA803 and RecA(wt) Proteins[a]

	salt titration midpoint (mM NaCl)		relative fluorescence increase	
nucleotide	wild-type RecA protein	RecA803 protein	wild-type RecA protein	RecA803 protein
none	270	230	1.7	1.9
ATP	850	500	2.3	2.3
ADP	145	135	1.8	1.9

[a] All reactions were performed at 37 °C in buffer containing 10 mM $MgCl_2$, 1.0 μM RecA protein, and 3 μM etheno M13 DNA. Either 250 μM ADP or 500 μM ATP was present when indicated. An ATP regenerating system containing 1 mM phosphoenolpyruvate and 2 units/mL pyruvate kinase was present when ATP was used. The uncertainty of the salt titration midpoint values is about 20 mM. The relative fluorescence increase is the ratio of the fluorescence of the RecA protein–etheno M13 DNA complex relative to that of the dissociated complex (Menetski et al., 1988).

Equilibrium and Kinetic Properties of the RecA803 Protein–ssDNA Complex. The preceding data suggested that the binding affinity of the two RecA proteins ssDNA might be different. To examine this possibility, the equilibrium binding affinity of each protein for etheno M13 DNA was measured in the absence and in the presence of nucleotides. Menetski and Kowalczykowski (1985) showed that the salt concentration required to disrupt RecA protein–DNA complexes is related to the equilibrium affinity of RecA protein for ssDNA. They also demonstrated that both the relative increase in fluorescence upon complex formation (i.e., per mole of complex) and the salt concentration required to dissociate half of the RecA protein–etheno DNA complexes (previously defined as the "salt titration mid-point") are affected by nucleotide cofactors.

Titration of etheno M13 DNA with the RecA803 protein showed that it saturates the DNA at the same stoichiometry as wild-type protein (data not shown). In the absence of added nucleotide, the salt titration midpoint for RecA803 protein (230 mM) is slightly lower than that of wild-type RecA protein (270 mM) (Table II). Addition of ATP to a complex of RecA protein and etheno M13 ssDNA increases the apparent DNA binding affinity for both proteins, and the apparent affinity of the wild-type protein remains greater than that of RecA803 protein (see Table II). Finally, ADP, which has been shown to decrease the equilibrium binding affinity of RecA protein (Menetski & Kowalczykowski, 1985), also reduces the equilibrium DNA binding affinity of the mutant protein; again, however, the apparent affinity of the wild-type protein remains slightly greater than that of RecA803 protein (Table II). These results indicate that the affinity of RecA803 protein for ssDNA is not greater than that of wild-type RecA protein, implying that the enzymatic differences between the two proteins cannot be explained by differences in equilibrium DNA binding affinity.

It is possible that an alteration in the interaction of RecA803 protein with ssDNA might be experimentally undetectable in the equilibrium experiments, which can be quantitatively analyzed only at elevated salt concentrations, but could be manifest in the kinetic properties of the protein–ssDNA complex. Therefore, we compared the rate at which RecA803 protein transferred from etheno M13 DNA complexes to an excess of poly(dT). The transfer reaction does not occur by simple dissociation but rather proceeds through a ternary intermediate consisting of etheno M13 DNA, RecA protein,

Table III: Transfer of RecA Protein from Etheno M13 DNA to Poly(dT)[a]

	no cofactor		ATP		ADP	
	RecA(wt) protein	RecA803 protein	RecA(wt) protein	RecA803 protein	RecA(wt) protein	RecA803 protein
(a) fast component						
rate, k_1 (s^{-1})	71	77	22	30	>100	>100
fraction transferred	0.5	0.4	0.4	0.4	0.3	0.3
(b) slow component						
rate, k_2 (s^{-1})	3.0	3.3	2.2	1.8	17	18
fraction transferred	0.5	0.6	0.6	0.6	0.7	0.7

[a] All reactions were conducted at 25 °C and contained 4 mM $MgCl_2$, 0.56 μM RecA protein, 4.2 μM etheno M13 DNA, and either 250 μM ADP or 500 μM ATP as indicated. Poly(dT) was added at time zero to a final concentration of 25 μM.

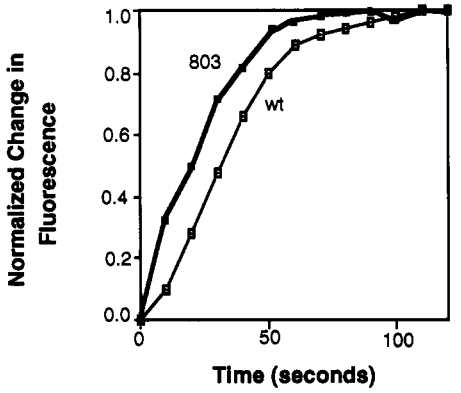

FIGURE 3: Association of RecA protein with etheno M13 DNA. Reactions were performed at 25 °C in buffer containing 4 mM $MgCl_2$, 150 mM NaCl, 500 μM ATP, and 6 μM etheno M13 DNA; 0.1 μM RecA protein was added at zero time. Open squares represent wild-type RecA protein, and closed squares represent RecA803 protein.

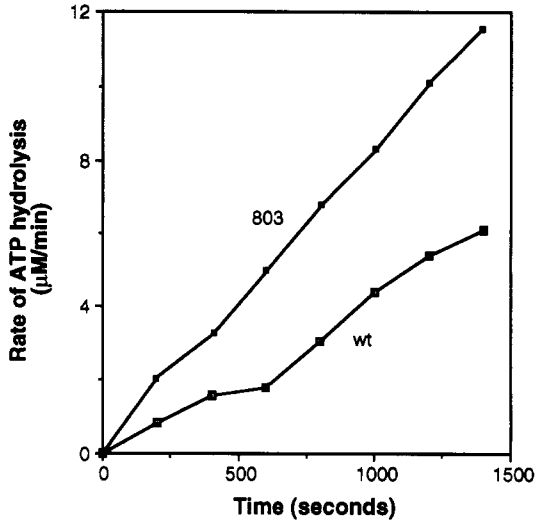

FIGURE 4: Double-stranded DNA-dependent ATPase activity. Reactions were performed in buffer containing 8.4 μM dsDNA, 3 μM RecA protein, 1 mM ATP, and 10 mM $MgCl_2$. The change in NADH concentration at 200-s intervals was converted to units of micromolar ATP hydrolyzed per minute. Open squares represent wild-type RecA protein, and closed squares represent RecA803 protein.

and poly(dT); in addition, the reaction displays multicomponent behavior, with one fraction of bound protein transferring rapidly and a second fraction transferring slowly (Menetski & Kowalczykowski, 1987). Our results (Table III) indicate that both the rates of transfer and the relative proportions of kinetic species for RecA803 protein are very similar to those for wild-type RecA protein, regardless of whether nucleotide cofactors are present or not. Similar results were obtained at 37 °C (data not shown). These results suggest that the kinetic stability of RecA803 protein–ssDNA complexes is not greater than that of the wild-type RecA protein–ssDNA complexes.

Since neither the equilibrium data nor the kinetic transfer data offered an explanation for the enhanced ability of RecA803 protein to compete with SSB protein for ssDNA binding, the rate of association of RecA protein with ssDNA was examined (Menetski & Kowalczykowski, 1990). Figure 3 shows that RecA803 protein binds more rapidly to etheno M13 DNA than does wild-type RecA protein. After about 90 s, however, the extent of binding is approximately equal for the two proteins, consistent with the equilibrium results. Thus, although RecA803 protein shows no alteration in its equilibrium binding affinity or in its kinetic stability, it associates with ssDNA more rapidly than does wild-type protein; similar behavior was observed for RecA441 protein, a mutant protein that also displays an enhanced ability to compete with SSB protein (Lavery & Kowalczykowski, 1988, 1990).

dsDNA-Dependent ATP Hydrolysis by RecA803 Protein. At pH 7.5, ATP hydrolysis by wild-type RecA protein in the presence of dsDNA displays a significant lag before attainment of steady-state ATP hydrolysis; the rate-limiting step in this reaction is a slow nucleation of RecA protein onto the dsDNA (Kowalczykowski et al., 1987; Pugh & Cox, 1987). Since RecA803 protein associates with ssDNA more rapidly than wild-type protin, we examined the possibility that nucleation with dsDNA is also faster for RecA803 protein. RecA803 protein shows a higher rate of dsDNA-dependent ATP hydrolysis at all times (Figure 4) and a greater rate of acceleration in ATP hydrolysis than does wild-type RecA protein. The concentration of $MgCl_2$ affects the rate of dsDNA-dependent ATPase activity of RecA protein, with the lag being shorter at lower $MgCl_2$ concentrations (Kowalczykowski & Krupp, 1987). At both 1 and 4 mM $MgCl_2$, RecA803 protein also showed higher rates of ATP hydrolysis than wild-type RecA protein at all time points (data not shown).

DISCUSSION

The *recA803* mutation partially suppresses the defects in DNA recombination and repair that result from *recF* mutations (Volkert & Hartke, 1984; Madiraju et al., 1988). In vitro, the mutant RecA803 protein promotes the formation of joint molecules more rapidly than wild-type RecA protein under two suboptimal conditions (Madiraju et al., 1988): (1) when SSB protein is bound to the ssDNA prior to RecA protein and (2) in the absence of SSB protein, when conditions favor the formation of DNA secondary structure. In this study, we have used ssDNA-dependent ATPase activity as a measure of ternary complex (ATP–RecA protein–ssDNA) formation and have concluded that RecA803 protein utilizes DNA secondary structures more effectively than does wild-type RecA protein. In addition, the ATPase activity of RecA803

protein is less susceptible to inhibition by SSB protein than is that of wild-type RecA protein, and RecA803 protein can displace SSB protein from ssDNA more effectively than the wild-type protein.

The enhanced activities of RecA803 protein could result from a number of physical alterations. One possibility is that the protein–DNA interaction properties of RecA803 protein are altered, resulting in a higher binding affinity for ssDNA. However, our results show that the protein–DNA interaction properties of both mutant and wild-type proteins are similar. At equilibrium, both proteins bind to ssDNA with the same stoichiometry. The affinity of RecA803 protein for ssDNA is not greater than that of wild-type RecA protein, and, in fact, the stabilities of RecA803 protein–ssDNA complexes are somewhat lower than those of the wild-type RecA protein–ssDNA complexes. In addition, both proteins showed similar rates of dissociation as measured by the rate of transfer to another competing DNA. Thus, these results do not support the notion that RecA803 protein has higher affinity for DNA.

A second possibility is that interactions between RecA protein monomers, i.e., protein–protein interactions, are affected without actually influencing the intrinsic binding affinity of RecA803 protein for ssDNA. Although we have no direct evidence for altered protein–protein interactions, this interpretation is consistent with the observation that RecA803 protein associates with ssDNA faster than wild-type RecA protein. Unfortunately, the molecular basis for this enhanced rate of association is unclear, due to the complexity of the association process. The association of RecA protein with ssDNA does not occur by a diffusion-limited bimolecular pathway but, instead, involves a rate-limiting first-order nucleation process (Chabbert et al., 1987). While the composition of the rate-limiting kinetic intermediate is unknown, kinetic data (e.g., dependence on the Mg^{2+} ion concentration) suggest that a protein–protein aggregate is involved. Thus, the rate-limiting step in the association process either may involve the slow formation of a specific protein aggregate (a nucleus) or, alternatively, may require the slow disassembly of a protein aggregate that is unable to bind ssDNA directly [see Kowalczykowski (1991a,b) for discussion]. The implication of either view is that RecA803 protein is altered in the protein–protein interactions crucial to this important kinetic process. This conclusion is consistent with the absence of an effect either on the equilibrium binding affinity or on the rate of dissociation; however, either direct self-association studies or a more detailed kinetic analysis of the association reaction is required to confirm this interpretation. This conclusion is also consistent with recent structural data showing that the site of the *recA803* mutation (residue 37) is involved in protein–protein contacts between RecA protein filaments (Story et al., 1992). Our data demonstrate that mutation at this contact site results in a protein with an increased rate of association with ssDNA that, in turn, results in an increased probability of RecA803 protein occupying a vacant ssDNA binding site relative to the probability of occupancy by SSB protein, under conditions where net RecA protein–ssDNA complex formation is favored. This enhanced physical property may suffice to explain the increased ability of RecA803 protein to compete with SSB protein for limited ssDNA binding sites.

The increased ability of RecA803 protein to compete with SSB protein for ssDNA binding is particularly noteworthy given the correlation between this biochemical behavior of mutant RecA proteins and their in vivo phenotype (Kowalczykowski, 1991b). All mutant RecA proteins that are defective for cellular function fail to displace SSB protein from ssDNA, whereas those that are enhanced for a cellular function display an increased ability to displace SSB protein from ssDNA. One protein in the latter class is the RecA441 protein. The *recA441* allele also partially suppresses *recF* mutations, although differently than *recA803* (e.g., *recA441* suppresses the regulatory defect due to the *recF* mutation whereas *recA803* does not; Thomas & Lloyd, 1983; Volkert et al., 1984). One of the *recA441* mutations is at codon 38 [resulting in a change from glutamic acid to lysine (Knight et al., 1984)], which is adjacent to the *recA803* mutation at codon 37 [valine to methionine (Madiraju et al., 1988)]. What makes this comparison all the more interesting is that in vitro, the RecA441 protein shows both enhanced DNA strand exchange activity under suboptimal conditions and enhanced ability to compete with SSB protein for ssDNA binding (Lavery & Kowalczykowski, 1988, 1990). As with RecA803 protein, the only physical property that could explain the enhancement of RecA441 protein activity is an increased rate of association with ssDNA (Kowalczykowski, 1991b). It is likely that the heightened activities common to these two proteins result from the same root cause.

Assuming that the rate with which the RecA803 protein associates with ssDNA is responsible for the in vivo alterations, our data offer a possible explanation for the suppression of the *recF* phenotype by the *recA803* mutation. Thoma and Wackernagel (1988) reported that *recJ* function is required for suppression of the UV sensitivity of a *recF* mutation by suppressors that map in *recA*. *recJ* protein encodes a 5′ to 3′ ssDNA exonuclease activity (Lovett & Kolodner, 1989). These observations imply that the *recF* suppressors which encode mutant RecA proteins utilize ssDNA that is generated by *recJ* protein in the absence of RecF protein. Therefore, in wild-type cell, RecF protein may assist RecA protein in the utilization of such ssDNA. Since the nucleation of RecA protein onto ssDNA is slow and stochastic [see Kowalczykowski (1991a)], ssDNA that is being rapidly exposed by *recJ* protein may possibly contain discontinuous RecA protein filaments. Such newly formed ssDNA may be a potential binding site for SSB protein, may base-pair, generating secondary structure, or, finally, may be degraded by other cellular nucleases. All of these possibilities would limit the binding of RecA protein to ssDNA, and thereby affect both recombination and repair processes in vivo. An enhanced rate of association with ssDNA would allow RecA803 protein to compete more effectively with SSB protein, to disrupt secondary structures, and to prevent degradative loss of ssDNA. Therefore, it is logical to hypothesize that in vivo RecF protein either assists RecA protein in the nucleation process and/or binds to ssDNA, thereby limiting the loss of ssDNA by exonucleolytic degradation. Griffin and Kolodner (1990) and Madiraju and Clark (1991) showed that RecF is a single-stranded DNA binding protein, and in vivo data suggest that it may compete with SSB protein (Moreau, 1987, 1988). Detailed characterization of the DNA binding properties of RecF protein and of its effects on the binding of RecA protein to ssDNA would promote understanding of how the recombinational repair process occurs in cells. In summary, increased resistance of RecA803 protein to inhibition by SSB protein and the ability of RecA803 protein to utilize ssDNA containing secondary structures are properties of RecA803 protein which could plausibly explain the suppression of *recF* mutations.

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