Title of Senior Honors Thesis

Inhibition of the *Escherichia coli* replication cycle by a mutant RecA protein

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A conserved [K/R]X[K/R] motif is found at the subunit-subunit interface in bacterial RecA protein filaments. In the *Escherichia coli* RecA protein, this motif is made up of residues K248 and K250. Both of these residues have a role in ATP hydrolysis and in the transmission of conformational information across the interface. A K250R mutation creates a RecA protein that promotes both ATP hydrolysis and DNA-strand exchange at a six-fold lower rate than the wild type protein. *E. coli* strains overexpressing this mutant RecA protein also grow much slower than wild type strains. Suppressor mutations appear quickly, and most suppressor mutations inactivate RecA. One suppressor mutation, A11V, did not inactivate the RecA protein and produced a RecA variant that has wild type growth rate, while partially restoring UV resistance. We found that this suppressor mutation has no effect on the rate of ATP hydrolysis and strand exchange activity of the mutant RecA K250R protein. We also found that RecA K250R A11V has a higher rate of disassembly than RecA K250R, showing that this suppression mutation allowed the double mutant RecA nucleoprotein filament to be more dynamic.

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Senior Honors Thesis

Cover Sheet

TITLE: **Inhibition of the *Escherichia coli* replication cycle by a mutant RecA protein**

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Inhibition of the *Escherichia coli* replication cycle by a mutant RecA protein

Abstract:

A conserved [K/R]x[K/R] motif is found at the subunit-subunit interface in bacterial RecA protein filaments. In the *Escherichia coli* RecA protein, this motif is made up of residues K248 and K250. Both of these residues have a role in ATP hydrolysis (in trans across the interface) and in the transmission of conformational information across the interface. A K250R mutation creates a RecA protein that promotes both ATP hydrolysis and DNA-strand exchange at a six-fold lower rate than the wild type protein. *E. coli* strains overexpressing this mutant RecA protein also grow much slower than wild type strains. Suppressor mutations appear quickly, and most suppressor mutations inactivate RecA (leading to high levels of UV sensitivity). One suppressor mutation, A11V, did not inactivate the RecA protein, but instead produced a RecA variant that allowed normal rates of growth under normal growth conditions, while partially restoring UV resistance. We found that this suppressor mutation has no effect on the rate of ATP hydrolysis of the mutant RecA K250R protein, but has slightly better strand exchange activity than RecA K250R. We also found that RecA K250R/A11V has a higher rate of disassembly than RecA K250R, showing that this suppression mutation allowed the double mutant RecA nucleoprotein filament to be more dynamic. Our hypothesis is that the [KR]x[KR] motif, especially RecA residue K250 is directly involved in RecA functions. Through mutational studies, we can get a better understanding of the role of this residue in RecA-catalyzed reactions.

Introduction:

The RecA protein family consists of recombinases that promote DNA-strand exchange between two homologous DNA molecules. RecA and its homologues have an important

function in DNA metabolism, facilitating DNA-strand exchange during recombinational DNA repair (Cox, 2007). Recombination is crucial in promoting repair and allowing replication to restart after a replication fork stalls due to DNA damage. RecA thus plays an important role in maintaining genome integrity.

E.coli RecA forms filament on DNA by the association of ATP-liganded RecA protomers (Cox, 2007). RecA contains a simple motif, called the [KR] x [KR] motif at the subunit-subunit interface. This motif has been implicated in the catalysis of ATP hydrolysis in trans (Leipe et al., 2000). Also, the [KR]x[KR] motif is absent from the archaea or eukaryotic homologues of RecA, and these homologues cannot promote some of the ATP-dependent functions of the bacterial RecA protein (Cox, 2006). In the *E. coli* RecA protein, the residues in the [KR]x[KR] motif are K248 and K250 (Figure 1). In a new examination of active filaments, it is strongly suggested that the ATP hydrolytic site is at the subunit-subunit interface and that residues K248 and K250 are in very close proximity (VanLoock et al., 2003).

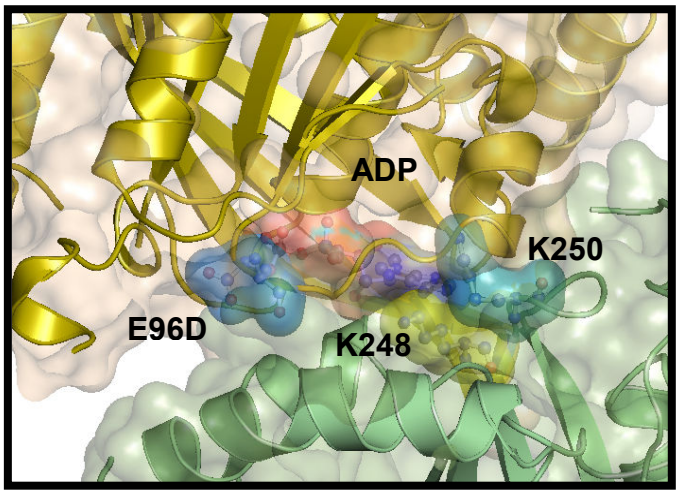


FIG 1. Model of the active form of RecA with ADP bound between 2 adjacent RecA subunits in the active nucleoprotein filament (Protein Data Bank entry 1N03). K248, K250R and E96D are near the ADP molecule at the interface of two RecA monomers.

To understand more about the properties of residue K250 and its role in RecA functions, we examined K250 using mutational studies. We constructed two less conservative mutations, lysine to asparagines (K250N) and lysine to glutamine (K250Q). A more conservative mutation, K250R was also constructed and is of particular interest since a lysine to arginine mutation should conserve most of the catalytic functions of RecA.

Also, we studied the suppressor mutations that arise when K250R is overexpressed in *E.coli* cells. This allows us to understand how a mutation in K250 can be salvaged, and hence bring further insight into the properties of the RecA residue K250.

We propose that the [KR]x[KR] motif, especially the residue K250 is directly involved in RecA functions. Through mutational studies, we can get a better understanding of the role of this residue in RecA-catalyzed reactions.

Previous Research

We overexpressed and purified K250N, K250Q and K250R in *Escherichia coli*. The rate of ATP hydrolysis is then determined for each of the mutants. The results are summarized as follows.

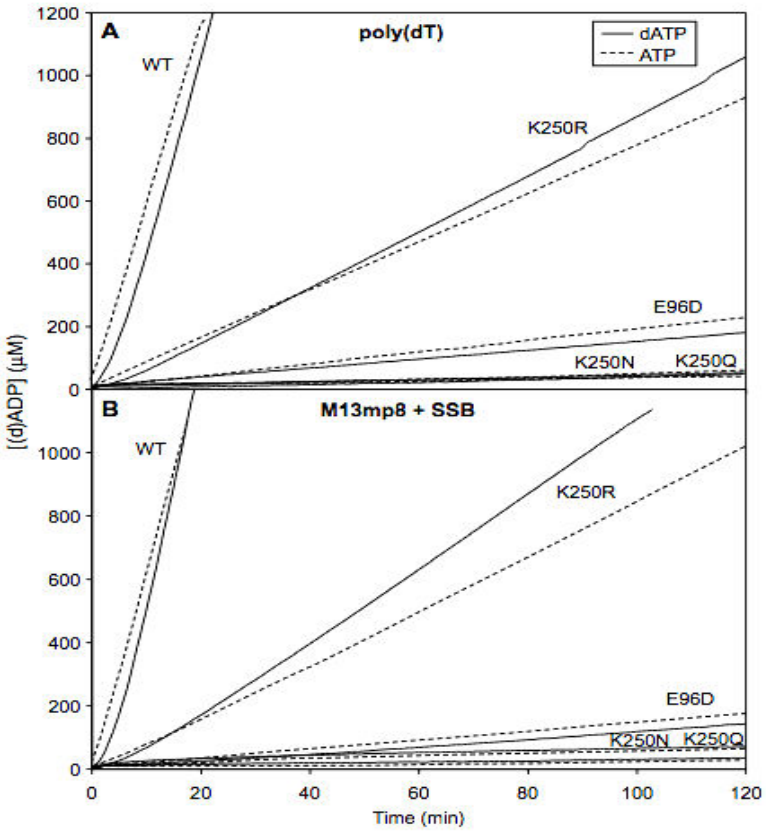


FIG 2. Mutations at residue K250 affect (d)ATP hydrolysis catalyzed by the RecA protein. RecA protein-catalyzed (d)ATP hydrolysis was monitored. The RecA protein was pre-incubated with the DNA for 10 min before (d)ATP(+SSB) was added. Time 0 corresponds to the time of (d)ATP addition. The average observed rates of (d)ATP hydrolysis are reported in Table 1. *(A)* Reactions included 4 μM RecA protein and 6 μM poly(dT) linear ssDNA at pH 7.5. A *solid* line indicates reactions with dATP, and reactions with ATP are indicated with a *dashed* line. *(B)* Reactions included 4 μM RecA protein, 6 μM M13mp8 circular ssDNA, and 0.6 μM SSB protein at pH 7.5. A *solid* line indicates reactions with dATP, and reactions with ATP are indicated with a *dashed* line. (JM Cox, unpublished)

RecA	poly(dT) dATP	Std Dev	*n*	% of WT	poly(dT) ATP	Std Dev	*n*	% of WT	M13mp8 dATP	Std Dev	*n*	% of WT	M13mp8 ATP	Std Dev	*n*	% of WT
WT	62.761 (+/-)	2.563	29	100.0	54.804 (+/-)	2.667	8	100.0	79.495 (+/-)	2.192	8	100.0	63.283 (+/-)	2.657	11	100.0
K250R	9.618 (+/-)	0.268	8	15.3	7.193 (+/-)	0.534	8	13.1	11.798 (+/-)	0.195	4	14.8	9.126 (+/-)	0.631	7	14.4
K250N	0.449 (+/-)	0.044	7	0.7	0.289 (+/-)	0.048	4	0.5	0.289 (+/-)	0.048	4	0.4	0.248 (+/-)	0.000	4	0.4
K250Q	0.434 (+/-)	0.041	4	0.7	0.331 (+/-)	0.138	6	0.6	0.351 (+/-)	0.041	4	0.4	0.372 (+/-)	0.045	6	0.6
E96D	1.405 (+/-)	0.117	4	2.2	1.749 (+/-)	0.062	6	3.2	1.384 (+/-)	0.079	4	1.7	1.543 (+/-)	0.179	6	2.1
K250R/E96D	3.515 (+/-)	0.401	15	5.6	5.436 (+/-)	0.421	9	9.9	2.572 (+/-)	0.384	8	3.2	4.061 (+/-)	1.175	7	6.4
E96D+K250R	20.397 (+/-)	0.419	5	32.5	nd				nd				nd			
E96D+K250N	10.165 (+/-)	0.973	5	16.2	nd				nd				nd			
E96D+K250Q	1.736 (+/-)	0.165	7	2.8	nd				nd				nd			

RecA	no DNA dATP	Std Dev	*n*	% of WT	no DNA ATP	Std Dev	*n*	% of WT
WT	0.744 (+/-)	0.000	2	100.0	0.372 (+/-)	0.058	2	100.0
K250R	0.661 (+/-)	0.117	2	88.9	0.372 (+/-)	0.058	2	100.0
K250N	0.331 (+/-)	0.000	2	44.4	0.248 (+/-)	0.000	2	66.7
K250Q	0.620 (+/-)	0.175	2	83.3	0.413 (+/-)	0.000	2	111.1
E96D	1.281 (+/-)	0.292	2	172.2	1.818 (+/-)	0.351	2	488.9
K250R/E96D	2.963 (+/-)	0.263	7	398.4	4.339 (+/-)	0.372	6	1166.7

TABLE 1. Rates of (d)ATP hydrolysis catalyzed by wild-type and mutant RecA proteins. RecA protein-catalyzed (d)ATP hydrolysis was monitored as described in *FIG* 2. Characteristic reactions for each solution condition are those presented in *FIG* 2. Rates are in units of μM/min. (Cox, unpublished)

From figure 2, we can see that all mutant proteins catalyze ATP and dATP hydrolysis at a reduced rate relative to wildtype RecA protein when bound to either linear poly (dT) ssDNA

or M13mp8 circular DNA. This decrease in rate of ATP hydrolysis is especially significant in the mutants K250N and K250Q, as these two proteins show a very low rate of ATP hydrolysis. The K250R mutation resulted in a much less attenuated rate of ATP and dATP hydrolysis. As shown in Table 1, the K250R mutant protein catalyzed (d)ATP hydrolysis at a rate that was 13.1% to 15.3% of the wildtype rate. Since the lysine to arginine mutation is more conservative than the lysine to asparagine or lysine to glutamine mutation, this result is within expectation. From this experiment, we can see that residue K250 is important in ATP hydrolysis of RecA.

In order to investigate the effect of the mutation on strand exchange activity of RecA, in-vitro DNA strand exchange reactions were set up. The results are shown in figure 3.

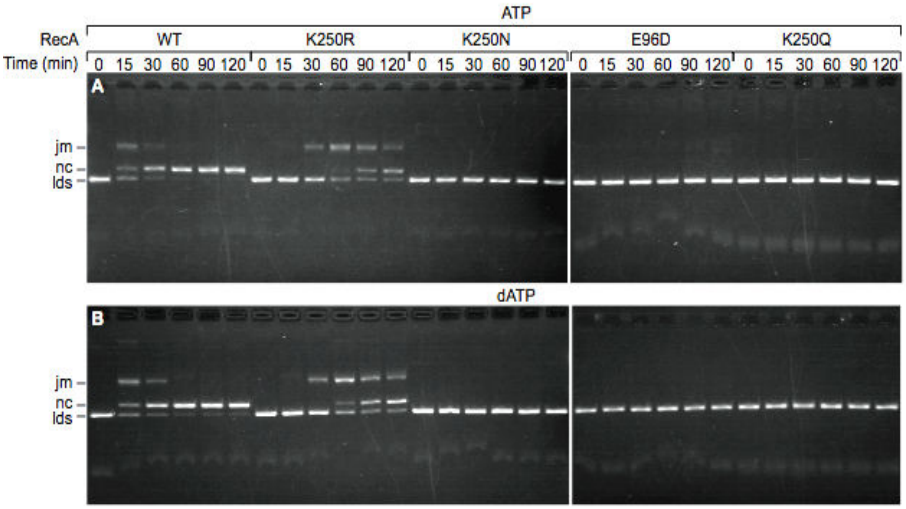


FIG 3. DNA Three-strand exchange catalyzed by wild-type and mutant RecA proteins. Reactions were carried out as described in Experimental Procedures. *(A,B)* DNA three-strand exchange at pH 7.5 with ATP (A) or dATP (B); lds, linear dsDNA reactant; jm, joint molecule intermediate; nc, nicked circular product. Reaction time points for each RecA protein were taken at 0, 15, 30, 60, 90, and 120 min. Only wild-type and K250R mutant RecA proteins were able to catalyze complete DNA three-strand exchange at pH 7.5. *(C,D)* DNA three-strand exchange with dATP at pH 6.6 (C) or pH 8.5 (D); lds, linear dsDNA reactant; jm, joint molecule intermediate; nc, nicked circular product. Reaction time points for each RecA protein were taken at 0 and 120 min. Only wild-type and K250R mutant RecA proteins were able to catalyze complete DNA three-strand exchange at pH 6.6 and pH 8.5.(Cox, unpublished)

From figure 3, we can see that there is no formation of nicked circular product for K250N and K250Q. Wildtype RecA protein catalyzed complete DNA three-strand exchange to form nicked circular product with the first nicked circular product forming within 15min. The RecA K250R mutant protein also catalyzed DNA strand exchange to some extent. However, it catalyzes strand exchange at a rate much lower than wildtype, with initial product formation at about 60min. This reduced rate of strand exchange was approximately proportional to the reduced rate if ATP hydrolysis catalyzed by K250R relative to wildtype.

From the ATP hydrolysis and DNA-strand exchange data, we can conclude that RecA K250R appears to be fully functional in carrying out the catalytic activities of wildtype RecA. However, RecA K250R performs these activities as a much lower rate.

To further explore the effect of the K250R mutation on the growth of *E.coli*, we overexpressed RecA K250R in *E.coli* cells.

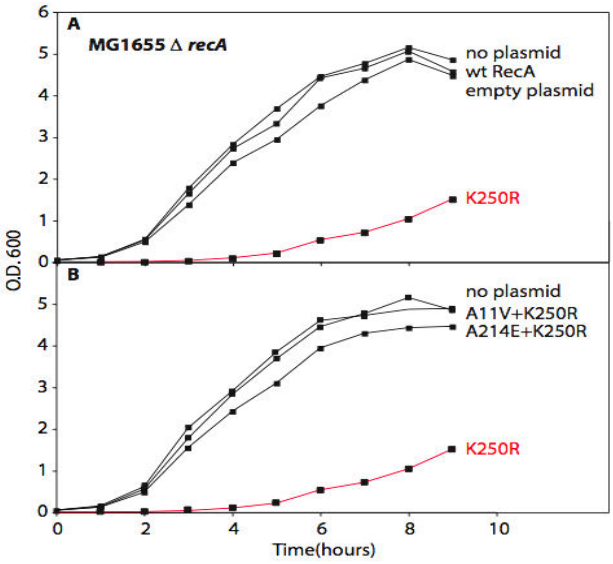


FIG 4. *recA* K250R is inserted into pEAW plasmid and transformed into *E.coli* MG 1655. (A) Cells expressing RecA K250R mutant protein display a slow growth phenotype. (B) Several suppression mutations arise to rescue this slow growth phenotype.

E.coli cells overexpressing RecA K250R grow at a much slower rate than cells expressing wildtype RecA. This shows that the K250R mutation is probably limiting growth in some way. To further explore how the K250R mutant is reducing the growth of *E.coli*, we expressed RecA K250R in cells that lack either RecF, RecO or RecR.

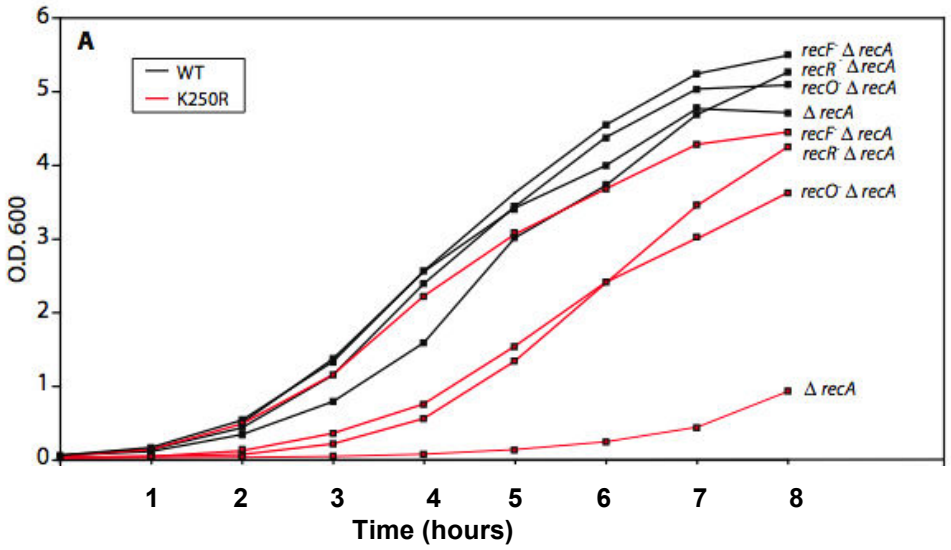


FIG 5.Growth rate is recovered in RecFOR knockout strains. Growth curves of wildtype *E.coli* Δ*recA* with the indicated mutations are in black. Growth curve of RecA K250R *E.coli* with the indicated mutations are in red. Cells are grown for 8 hours in LB medium. The O.D 600 is taken as a measure of cell density. Cells expressing RecA K250R have a rescued growth rate when RecF, O or R mutation is present.

When RecA K250R is expressed in *E.coli* cells with RecFOR knocked out, we find that cell growth is significantly rescued. RecFOR are involved in loading RecA onto regions of single-stranded DNA at replication fork for recombinational repair (Morimatsu, 2003). When Rec F, O or R is absent, RecA is no longer loaded onto the DNA at a replication fork. This shows that the absence of the mutant RecA K250R at the replication fork allowed growth recovery of *E.coli* cells. As such, we concluded that the defect caused by RecA K250R mutation is acting at the replication fork.

To further characterize the K250R mutation, we isolated several suppressor mutations. When RecA K250R is overexpressed in *E.coli*, suppressor mutations arise quickly so that growth rate can recover. Most of these mutation inactivate RecA (Table 2).

# of suppressor colonies	Mutants of RecA K250R plasmid pEAW451 mutation
11	IS10 insertion between D100 and H97
1	IS10 insertion between H97 and D94
1	IS10 insertion between S117 and L114
1	deletion between the *recA* promoter and N236
1	deletion after G59 to beyond the end of the gene
1	Q173 change to stop codon
5	missing first base of aa 123=frameshift after G122
1	A214E+K250R
2	E154A+K250R
4	A11V+K250R
3	L99R+K250R
1	A147V+K250R
2	I195N+K250R

TABLE 2. List of suppressor mutations that arise when RecA K250R is expressed in *E.coli* MG1655 cells. Cells with these suppressor mutations display normal growth phenotype. However, most inactivate RecA so that growth can be rescued. These changes are determined by sequencing these strains across the *recA* gene.

However, one suppressor mutation A11V did not inactivate RecA. RecA K250R/A11V shows a normal growth phenotype (*FIG* 4) and retains limited RecA activity, as shown in *FIG* 6.

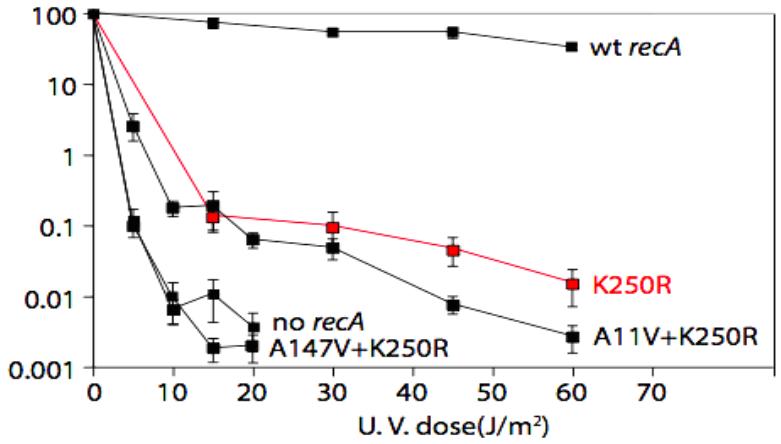


FIG 6. RecA K250R/A11V retains limited RecA activities. *E.coli* MG1655 cells expressing RecA proteins are exposed to increasing amount of UV radiation. UV radiation causes dimerization of thymine, leading to single stranded breaks. Functional RecA protein is able to promote recombination repair to restore the genome. Both RecA K250R and K250R/A11V shows some resistance UV radiation, though both are less UV resistant than wildtype RecA.

The comparable UV sensitivity of RecA K250R and K250R/A11V shows that the double mutant still has some ability to carry out recombination repair of single stranded breaks.

Based on these results, we turned our focus to RecA K250R/A11V. In this thesis project, I will explore how the A11V suppressor mutation allows *E.coli* cells to recover their growth rate in the presence of the RecA K250R mutant protein. We hypothesize that the A11V mutation is directly involved in suppressing the slow growth phenotype of RecA K250R. Through this study, we hope to get a better understanding on the importance of the K250 residue in the [KR]x [KR] motif.

Materials and Methods

Enzymes and Biochemicals—The *Escherichia coli* SSB protein was purified as previously described (Shan, 1996). The *E. coli* wild-type RecA protein was purified as previously described (Lusetti, 2003) with the following exceptions: the polyethyleneimine pellet was washed with 100 ml of R Buffer (20 mM Tris-Cl buffer (80% cation, pH 7.5), 10% (w/v) glycerol, 1 mM dithiothreitol) plus 150 mM ammonium sulfate, protein was extracted from the polyethyleneimine pellet twice with 75 ml of R Buffer plus 300 mM ammonium sulfate, the ammonium sulfate pellet was resuspended in R Buffer plus 100 mM KCl, the wild-type RecA protein was then loaded onto the DEAE Sepharose column and washed with two column volumes of R Buffer plus 100 mM KCl and then two column volumes of R Buffer plus 1 M KCl, and flow-through peak fractions and peak fractions eluted in R Buffer plus 1 M KCl were identified by SDS-PAGE and pooled together before dialyzing versus 20 mM phosphate buffer (10 mM KH_2PO_4, 10 mM K_2HPO_4, 10% (w/v) glycerol, 1 mM dithiothreitol) in preparation for the hydroxyapatite column.

The RecA K250R mutant protein was purified by the same means as the wild-type RecA protein with the following exceptions: the plasmid encoding the mutant RecA gene was transformed into the nuclease-deficient strain STL2669 (described in [Lusetti, 2003), the DEAE Sepharose column was washed with two column volumes of R Buffer plus 100 mM KCl and protein was eluted in two column volumes of R Buffer plus 1 M KCl, only peak fractions eluted in R Buffer plus 1 M KCl were identified by SDS-PAGE and pooled together before dialyzing versus 20 mM phosphate buffer in preparation for the hydroxyapatite column. Peak fractions from the hydroxyapatite column were identified by SDS-PAGE and pooled and dialyzed against R Buffer and then loaded onto a PBE-94 column (Amersham Biosciences), the PBE-94 column was washed with 2 column volumes of R Buffer, the RecA K250R mutant protein was eluted from the PBE-94 column with a linear gradient of R Buffer to R Buffer plus 1 M KCl, and the peak and nuclease-free fractions were pooled.

The RecA K250R/A11V mutant protein was purified by the same means as the wild-type RecA protein with the following exceptions: the plasmid encoding the mutant RecA gene was transformed into the nuclease-deficient strain STL2669/pT7pol26, the Phenyl Sepharose column was washed with two column volumes of R Buffer plus 1M ammonium sulfate and protein was eluted by running a linear gradient using eight column volumes of R Buffer plus 1 M ammonium sulfate to R Buffer. Only peak fractions eluted in R Buffer plus 1 M ammonium sulfate were identified by SDS-PAGE and pooled together before dialyzing versus R Buffer in preparation for

the Source Q (Amersham Biosciences) column. The Source Q column was washed with 2 column volumes of R Buffer plus DTT, and the protein was eluted by running a linear gradient using ten column volumes of R Buffer plus DTT to R buffer plus 1M KCl and DTT. Peak fractions from the Source Q column were identified by SDS-PAGE and pooled and dialyzed against P Buffer (20mM phosphate plus DTT) and then loaded onto a Ceramic hydroxyapatite (HAP) Column (Bio-Rad Laboratories). The Ceramic HAP column was washed with 2 column volumes of P Buffer, the RecA K250R/A11V mutant protein was eluted from the Ceramic HAP column with a linear gradient of ten column volumes of P Buffer to 1M phosphate buffer. The peak fractions were pooled and dialyzed into R buffer. The protein fractions were then tested for nucleases and nuclease-free fractions were pooled.

The purified RecA proteins and SSB protein concentrations were determined by absorbance at 280 nm, using extinction coefficients of $\varepsilon_{280} = 2.23 \times 10^4\ M^{-1}\ cm^{-1}$ (Craig, 1981) and $\varepsilon_{280} = 2.38 \times 10^4\ M^{-1}\ cm^{-1}$ (Lohman, 1985), respectively. RecA proteins and SSB preparations were free of detectable endo- and exonuclease activities on double-stranded DNA and single-stranded DNA. Unless otherwise noted, all reagents were purchased from Fisher (Pittsburgh, Pennsylvania, United States). Ammonium sulfate was purchased from MP Biomedicals (Solon, Ohio, United States), formerly ICN Biomedicals (Irvine, California, United States). Phosphoenolpyruvate (PEP), pyruvate kinase, lactate dehydrogenase, phosphocreatine, creatine kinase, ATP, dATP, and NADH were purchased from Sigma (St. Louis, Missouri, United States. Restriction enzymes were purchased from Fermentas Life Sciences (Hanover, Maryland, United States). Dithiothreitol was purchased from Research Organics (Cleveland, Ohio, United States).

DNA Substrates—Bacteriophage ΦX174 circular ssDNA and replicative form I circular duplex DNA were purchased from New England Biolabs (Ipswich, Massachusettes, United States) and Invitrogen (Carlsbad, California, United States), respectively. Linear duplex DNA (dsDNA) was generated by digesting ΦX174 replicative form I DNA (5386 bp) with the XhoI endonuclease. Concentrations of ssDNA and dsDNA were determined by absorbance at 260 nm using 108 and 151 $\mu M\ A_{260}^{-1}$, respectively, as conversion factors. All DNA concentrations are given in terms of total nucleotides.

ATP Hydrolysis Assays—A coupled spectrophotometric assay (Lindsley, 1990; Morrical,1986) was used to measure the (d)ATP hydrolysis catalyzed by the RecA protein (Lusetti, 2003). The regeneration of ATP or dATP from ADP and PEP was coupled to the oxidation of NADH and monitored by the decrease in absorbance of NADH at 380 nm. The 380-nm wavelength was used instead of the absorption maximum at 340 nm so that the signal would remain within the linear range of the spectrophotometer for the duration of the experiment. The assays were executed using a Varian Cary 300 (Varian, Palo Alto, California, United States) dual beam spectrophotometer equipped with a temperature controller and 12-position cell changer. The cell path length and band pass were 1.0 cm and 2 nm, respectively. The NADH extinction coefficient at 380 nm of $1.21\ mM^{-1}\ cm^{-1}$ was used to calculate rates of ATP or dATP hydrolysis. All reactions were carried out at 37°C in 25 mM Tris-acetate buffer (80% cation; final pH after addition of all components was 7.56), unless otherwise stated. All reactions also included 10 mM magnesium acetate, 3 mM potassium glutamate, 5% (v/v) glycerol, 1 mM dithiothreitol, 3 mM ATP , 0.8μM SSB, an ATP regenerating system (10 units/ml pyruvate kinase and 3.5 mM PEP), and concentrations of DNA and RecA protein as described below and in *fig*ure legends. The coupled spectrophotometric assay to monitor ATP hydrolysis catalyzed by the RecA proteins also contained 3 mM NADH and 10 units/ml lactate dehydrogenase. Reactions were incubated for 10 min before ATP(and single-stranded DNA-binding protein of *E. coli* (SSB) when included) was added to start the reaction.

DNA Three-strand Exchange Reactions—DNA three-strand exchange reactions were carried out at 37°C in 25 mM Tris-acetate buffer (80% cation, pH 7.5), 25 mM Tris-acetate buffer (30% cation, pH 8.5), or 25 mM 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer (76% anion, pH 6.6). RecA protein (to 4 μM) was pre-incubated with 4 μM ΦX174 circular ssDNA for 10 min. Then ATP and SSB (to 3 mM and 1μM, respectively) were added to the reaction, followed by another incubation for 10 min. The reactions were initiated with the addition of ΦX174 linear dsDNA to 10 μM. Reactions were incubated for a total of 120 min with a 12 μl aliquot taken at 0,5,10,15, 20, 30, 40, 60, 90, and 120 min for wildtype RecA , and 0, 30 ,40, 45, 50, 60, 75, 90 and 120 min for RecA K250R and K250R/A11V. To stop the reaction, the 12 μl aliquot was added to 6 μl of a solution composed of 30% (w/v) glycerol, 72 mM EDTA, 0.12% (w/v) bromophenol blue, and 4% (w/v) sodium dodecyl sulfate (SDS). Reactions were analyzed by agarose gel electrophoresis as previously described (Drees,

2004).Gel images were captured with a Fotodyne FOTO/Analyst ® CCD camera, PC Image acquisition software, and FOTO/Convertible dual transilluminator. The intensity of DNA bands was measured using the software package TotalLab version TL100 from Nonlinear Dynamics.

DNA Strand Exchange monitored in the spectrophotometer— DNA three-strand exchange reaction was set up as described above with the following changes: 4μM circular single stranded DNA is used, reaction contains 4.5μM NADH, 4.5μM PEP, 10μM pyruvate kinase and 10μM lactate dehydrogenase for coupled spectrophotometric assay. 2.5 μM of RecA protein was preincubated with M13 css DNA. ATP and SSB (to 3mM and 0.4μM respectively) were then added to the reaction, followed by incubation for 20 mins. The strand exchange reactions were initiated with the addition of 4μM M13 linear double stranded DNA. Reaction is monitored in the Cary 300 Spectrophotometer as described above. For strand exchange reactions using K72R or RecA K72R/E38K as challenge, 5μM of each respective RecA protein is used for challenge.

Results

RecA K250R and K250R/A11V hydrolyze ATP at similar rates

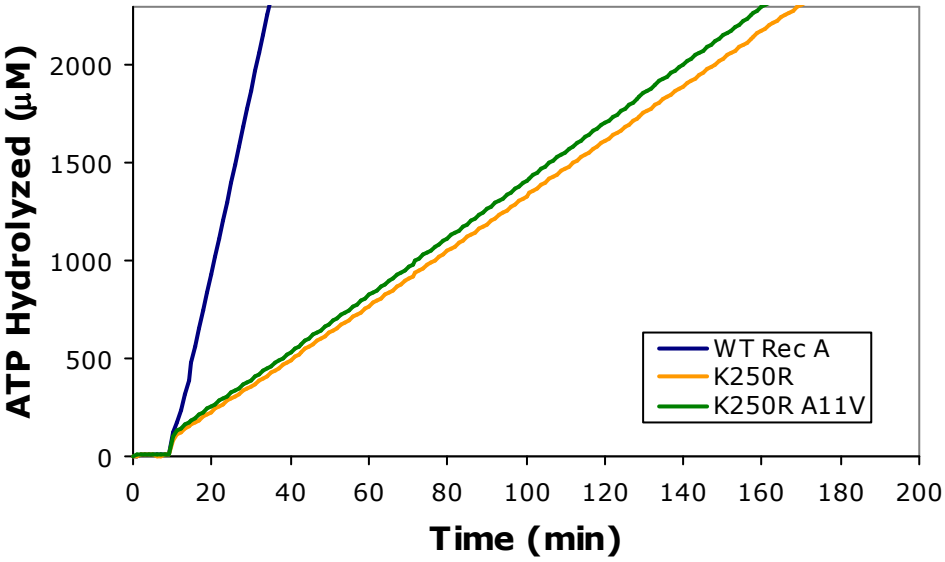


FIG 7. RecA K250R and K250R/A11V mutations affect rate of ATP hydrolysis. RecA protein-catalyzed ATP hydrolysis was monitored. 5μM RecA protein was preincubated for 10 minutes before ATP and SSB are added. The average observed rates of ATP hydrolysis is reported in TABLE 3.

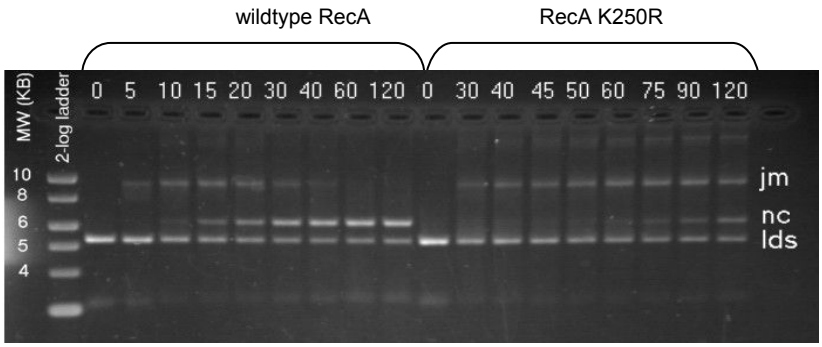
	Kcat	*n*	SD
WT RecA	34.84	6	2.17
K250R	4.62	6	0.34
K250R/A11V	4.88	6	0.35

TABLE 3. Rates of ATP hydrolysis by wildtype and mutant RecA proteins. RecA protein-catalyzed ATP hydrolysis is monitored as described in FIG 7. Rates are in units μM/min.

The rate of ATP hydrolysis is determined as described under experimental procedures. From Table 3, we can see that RecA K250R and K250R/A11V hydrolyze ATP at comparable rates

suggesting that the A11V mutation did not change the rate of ATP hydrolysis significantly. The A11 residue is not near the site of ATP binding and hydrolysis on RecA monomers and this perhaps accounts for the reason why rate of ATP hydrolysis is not changed appreciably. As such, we can conclude that the rescue in cell growth in the RecA K250R/A11V mutant is not due to an increase in ATP hydrolysis by RecA.

RecA K250R and K250R/A11V both catalyze DNA-strand exchange slower than wildtype RecA.



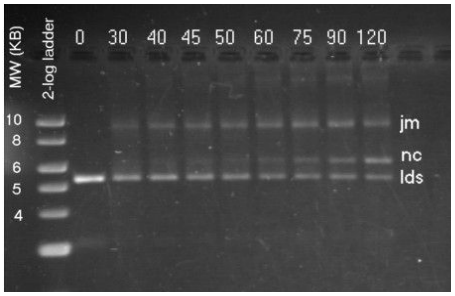


FIG 8. DNA-strand exchange of wildtype, K250R and K250R/A11V. 4μMRecA protein is preincubated with PhiX174 circular single stranded DNA for 10 minutes before the addition of ATP/SSB. PhiX174 linear double stranded DNA is then added after a 10 min incubation. Time points are taken as shown in figure and analyzed by agarose gel electrophoresis; lds, linear dsDNA reactant; jm, joint molecule intermediate; nc, nicked circular product.

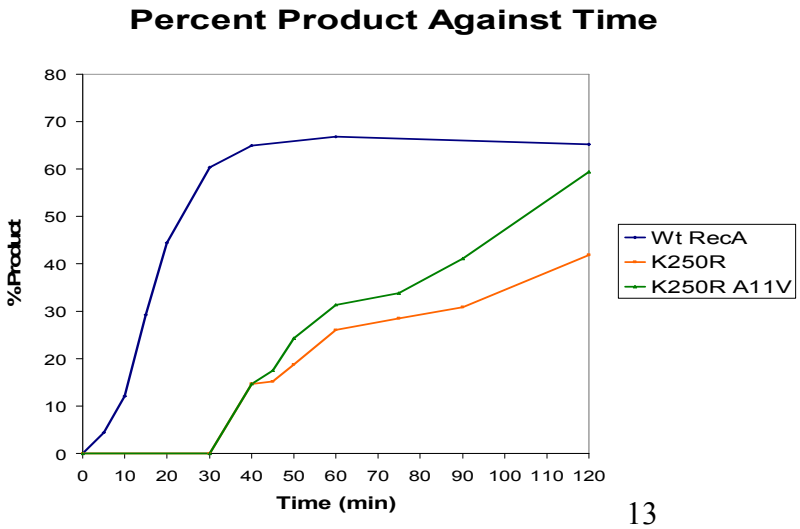


FIG 9. RecA K250R and K250R/A11V show similar rates of strand exchange. Percentage product is based on the amount of nicked circular product formed. The agarose gel in Figure 8 is analyzed using TotalLab software. Both RecA K250R and K250R/A11V forms initial nicked circular product later than wildtype RecA. RecA K250R/A11V has slightly higher percent product formation than RecA K250R at each time point, showing that the double mutant can carry out strand exchange slightly better than RecA K250R, although the overall rates are very similar.

Both RecA K250R and K250R/A11V catalyze DNA-strand exchange at a slower rate than wildtype RecA. Wildtype RecA protein catalyzed complete DNA-strand exchange to form nicked circular product, with the first nicked circular product forming at around 10min. Both RecA K250R and K250R/A11V are able to catalyze complete strand exchange. However, both catalyze at a rate much slower than wildtype RecA, with the first nicked circular product forming at around 40min.

We have observed that RecAK250R/A11V consistently forms slightly more nicked circular product than RecA K250R at any given time point. However, both K250R and K250R/A11V seems to be catalyzing strand exchange at similar rates.

When the strand exchange reaction is monitored in the Cary 300 spectrophotometer, we also observe very little difference in rate of ATP hydrolysis between RecA K250R and K250R/A11V (figure 9). When linear double stranded DNA is added, both mutants show a decrease in rate of ATP hydrolysis. RecA K250R/A11V tends to show a more significant drop in rate of ATP hydrolysis than K250R, and this probably corresponds to the slightly better strand exchange activity of this double mutant.
As such, we conclude that there is no significant difference between the strand exchange activity of RecA K250R and K250R/A11V.

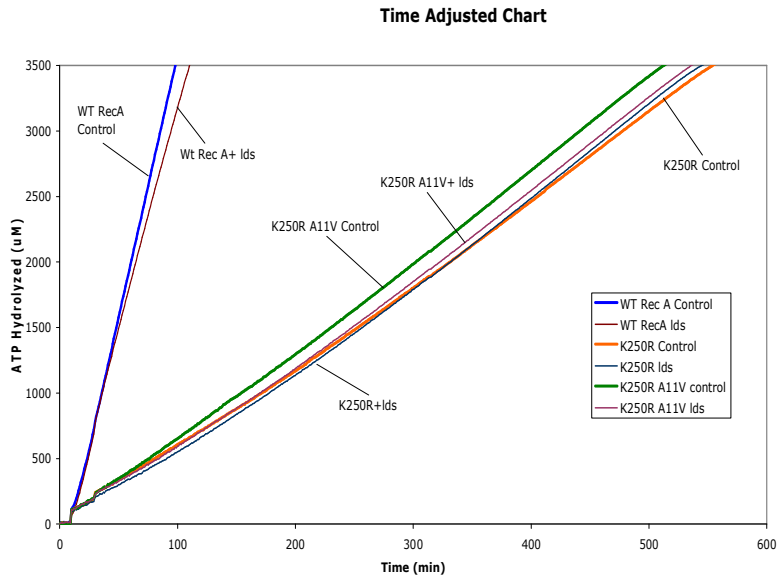


FIG 10. RecA K250R and K250R/A11V show similar drop in ATP hydrolysis during DNA-strand exchange. Strand exchange is monitored in the Cary 300 spectrophotmometer. 2.5μM RecA protein is preincubated for 10 mins before addition of ATP/SSB. Strand exchange is initiated after an additional 20 mins by the addition of 4μM M13 linear double stranded DNA. The rateate of ATP hydrolysis decreases when linear double stranded DNA is added.

RecA K250R/A11V disassembles better than RecA K250R during strand exchange

In this experiment, we use RecA K72R as a tool to monitor disassembly of RecA monomers during strand exchange. RecA K72R binds ATP and DNA, but does not hydrolyze ATP and hence does not disassemble (Shan, 1997). RecA K72R monomers bind DNA at sites recently vacated by wildtype or mutant RecA. Over time, the rate of ATP hydrolysis decreases as more K72R is bound to DNA without disassembling. This decline in ATP hydrolysis gives

us an indirect measure of disassembly of the RecA monomers of interest.

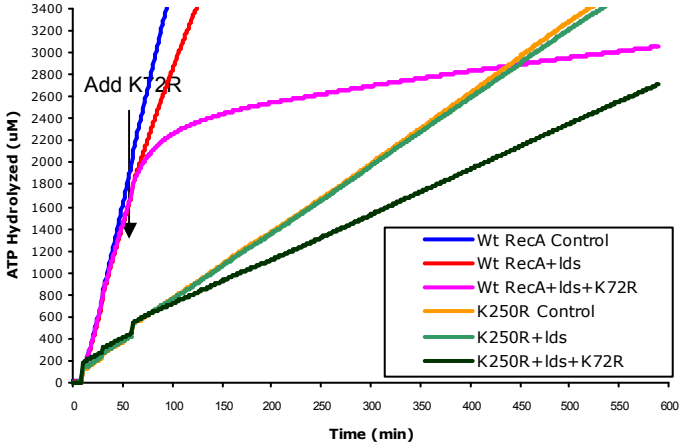


FIG 11. RecA K250R does not disassemble well. 2.5μM RecA protein was pre-incubated for 10 mins before addition of ATP/SSB. 4μM M13 linear double stranded DNA was added after an additional 20 mins to initiate strand exchange. To monitor disassembly of RecA filaments, 5μM of RecA K72R was added after a further 30mins. The concentration of RecA K72R was double of wildtype and K250R so as to effectively challenge strand exchange. Rate of ATP hydrolysis attenuates almost immediately after addition of RecA K72R. Rate of ATP hydrolysis only decreases a little after strand exchange using RecA K250R is challenged by RecA K72R.

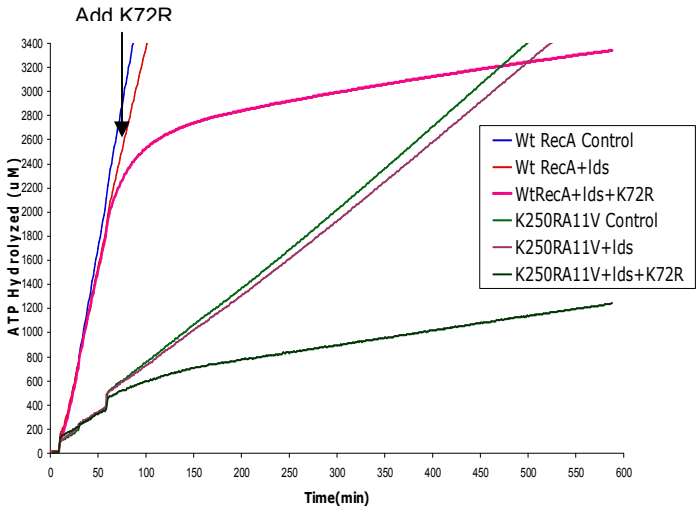


FIG 12. RecA K250R/A11V restores wildtype disassembly of RecA filaments. The reaction were carried out as in Figure 11. ATP hydrolysis for both wildtype and RecA K25R/A11V show sharp attenuation when RecA K72R is added as a challenge to strand exchange. This shows that RecA K250R/A11V can disassemble as well as wildtype RecA.

When strand exchange catalyzed by wildtype RecA is challenged by RecAK72R, the rate of ATP hydrolysis decreases markedly. Disassembly of *E.coli* RecA nucleoprotein filament is necessary for completion of DNA strand exchange (Britt, unpublished). Since RecA K72R cannot hydrolyze ATP and thus cannot disassemble, this prevents strand exchange from taking place. From Figure 10, we can see that even when RecA K72R is added, the rate of

ATP hydrolysis of RecA K250R does not decrease as significantly as for wildtype RecA. This shows that RecA K250R nucleoprotein filament does not disassemble well from DNA during strand exchange.

On the other hand, RecA K250R/A11V shows similar disassembly as wildtype RecA. When RecA K72R is added during strand exchange catalyzed by RecA K250R/A11V, the rate of ATP hydrolysis decreases markedly. This shows that the double mutant is probably as dynamic as wildtype RecA. Also, the A11V mutation allows RecA monomers to disassemble more readily from the nucleoprotein filament.

RecA K72R/E38K is not a good tool to monitor disassembly

The DNA-strand exchange activities of both mutants are also challenged with RecA K72R/E38K. RecA K72R/E38K also does not hydrolyze ATP, but forms better filaments on single stranded DNA than RecA K72R (Gruenig et al., submitted). As such, we initially thought that RecA K72R/E38K might be a better tool to study disassembly.

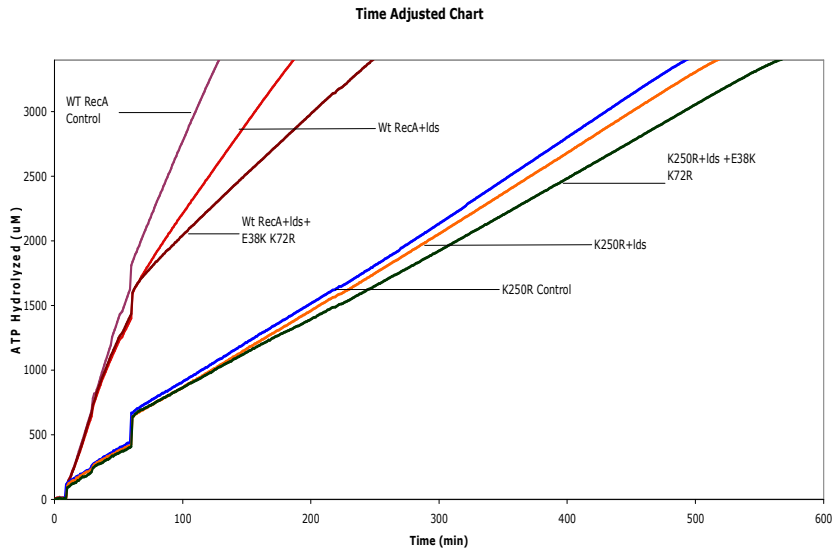


FIG 12. Strand exchange of RecA K250R using RecA K72R/E38K as a tool to monitor disassembly. Reaction condition and reagent concentrations are the same as that used for RecA K72R as a challenge (Figure 10). 5μM of RecA K72R/E38K is used. Even for wildtype RecA, ATP hydrolysis decreases slightly when RecA K72R/E38K is added.

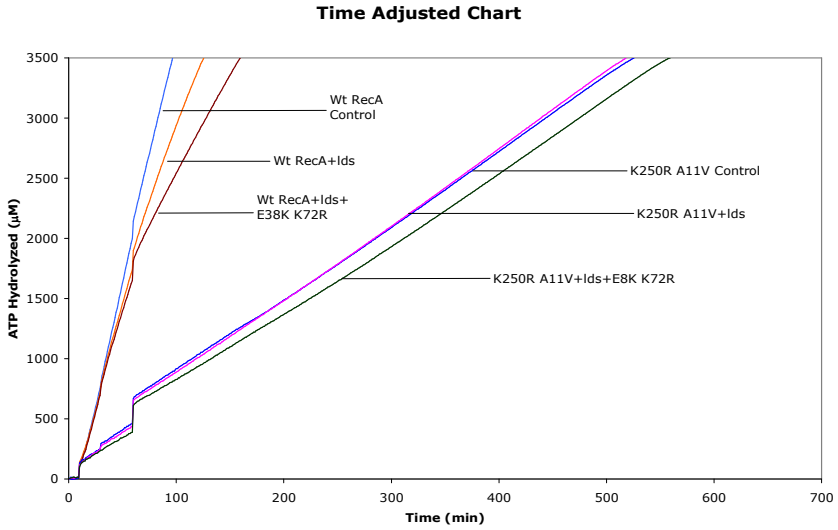


FIG 13. Strand exchange of RecA K250R/A11V using RecA K72R/E38K as a tool to monitor disassembly. Reaction conditions and reagent concentrations are as decribed in Figure 10. Rate of ATP hydrolysis only decreases slightly for both wildtype and RecA K250R/A11V when RecA K72R/E38K is added as a challenge. Neither protein shows the near total attenuation of ATPase activity when the challenge is added, as observed in Figure 11.

From Figure 12 and 13, we can see that the rate of ATP hydrolysis did not decrease much for wildtype RecA, RecA K250R and RecA K250R/A11V when RecA E38K/K72R is added to challenge the strand exchange of the RecA protein. There are very little differences in rates of ATP hydrolysis when the strand exchange reaction is challenged. As such, RecA E38K/K72R is not a good tool under these conditions to monitor RecA disassembly. Further studies will be performed to investigate this.

Discussion

We conclude that the [KR]x[KR] motif, especially RecA residue K250, is directly involved in the ATP hydrolytic activity of *E.coli* RecA. This motif also has a role in coupling RecA-mediated ATP hydrolysis to DNA-strand exchange.

Using mutational studies, we have shown that substituting the lysine 250 residue by a polar but uncharged residue (asparagine and glutamine) causes the mutant RecA to lose much of its catalytic functions. As such, we conclude that K250 is important in RecA function. A more conservative mutation, K250R retains most of RecA's catalytic functions. However, RecA K250R performs these activities at a much slower rate than wildtype RecA.

E.coli cells expressing K250R grows at a much slower rate than wildtype *E.coli*. This slow growth phenotype is rescued when we knockout RecFOR. The RecFOR proteins are involved in loading RecA onto single stranded DNA for recombinational repair during replication. From this we conclude that the defect caused by the K250R mutation involves loading RecA protein at the replication fork. From the DNA-strand exchange experimeny using RecA K72R as a challenge, we observed that RecAK250R does not disassemble well from DNA and is less dynamic than wildtype RecA. Hence, we propose that this limit to replication of *E.coli* is due to the RecA K250R not disassembling fast enough from the replication fork for replication to proceed. RecA K250R thus slows down replication of the *E.coli* genome, and this in turn limits growth.

When RecA K250R is overexpressed in *E.coli* cells, suppression mutations quickly arise to recover the slow growth phenotype. We observed that most cells recover growth by inactivating this mutant RecA. One suppressor mutation, RecA K250R/A11V allows for normal growth and still retains limited RecA functions. We focused on characterizing this double mutant to investigate how the A11V suppressor mutation allows for growth recovery.

We have shown from the ATP hydrolysis assays and DNA-strand exchange reactions that RecA K250R and RecA K250R/A11V have similar rates of ATPase and intro recombination activities. Both mutant proteins promote ATP hydrolysis and strand exchange at rates six times slower than wildtype RecA. From this we conclude that the A11V mutation does not change the catalytic activities of RecA significantly.

From the strand exchange reaction using K72R as a challenge, we observed that RecA K250R/A11V disassemble at a rate similar to that of wildtype RecA. Hence, the main difference between RecA K250R and K250R/A11V that we have observed is this difference in rate of disassembly from the nucleoprotein filament. Both wildtype and K250R/A11V RecA are dynamic, and disassembles readily. RecA K250R is less dynamic than wildtype and K250R/A11V. We propose that this rescue in rate of disassembly of the RecA nucleoprotein filament is important in suppressing the slow growth phenotype of RecA K250R. RecA K250R blocks replication at the replication fork and limits cell growth. Although RecA K250/A11V does not significantly change the catalytic functions of the mutant RecA, it allows the RecA monomers to be more dynamic. RecA proteins can now

disassemble easily from the filament, and will not be blocking the replication fork. As such, replication can proceed at normal rate, and cell growth is rescued.

From this study, we conclude that K250 residue in the [KR]x[KR] motif is important in RecA function. Mutations in K250 affect ATP hydrolysis, strand exchange and disassembly of the RecA filament. The suppressor mutation RecA K250R/A11V does not significantly alter catalytic functions of RecA, but promotes better disassembly of RecA filaments. We are unclear how the A11V mutation helps to promote disassembly and more studies will be done on this. The alanine 11 residue is involved in docking RecA monomer to the adjacent monomer, and we speculate that a mutation in this residue possibly alters the interactions between RecA monomers.

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