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A recombinase paralog from the hyperthermophilic crenarchaeon *Sulfolobus solfataricus* enhances SsoRadA ssDNA binding and strand displacement

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Abstract

Homologous recombination (HR) is a major pathway for the repair of double-strand DNA breaks, a highly deleterious form of DNA damage. The main catalytic protein in HR is the essential RecA-family recombinase, which is conserved across all three domains of life. Eukaryotes and archaea encode varying numbers of proteins paralogous to their main recombinase. Although there is increasing evidence for the functions of some of these paralog proteins, overall their mechanism of action remains largely unclear. Here we present the first biochemical characterization of one of the paralog proteins, SsoRal3, from the crenarchaeon *Sulfolobus solfataricus*. The SsoRal3 protein is a ssDNA-dependent ATPase that can catalyze strand invasion at both saturating and subsaturating concentrations. It can bind both ssDNA and dsDNA, but its binding preference is altered by the presence or absence of ATP. Addition of SsoRal3 to SsoRadA nucleoprotein filaments reduces total ATPase activity. Subsaturating concentrations of SsoRal3 increase the ssDNA binding activity of SsoRadA approximately 9-fold and also increase the persistence of SsoRadA catalyzed strand invasion products. Overall, these results suggest that SsoRal3 functions to stabilize the SsoRadA presynaptic filament.

Keywords

Archaea; Recombination; DNA binding; ATPase; Strand invasion

1. Introduction

Double-strand breaks (DSB) are highly deleterious to the cell and when left unrepaired can result in not only cell death but also genetic instability, gross chromosomal rearrangements, and cancer in higher eukaryotes (Hada and Georgakilas, 2008; Shikazono et al., 2009; Ward, 1985). These breaks can be programmed, result from replication fork collapse, or arise through damage by UV, IR, or DNA crosslinking agents. Repair of these breaks is often through homologous recombination (HR), a high fidelity, universally conserved mechanism. In HR, strand invasion and strand exchange are catalyzed by the RecA family of

recombinases that includes RecA in bacteria and Rad51 in eukaryotes. These ATPases form dynamic nucleoprotein filaments with ssDNA that are competent for strand invasion and exchange. Pre-synaptic filament formation is thought to be facilitated by the action of recombination mediator proteins, including the recombinase paralogs (Carreira and Kowalczykowski, 2011; Liu et al., 2006, 2011a, 2011b; Morimatsu and Kowalczykowski, 2003).

In *Saccharomyces cerevisiae*, Rad52 and the Rad55 and Rad57 paralog proteins assist Rad51 protein in the competition imposed by RPA binding to ssDNA (Pâques and Haber, 1999; Sung, 1997; Sung et al., 2000). Rad55 and Rad57 form a functional heterodimer that stimulates Rad51 pairing reactions, contributes to nucleoprotein filament formation, and assembly of recombination complexes (Gasior et al., 1998; Hays et al., 1995; Johnson and Symington, 1995; Lisby et al., 2004; Sung, 1997). Most recently Rad55/57 has been shown to stabilize the Rad51 presynaptic filament through prevention of filament disassembly by the anti-recombinase Srs2 (Liu et al., 2011b). Six human Rad51 paralogs have been identified: the meiosis recombinase DMC1 as well as the poorly understood Rad51B, Rad51C, Rad51D, XRCC2, and XRCC3 proteins (Takata et al., 2001). There is very little biochemical information for the non-DMC1 proteins, but it has been shown that they form various heterodimeric complexes and at least one heterotetramer (Braybrooke et al., 2000; Kurumizaka et al., 2001; Masson et al., 2001a, 2001b; Miller et al., 2002; Schild et al., 2000; Sigurdsson et al., 2001; Wiese et al., 2002). Two complexes, XRCC2/Rad51D and XRCC3/Rad51B, have been shown to catalyze strand displacement and pairing, suggesting that these paralogs have a role in early recombination steps (Kurumizaka et al., 2001, 2002).

Archaea comprise the third branch in the phylogenetic tree of life, and while these microbes physiologically resemble bacteria, their genomic information processing mechanisms are remarkably eukaryotic in nature. Archaeal HR is one key process that involves proteins with significant eukaryotic homology. *Sulfolobus solfataricus* is a hyperthermophilic acidophile which grows at temperatures ranging from 70 to 85 °C in terrestrial hot springs that has emerged as a robust model system for biochemical studies of HR mechanisms. This archaeon encodes a Rad51 homolog, SsoRadA, as well as three paralogs that are members of the aRadC family: SsoRal1, SsoRal2, and SsoRal3 (Haldenby et al., 2009; McRobbie et al., 2009; Rolfsmeier et al., 2010). While it is not known if any of these paralogs are essential, our laboratory has recently shown that their transcripts are strongly upregulated in response to chromosomal damage caused by IR, suggesting a role for these proteins in repair of DSBs (Rolfsmeier et al., 2011). Biochemical characterization of the *S. solfataricus* paralogs has been limited to only the SsoRal1 protein, which can function as an anti-recombinase (McRobbie et al., 2009). Examination of post-synaptic function of the *Sulfolobus tokodaii* StRadC2 protein, a paralog with similarity to SsoRal3 protein, suggests that it can also act as an anti-recombinase with primary involvement in post-synapsis where it directly interacts with the cognate RadA and Holliday junction resolvase proteins (Wang et al., 2012). In this work we present the purification and first biochemical characterization of the *S. solfataricus* SsoRal3 protein and propose a role for this paralog during the presynaptic and synaptic steps of HR.

2. Materials and methods

2.1. Expression and purification of proteins

Open reading frame Sso1861 was PCR amplified using total genomic DNA from *S. solfataricus* strain P2 obtained from the American Type Culture Collection as a template. Forward (5'-CGCGGATCCGT TTATTAAATAACTAACTCTACTCC-3') and reverse (5'-CTTATGGCTGC AGTAAAGACATATATACCTGGG-3') primers were designed to be complimentary to the beginning and end of the open reading frame, respectively, and included restriction sites for ligation of the product into pET3a (Novagen) digested with NheI and BamHI (New England Biolabs). For heterologous expression, this construct was transformed into the Rosetta strain (Novagen) which was grown in LB medium at 37 °C with shaking to late log phase. Protein production was induced by the addition of IPTG (Sigma) to a final concentration of 1 mM. Expression was allowed to continue for 3 h, after which cells were harvested by centrifugation and stored at -80 °C until processing. Lysates were prepared by sonicating cells resuspended at a density of 4 ml buffer/g cells in 20 mM Tris-Cl (pH 7.5), 500 mM NaCl, and 10% glycerol (sonication buffer). Crude sonicate was heated for 20 min at 80 °C and precipitated protein was removed by centrifugation for 15 min at 16,300 ×g at room temperature. Soluble material was subjected to dialysis for 4 h with two changes of buffer using 20 mM Tris-Cl (pH 7.5), 100 mM NaCl, 10% glycerol (equilibration buffer) and then filtered using a 0.45 µm filter. Dialyzed protein was loaded onto a Blue Sepharose column (GE Healthcare) pre-equilibrated with the same buffer. The column was developed using a gradient of NaCl from 100 to 800 mM. SsoRal3 protein containing fractions were dialyzed overnight against 20 mM Tris-Cl (pH 7.5), 50 mM NaCl, and 10% glycerol and then loaded onto a MonoQ column (GE Healthcare) equilibrated with the same buffer. Protein-containing fractions were pooled and concentrated with polyethylene glycol 20,000 (JT Baker), flash frozen, and stored at -80 °C. The SsoRadA, SsoSSB, *Thermotoga maritima* pyruvate kinase (*tmPK*), and *T. maritima* lactate dehydrogenase (*tmLDH*) proteins were from laboratory stocks and prepared as previously described (Haseltine and Kowalczykowski, 2009; Rolfsmeier and Haseltine, 2010). Protein concentrations were determined using optical density at 280 nm and the corresponding extinction coefficient (SsoRal3, 33,920 M⁻¹ cm⁻¹; SsoRadA, 17,420 M⁻¹ cm⁻¹; SsoSSB, 12,660 M⁻¹ cm⁻¹). Tricine SDS/PAGE gels to monitor protein purification were prepared with a 4% stacking gel and a 10% separating gel as described (Price and Shand, 2000).

2.2. ATPase assays

ATPase activity was determined using the thermophilic continuous coupled spectrophotometric assay previously described (Rolfsmeier and Haseltine, 2010; Seybert et al., 2002). Assays were performed at the indicated temperatures using a Beckman Coulter DU-800 spectrophotometer fitted with a Peltier device. Conversion of NADH (Sigma) into NAD was measured at 340 nm. Typical reactions contained 40 mM HEPES (pH7.0), 20 mM MgOAc, 300 µM NADH, 2 mM phosphoenolpyruvate (Sigma), 15 U *tmLDH*, 15 U *tmPK*, 3 mM ATP, with DNA, SsoRal3, SsoRadA, and SsoSSB at the concentrations indicated in a 120 µl volume. Reactions were incubated 3 min at the indicated temperature before addition of the initiating component (s). In cases where SsoRal3 and SsoRadA were added at different times, the second protein was added after 6 min. Where SsoSSB was included, 0.04

μ M SsoSSB protein was incubated for 3 min at the indicated temperature after which SsoRal3, SsoRadA, or both SsoRal3 and SsoRadA were added. All assays were performed a minimum of three times. The extent of ATP hydrolysis was determined by measuring NADH oxidation with an extinction coefficient of $6220 \text{ M}^{-1} \text{ cm}^{-1}$. Values were adjusted by subtraction to account for spontaneous oxidation of NADH. Means, standard deviations, and linear regression were calculated using GraphPad Prism 4 to determine the reaction velocity for the data points. Final reaction yields were determined after 30 min.

2.3. Electrophoretic mobility shift assays

A 63 nucleotide oligonucleotide (5' ACAGCACCAATGAAATCTATT AAGCTCCTCATCGTCCGCAAAAATATCGTCACCTCAAAAGGA 3') was labeled at the 5' end with [γ - ^{32}P]ATP using 10 U of T4 polynucleotide kinase (NEB) for 1 h at 37 °C using the manufacturer's recommended buffer, followed by inactivation of the kinase by incubation for 10 min at 75 °C. Assay reactions were performed at 70 °C for 30 min in an Eppendorf Master Cycler Gradient Thermal Cycler fitted with a heated lid. Typical reactions were 20 μ l and contained 30 mM HEPES (pH 7.0), 10 mM MgOAc, 50 μ g/ml bovine serum albumin, 50 mM NaCl (reaction buffer), varying concentrations of SsoRal3 and SsoRadA, and 5 μ M nucleotide-labeled oligonucleotide. ATP was included at a concentration of 3 mM where indicated. dsDNA substrates were prepared by annealing the radiolabeled 63-mer to its unlabeled complement. Equimolar concentrations of the oligonucleotides were boiled for 10 min and then slowly cooled over a period of 2 h to room temperature. Following incubation of the reactions, 4 μ l of loading dye (0.025% bromophenol blue in 30% glycerol) was added and samples were loaded on a 130 cm 7% acrylamide 1 $\times$ TBE gel that had been prerun for 15 min at 45 mA. Samples for salt midpoint experiments were incubated at 70 °C in reaction buffer containing the indicated concentration of NaCl. Following electrophoresis gels were dried, exposed to a phosphorimaging screen, and analyzed with a Storm 850 PhosphorImager (Molecular Dynamics). Shifted species were quantitated using the ImageQuant TL program. The amount of unbound oligonucleotide was assigned a value of 100% when measurements of two consecutive band intensities were equal or no longer increasing.

2.4. Displacement loop assay

To prepare the dsDNA substrate, DH5 α *E. coli* cells harboring pBR322 were grown to stationary phase in a 1 l volume of LB medium. Cells were collected by centrifugation for 15 min at 4 °C using an SLA-3000 rotor at 9000 rpm. The cells were washed using cold M9 buffer (22 mM KH₂PO₄, 42 mM Na₂HPO₄, 19 mM NH₄Cl) and then resuspended in 40 ml of cold lysis buffer (50 mM Tris-Cl (pH 8.0), 10 mM EDTA, 10% sucrose). 58 μ g of lysozyme (Fischer Biochemicals) was added and the lysis buffer was adjusted to 0.9% N-lauroylsarcosine (MP Biomedicals) and 1 M NaCl. The resulting crude lysate was subjected to centrifugation for 45 min at 4 °C using a Ti 50.2 rotor at 34,400 rpm. Soluble material was then extracted with an equal volume of phenol:chloroform twice, and then DNA was precipitated by the addition of 1/10th volume of 5 M NaCl and 2 volumes 100% ethanol followed by centrifugation for 15 min at 16,300 $\times g$ at room temperature. The resulting pellet was then resuspended in 2 ml of TE (10 mM Tris-Cl (pH 8.0), 1 mM EDTA). 100 μ g of RNaseA was added, and then incubation was permitted to continue for 30 min on ice. The

solution was adjusted to a final concentration of 3 M LiCl and incubated a further 30 min on ice, followed by centrifugation at 12,000×g for 15 min at 4 °C. The supernatant was transferred to a fresh tube and ethanol precipitated. The resulting DNA pellet was resuspended in 1 ml of TE and 0.5 ml of a 40% PEG 8000 (JT Baker), 30 mM MgCl₂ was added. DNA was collected by centrifugation for 30 min at 16,300×g at room temperature, and the pellet was washed twice with 70% ethanol before resuspension in 0.5 ml TE. This material was loaded on the top of a 5–20% sucrose gradient which was subjected to centrifugation at 25,600 rpm using an SW 41 Ti rotor for 18 h at 4 °C. Fractions of approximately 0.5 ml were collected by needle puncture of the centrifuge tube and were evaluated by electrophoresis on a 1% agarose TBE gel. Fractions containing covalently closed circular DNA that were free of nicked circular species were pooled, ethanol precipitated, and the pellet was resuspended in TE. The ssDNA substrate was prepared by end-labeling a 101-mer oligonucleotide (5'

TGGCCTGCAACGCGGGCATCCCCATGCCGCCGGAAG

CGAGAAGAATCATAATGGGGAAGGCCATCCAGCCTCGCGTCCGGAACGCC

AGCAAGACGTAGCCC 3') at the 5' end with [γ -³²P]ATP using 10 U of T4 polynucleotide kinase (NEB) for 1 h at 37 °C using the manufacturer's recommended buffer, followed by inactivation of the kinase by incubation for 10 min at 75 °C. Displacement loop reactions contained 25 mM TrisOAc (pH 7.5), 10 mM MgOAc, 50 µg/µl BSA, 2 mM ATP, 0.9 µM (nts) 101-mer radiolabeled oligonucleotide, and either 0.6 µM (saturating) or 0.12 µM (sub-saturating) SsoRadA and/or SsoRal3 were incubated for 5 min at 65 °C. Second protein additions were after this incubation step in the indicated reactions, which were further incubated for 5 min at 65 °C. In reactions including SsoSSB, 0.09 µM SsoSSB protein was initially incubated with assay components for 5 min at 80 °C. SsoRal3, SsoRadA, or SsoRal3 and SsoRadA were then added and reactions were incubated for 5 min at 65 °C. In order of addition reactions the second protein was added next, followed by an additional 5 minute incubation at 65 °C. To initiate the reaction 30 µM pBR322 (bp), prepared without alkaline lysis as detailed above, was added. Samples were then incubated for the indicated times at 65 °C. Reactions were stopped with the addition of 0.5 µl 0.5 M EDTA, 0.5 µl 20 mg/ml Proteinase K (NEB), and 1.5 µl 10% SDS followed by incubation at 65 °C for 30 min. Loading dye (0.025% bromophenol blue in 30% glycerol) was added and samples were subjected to electrophoresis on a vertical 1.5% agarose 1× TAE gel for 150 min at 75 V (gels were pre-run 15 min at 75 V). Gels were subsequently dried, exposed to a phosphorimaging screen, and analyzed with a Storm 850 PhosphorImager (Molecular Dynamics). Quantitation was performed using the ImageQuant TL program.

3. Results

3.1. Expression and purification of SsoRal3

The SsoRal3 protein has been previously identified as a paralog of SsoRadA, the main *S. solfataricus* strand exchange protein (Haldenby et al., 2009; Rolfsmeier et al., 2010). It is encoded by an 843 base pair ORF identified as SSO1861 in the *S. solfataricus* strain P2 genome (AE006641.1) and annotated as a conserved hypothetical protein. Since this ORF is not in an apparent operon conformation in the chromosome, the only indication of possible function is based on its homology to the main strand exchange protein, SsoRadA. The

SsoRal3 protein includes Walker A and B motifs, a conserved feature among the paralogs and SsoRadA protein. Aside from simple homology to homologous recombination related proteins, SsoRal3 has been implicated in repair of DNA damage as its transcript is upregulated in response to both UV and IR damage (Rolfmeier et al., 2010, 2011). Based on the gene sequence, the SsoRal3 protein has a predicted pI of 6.7 and a protein length of 283 amino acids (<http://www-archbac.u-psud.fr/projects/sulfolobus/r1600-1700k/>), which is smaller than the 342 amino acid SsoRadA protein. To better understand the function of SsoRal3 in DNA repair, we purified the protein for biochemical characterization (Fig. 1). The SSO1861 ORF was cloned into the pET3a bacterial expression vector and heterologously over-expressed in *Escherichia coli*. The bacterial strain used included a plasmid encoding rare tRNA genes to reduce translational pausing, since a number of codons in the gene are underrepresented in *E. coli*. The resulting expressed protein is largely soluble and was purified to near homogeneity using two chromatographic steps. Following over-expression, cells were lysed and this crude cell lysate was heated to 80 °C to denature mesophilic proteins and generate clarified sonicate (Fig. 1, lane 1). The clarified sonicate was then loaded on a blue sepharose column which was followed by anion exchange chromatography, which yielded protein that was approximately 97% pure (Fig. 1, lanes 2 and 3 respectively). The protein resolves as a single band of about 34.3 kDa in size by SDS-PAGE, which is in close agreement with the predicted molecular weight of 31.3 kDa as determined from the protein sequence.

3.2. SsoRal3 is a ssDNA-dependent ATPase

Since the SsoRal3 protein sequence includes Walker A and B motifs, it seemed likely that the protein could hydrolyze ATP. To determine general SsoRal3 activity characteristics, we used a spectrophotometric assay that couples ADP production through ATP hydrolysis to NADH oxidation, which is then directly measured in real time (Rolfmeier and Haseltine, 2010; Seybert et al., 2002) (Fig. 2, Table 1). SsoRal3 showed almost no ATP hydrolysis in the absence of DNA or when pBR322 was used as a substrate (Fig. 2a). Activity for this protein was apparent with ssDNA, however, as either ss ϕ X174 or poly(dT) used as a substrate resulted in measureable ATP hydrolysis. SsoRal3 protein is active over a broad temperature range, with an optimum of approximately 70 °C (Fig. 2b). Approximately 40% protein activity is observed as low as 60 °C and as high as 80 °C. We also determined the approximate site-size for SsoRal3 with ssDNA as a substrate (Fig. 2c). Here the concentration of ϕ X174 ssDNA was held constant at 0.6 μ M and the concentration of SsoRal3 protein was varied. At the saturation point, the apparent binding stoichiometry is one SsoRal3 monomer per 4 nucleotides. Based on this site-size, in all subsequent assays SsoRal3 was considered to be saturating at a stoichiometry of one monomer per 3 nucleotides in order to account for any inactive protein present in the preparation. These data show that SsoRal3 is a ssDNA-dependent ATPase that is active at physiologically relevant temperatures.

3.3. DNA binding preference is dependent on ATP

While the ssDNA-dependent ATPase activity of SsoRal3 suggested that the protein can bind ssDNA, this activity does not preclude possible binding to dsDNA. To determine the binding characteristics of the SsoRal3 protein with both ss- and dsDNA in the presence and

absence of ATP, we used EMSA analysis (Fig. 3). When ssDNA is used in the presence of ATP, SsoRal3 produces a gradual, non-cooperative gel shift product that increases in intensity with protein concentration (Fig. 3a). In the absence of ATP, however, a mild gel shift was detected only at the highest protein concentration, suggesting that the presence of ATP enhances the ability of SsoRal3 to bind ssDNA. The opposite effect was observed when dsDNA was used (Fig. 3b). In this case, a stronger non-cooperative gel shift product was apparent in the absence of ATP. These data suggest that SsoRal3 differentially binds DNA in response to the presence of an ATP nucleotide cofactor. The binding strength of SsoRal3 at a saturating concentration (1.7 μ M) was determined through salt midpoints using both ss- and dsDNA substrates under the best binding conditions in the presence or absence of ATP, respectively, as determined in Figs. 3a and b and shown in Figs. 3c and d. Protein-DNA binding was performed in the presence of increasing concentrations of NaCl and gel shifted products were quantified (Fig. 3e). The salt midpoints for SsoRal3 binding ss- and dsDNA under these conditions were similar, with a midpoint for ssDNA at approximately 170 mM and a midpoint for dsDNA at approximately 140 mM NaCl. We were unable to determine a midpoint for SsoRal3 binding ssDNA in the absence of ATP and SsoRal3 binding dsDNA in the presence of ATP since the binding was very weak (data not shown). These data suggest that SsoRal3 can bind both ss- and dsDNA, that binding preference depends on the presence or absence of ATP, and that when bound to either substrate, the strength of binding is nearly equivalent.

3.4. SsoRal3 can stabilize SsoRadA–ssDNA complexes

To further understand the function of the SsoRal3 paralog, we investigated the possibility that it might interact with the SsoRadA strand exchange protein. We were unable to detect a direct interaction between purified SsoRal3 and SsoRadA through co-immunoprecipitation efforts (data not shown), suggesting that if SsoRal3 functions with SsoRadA the effect could be DNA-mediated. We next used EMSA analysis to determine SsoRal3 behavior with SsoRadA nucleoprotein filaments (Fig. 4). Under conditions where SsoRadA is saturating and forms a complete nucleoprotein filament (Rolfmeier and Haseltine, 2010; Seitz et al., 1998), we did not detect a strong gel shift product either in the presence or absence of ATP (Fig. 4, lanes 3 and 9, upper band). No gel shift was detected under conditions when SsoRadA was at subsaturating concentrations (Fig. 4, lanes 4 and 10). This observation is similar to results reported for eukaryotic Rad51, where gel shift products are not detected in the absence of glutaraldehyde crosslinking and may reflect the dynamic filament character (Arenson et al., 1999; Forget and Kowalczykowski, 2010; Lee et al., 2004; Li et al., 2007; Mine et al., 2007; Robertson et al., 2009). The addition of subsaturating concentrations of SsoRadA had essentially no effect on the ability of SsoRal3 protein to bind ssDNA (Fig. 4, compare lower band in lanes 1 and 5, lanes 7 and 11). Addition of SsoRal3 at a subsaturating concentration, however, results in an approximate 9.2-fold increase in the SsoRadA gel shift product that is ATP-dependent (Fig. 4, compare upper band in lanes 3 and 6, lanes 9 and 12). These results suggested that the SsoRal3 protein could have a role in stabilizing the SsoRadA filament as it is forming or that SsoRal3 facilitates SsoRadA binding to ssDNA. In the absence of ATP, SsoRal3 showed little ssDNA binding and had no clear effect on SsoRadA–ssDNA binding (Fig. 4, lanes 7 and 8 lower band, lanes 9 and 12

upper band), suggesting that SsoRal3 ssDNA binding stabilizes the SsoRadA–ssDNA nucleoprotein complex only when ATP is present.

3.5. SsoRal3 alters SsoRadA ssDNA-dependent ATPase activity

Purified SsoRal3 and SsoRadA proteins have differential temperature optima; SsoRal3 is most active at 70 °C (Fig. 2b) while SsoRadA shows its highest activity at 80–85 °C (Rolfmeier and Haseltine, 2010; Seitz et al., 1998). We therefore examined the effect of both proteins in ATPase assays at 70 and 80 °C (Table 1). At 70 °C and at a saturating concentration, SsoRal3 reaction velocity was 2.4 ± 0.6 $\mu\text{mol ATP hydrolyzed/minute}$ with a yield of 69.1 ± 10.8 $\mu\text{mol of ATP hydrolyzed}$. SsoRadA is less active at this temperature and at a saturating concentration showed a reaction velocity of 0.8 ± 0.2 $\mu\text{mol ATP hydrolyzed/minute}$ and a yield of 22.1 ± 4.8 $\mu\text{mol of ATP hydrolyzed}$. At 80 °C, when SsoRal3 is at a saturating concentration the reaction velocity is nearly the same as that at 70 °C, but the yield of ATP hydrolyzed is reduced to 19.7 ± 4.0 μmol . When SsoRadA is at a saturating concentration at 80 °C, the reaction velocity is higher than at 70 °C at 1.2 ± 0.2 $\mu\text{mol ATP hydrolyzed/minute}$, but the yield is reduced to 11.1 ± 2.0 . We examined the effect the presence of both proteins in the coupled ATPase assay at both 70 °C and 80 °C using order of addition experiments (Fig. 5, Table 1). At 70 °C the presence of both proteins in the reaction resulted in a decrease in both reaction velocity and ATP hydrolysis yield in almost all cases, regardless of whether one was added before the other or both were added at the same time. When comparing reactions with subsaturating SsoRadA alone to those where subsaturating SsoRal3 was added second, we observed a slight increase in velocity and a similar ATP hydrolysis yield. At 80 °C, we again saw a similar trend where the presence of both proteins resulted in an overall reduction in velocity and yield relative to the first protein present in the reaction. The exception was when both proteins were present at a subsaturating concentration and the reaction was initiated by the addition of ssDNA. In this case we observed a stimulation of both reaction velocity and ATP hydrolysis yield that was above that which would be additive for the proteins together. Overall it seems unlikely that the observed reduction in velocity and yield of ATP hydrolysis is due to simple competition for binding sites on the ssDNA; in this situation the activity curves would be expected to closely match the single protein controls.

We investigated the effect of the *S. solfataricus* single-stranded DNA binding protein, SsoSSB on the velocity and yield of ATP hydrolysis in reactions containing SsoRal3 and SsoRadA (Fig. 6, Table 2). Overall, we found that our results were similar to those shown in the absence of SsoSSB in Fig. 5 but that activity was generally diminished. This observation is consistent with the suggestion that SsoSSB must be displaced over time to provide binding sites for SsoRal3 and SsoRadA. It is unlikely that SsoRal3 is actively removing SsoSSB from the ssDNA, as results would be expected to more closely resemble those lacking SsoSSB if this were the case. Also, SsoRal3 does not appear to work with SsoSSB to load SsoRadA, as is the case for Rad52 assisted Rad51 loading (Sugiyama and Kowalczykowski, 2002), as we did not observe an increase in ATPase activity upon addition of SsoRal3.

3.6. SsoRal3 can catalyze strand invasion and extends the persistence of SsoRadA-mediated D-loops

Our observation that SsoRadA ATPase activity is reduced and yield of SsoRadA–ssDNA gel shifted products is increased in the presence of SsoRal3 suggests that SsoRal3 has an effect on nucleoprotein filament formation. The ability of a functional filament to invade duplex DNA and the persistence of the resulting joint molecule can be directly measured in vitro using a strand invasion (or D-loop) assay. Dissociation of D-loops and the corresponding loss of the joint molecule is the result of branch migration promoted by the recombinase and is ATP-hydrolysis dependent (Shibata et al., 1982, 2001). As shown in Fig. 7 and Supplemental Fig. 1, SsoRal3 was found to catalyze strand invasion under both saturating and subsaturating concentrations, similar to SsoRadA (Fig. 7b, compare lanes 3, 8, 13, 18 to lanes 2, 7, 12, 17; Fig. 7c). Persistence of the invasion product was extended approximately 3-fold, from 10 min to 30 min (Fig. 7c), in the SsoRal3 alone condition when subsaturating concentrations were present. This could indicate that incomplete filaments are not only sufficient for strand invasion, but might be preferred. Order of addition experiments were used to further evaluate SsoRal3 and SsoRadA behavior during strand invasion (Figs. 7b,d-f). When both proteins were at saturating concentrations, the presence of SsoRal3 in the reaction prior to the addition of SsoRadA did not significantly affect D-loop formation and results resembled those of SsoRadA alone. At these same concentrations the addition of both proteins together or SsoRadA first yielded joint molecule formation that was not additive of SsoRal3 and SsoRadA together, but rather intermediate. When both proteins were at subsaturating concentrations, both SsoRal3 and SsoRadA first conditions resulted in extension of D-loop persistence, with the more dramatic extension occurring when SsoRadA was added prior to SsoRal3 and invasion products were still apparent at the 30 minute mark. The extension of the D-loop cycle under these conditions suggested that the presence of SsoRal3 could play a role in stabilization of the invasion product. The most striking results were obtained under conditions where SsoRadA was subsaturating and SsoRal3 was saturating. Here, sequential addition of SsoRadA and SsoRal3 in either order resulted in the greatest simulation of D-loop products and a nearly 7-fold increase in joint molecule production was observed. This could indicate that partial SsoRadA filaments are increased for invasion competency in the presence of SsoRal3. The second most stimulatory effect occurred when SsoRadA was saturating and SsoRal3 was subsaturating, where we observed a 3-fold increase in joint molecule production with sequential protein addition. In this case the presence of a small amount of SsoRal3 prior to addition of SsoRadA increased the persistence of D-loops relative to either protein alone. The addition of SsoSSB protein delayed D-loop formation in all cases and the addition of SsoRal3 did not significantly alter this delay, suggesting that SsoRal3 is not actively displacing SsoSSB.

4. Discussion

Based on its homology to the SsoRadA strand-exchange protein and upregulation in response to DNA damage (Rolfsmeier et al., 2010, 2011), we targeted the SsoRal3 paralog protein for biochemical characterization. Like the *S. cerevisiae* Rad55/57 heterodimer (Sung, 1997), SsoRal3 can bind ssDNA and is a ssDNA-dependent ATPase. In contrast to Rad55/57, SsoRal3 DNA-binding activity is altered depending on the availability of ATP.

SsoRal3 not only binds ssDNA but also dsDNA, and binding preference depends on the presence of ATP. ssDNA is favored in the presence of ATP but dsDNA is preferred when it is absent. Strength of binding for SsoRal3 to either ssDNA in the presence of ATP or dsDNA in the absence of ATP is similar as reflected by salt midpoint determination, suggesting that the protein can bind either substrate equally well and that preference is influenced by ATP. The SsoRal3 protein produced a more stable gel shift product with ssDNA than was seen with SsoRadA. This could indicate that SsoRal3 forms a less dynamic DNA–protein complex and that ATP hydrolysis may not necessarily correlate with dissociation from DNA. While high concentrations of SsoRal3 could successfully outcompete SsoRadA for available ssDNA-binding sites, lower amounts of SsoRal3 appear to enhance SsoRadA binding to ssDNA; this suggests a possible role for SsoRal3 in nucleoprotein filament formation or stabilization. Alternatively SsoRal3 could function as a recombinase loading mediator. This seems unlikely, however, given bacterial and eukaryotic precedents. Bacterial RecFOR protein addition causes an increase in RecA recombinase ATPase activity (Morimatsu and Kowalczykowski, 2003), behavior that we did not observe with SsoRal3. We also were unable to detect a direct interaction between SsoRal3 and the cognate strand exchange protein, where such an interaction would be expected if the SsoRal3 protein were analogous to the eukaryotic Rad52 recombinase loading protein (New et al., 1998; Sugiyama and Kowalczykowski, 2002; Wu et al., 2008).

Addition of SsoRal3 to ATPase reactions including SsoRadA did not result in strong enhancement of activity under the majority of conditions tested. Instead the presence of SsoRal3 produced a general reduction of observed ATPase activity. Notably, challenging a preformed SsoRadA nucleoprotein filament with SsoRal3 decreases both the velocity and hydrolysis yield. While this decreased activity could be interpreted as removal of SsoRadA from ssDNA and/or prevention of filament formation, it could also indicate filament stabilization through reduction of SsoRadA disassociation. Such a stabilization function is consistent with the gel shift results that show improved SsoRadA binding in the presence of SsoRal3. This stabilization effect is likely DNA-mediated, since the two proteins do not appear to interact directly. Filament stabilization could function through a mechanism similar to that described for the phage UvsY protein, which interacts with both the UvsX recombinase and ssDNA (Liu et al., 2006; Sweezy and Morrical, 1997; Yassa et al., 1997). In this case, elimination of the UvsY–UvsX interaction does not prevent the stabilization effect, which is likely to be through UvsY–ssDNA interactions (Yassa et al., 1997).

Strand invasion has traditionally been shown to be catalyzed by recombinase proteins and occurs when the presynaptic filament invades homologous dsDNA, creating a D-loop (McIlwraith et al., 2000; Shibata et al., 1979). It had been previously reported that the SsoRadA recombinase protein could efficiently form D-loops in vitro (Seitz et al., 1998). Unexpectedly, we found that the SsoRal3 paralog protein can also produce strand invasion products. At subsaturating concentrations, both SsoRal3 and SsoRadA are individually capable of catalyzing the strand invasion reaction, suggesting that incomplete filaments of either SsoRadA or SsoRal3 are sufficient for invasion. In the presence of both proteins when SsoRal3 was subsaturating, the persistence of the invasion product was noticeably increased, suggesting stabilization of the joint molecule. It is likely that SsoRadA stabilization on ssDNA is the result of a reduction of ATP hydrolysis, because ATP hydrolysis by

recombinases is known to correlate with protein dissociation from ssDNA (Arenson et al., 1999; Forget and Kowalczykowski, 2010; Haldenby et al., 2009; Li et al., 2007; Masson et al., 2001a; Robertson et al., 2009). Nucleoprotein filament stabilization has been shown to be essential in a number of other systems: UvsY stabilization of UvsX filaments as well as Ca²⁺ and BRCA2 stabilization of Rad51 filaments all occur through reduction of recombinase ATPase activity (Bugreev and Mazin, 2004; Carreira and Kowalczykowski, 2011; Liu et al., 2006). Our observation of increased SsoRadA strand invasion product persistence suggests a probable similar stabilization of the nucleoprotein filament.

Under conditions where SsoRal3 is allowed to interact at the same time as or prior to the addition of SsoRadA, the SsoRal3 protein is capable of mediating strand invasion. Taken together with the gel shift and ATPase activity analyses, the mediation effect of SsoRal3 most likely occurs by stabilization of the presynaptic filament. Since subsaturating concentrations of either protein can catalyze formation of strand invasion products, occupation of all binding sites on the ssDNA strand is not essential. Instead nucleoprotein filament patches are sufficient for both stabilization and invasion. Extended persistence of strand invasion products in the presence of both proteins indicates that a co-filament is more stable than a filament comprised of either protein alone. In vivo the SsoRadA protein is present at significantly higher levels than SsoRal3 protein (C. Haseltine, unpublished results), suggesting that SsoRal3 is not likely to be an alternate recombinase and instead serves as a mediator at lower stoichiometric concentrations. A model proposing the function of SsoRal3 protein in early HR is shown in Fig. 8. Following SsoSSB displacement from ssDNA, SsoRadA would be able to begin nucleoprotein filament formation (Fig. 8a). In the absence of SsoRal3, filament formation is slow as SsoRadA continues to hydrolyze ATP (Fig. 8b). As shown in Fig. 8c, SsoRal3 could be locally available to invading nucleoprotein filaments through “storage” in an inactive state on dsDNA, as has been suggested for SsoRadA (Yang et al., 2001). At a region near a DSB, SsoRal3 protein DNA-binding preference would shift from ds- to ssDNA through ATP binding, a process that may require the assistance of additional protein partners in vivo. Upon binding, SsoRal3 stabilizes the strand invasion intermediate by reducing dissociation of the SsoRadA recombinase from ssDNA (Fig. 8c). Key to this process is the alteration in DNA binding preference for SsoRal3, a process that likely requires the assistance of additional protein partners in vivo. While we examined a single SsoRadA paralog protein in this work, *S. solfataricus* also encodes two additional paralogs as well as a Rad54 protein. Future work addressing the involvement of these probable accessory factors is likely to further illuminate the mechanistic underpinnings of the presynaptic step of HR in this archaeon.

Supplementary Material

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Abbreviations

dsDNA	double-strand DNA
ssDNA	single-strand DNA
Gy	Gray
IR	ionizing radiation
UV	ultraviolet
DSB	double-strand break
HR	homologous recombination
bp	basepairs
EMSA	electrophoretic mobility shift assay
ORF	open reading frame

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Fig. 1. SDS-PAGE of SsoRal3 purification. Samples from major steps during purification were subjected to SDS-PAGE using a 10% acrylamide tricine gel and stained with Coomassie brilliant blue R250. Lane 1: clarified, heat treated crude sonicate; lane 2: pooled fractions after Blue-Sepharose; lane 3: final purified, concentrated SsoRal3 (5 µg). The position of the SsoRal3 protein is indicated by the arrow.

Fig. 2.

SsoRal3 is a ssDNA dependent ATPase with a DNA binding site-size of approximately four nucleotides. All data were obtained using the coupled ATPase assay as described (Rolfmeier and Haseltine, 2010; Seybert et al., 2002). Assays in (a) and (c) were performed at 70 °C. (a) All reactions contained 0.6 μM DNA and were initiated with 0.2 μM SsoRal3. DNA used was circular φX174 ssDNA (green squares), poly(dT) (purple triangles), and pBR322 dsDNA (red diamonds). Black inverted triangles indicate assays that contained no DNA. (b) Points represent percent activity of SsoRal3 at various temperatures. The activity at 70 °C was defined as 100%, and all other points are portions thereof. (c) The φX174 ssDNA concentration was held constant at 0.6 μM and reactions were initiated with varying concentrations of SsoRal3. Points represent the velocity obtained from the slope of the linear portion of a curve generated from each experiment. Error bars in all panels represent standard deviation of a minimum of at least three separate experiments.

Fig. 3.

ATP alters SsoRal3 DNA binding preference. The top two panels are representative EMSA autoradiographs of SsoRal3-ssDNA binding (a) and SsoRal3-dsDNA binding (b) in the presence or absence of ATP with 50 mM NaCl. Panels (c) and (d) are representative autoradiographs of NaCl titrations of SsoRal3 bound to ssDNA or dsDNA respectively. Reactions with ssDNA included ATP, while ATP was omitted for those with dsDNA. NP indicates no protein control reactions. (e) Quantitation of binding affinity through salt midpoint titration, where the mobility shift apparent at 50 mM NaCl was designated as 100%. Squares represent ssDNA shifts while triangles represent dsDNA shifts. The dashed lines indicate 50% binding. Error bars represent standard deviation from a minimum of three separate experiments.

Fig. 4.

SsoRal3 enhances SsoRadA binding to ssDNA in the presence of ATP. Representative EMSA autoradiograph showing shifts produced with varying concentrations of SsoRadA and SsoRal3 proteins either together (lanes 5, 6, 11, 12) or separately (lanes 1–4, 7–10). Lanes 1–6 represent reactions that included 3 mM ATP, while lanes 7–12 were reactions where ATP was omitted. All reactions contained 5 μ M ssDNA. Shifted products and ssDNA are indicated by arrows.

Fig. 5. Order of addition experiments suggest SsoRal3 alters the ATPase activity of SsoRadA. (a-d) Effects of SsoRal3 on SsoRadA activity was determined using the coupled ATPase assay at 70 °C (Rolfmeier and Haseltine, 2010; Seybert et al., 2002). In all panels, DNA was ssφX174. Symbols represent: SsoRal3 alone (purple triangles), SsoRadA alone (green squares), SsoRal3 added first (blue inverted triangles), SsoRadA added first (black circles), and SsoRal3 and SsoRadA added together (red diamonds). The vertical dashed line indicates the time at which the second protein was added, as applicable. (a) SsoRal3 and SsoRadA were saturating (0.2 μM). (b) SsoRadA was saturating (0.2 μM) and SsoRal3 was sub-saturating (0.08 μM). (c) SsoRadA was sub-saturating (0.08 μM) and SsoRal3 was saturating (0.2 μM). (d) Both SsoRadA and SsoRal3 were sub-saturating (0.08 μM). Error bars represent standard deviation of a minimum of three separate experiments. The y-axes for panels (b)–(d) have been adjusted to a smaller maximum than that for panel (a) in order to better show the data.

Fig. 6.

Order of addition experiments including the single-strand DNA binding protein SsoSSB. (a–d) Effects of SsoRal3 on SsoRadA activity in the presence of ssDNA substrate pre-bound with SsoSSB as determined using the coupled ATPase assay at 70 °C (Rolfmeier and Haseltine, 2010; Seybert et al., 2002). In all panels, DNA was ssφX174 and SsoSSB was used at a concentration of 0.04 μM. The symbols are: SsoRal3 alone (purple triangles), SsoRadA alone (green squares), SsoRal3 added first (blue inverted triangles), SsoRadA added first (black circles), and SsoRal3 and SsoRadA added together (red diamonds). The vertical dashed line indicates the time at which the second protein was added, as applicable. (a) SsoRal3 and SsoRadA were saturating (0.2 μM). (b) SsoRadA was saturating (0.2 μM) and SsoRal3 was sub-saturating (0.08 μM). (c) SsoRadA was sub-saturating (0.08 μM) and SsoRal3 was saturating (0.2 μM). (d) Both SsoRadA and SsoRal3 were sub-saturating (0.08 μM). Error bars represent standard deviation of a minimum of three separate experiments.

Fig. 7. SsoRal3 increases the persistence of SsoRadA catalyzed D-loops. (a) Schematic of the D-loop reaction. (b) Representative autoradiographs of D-loop time courses. Saturating (0.6 μM) and subsaturating (0.12 μM) concentrations of SsoRadA and SsoRal3 are indicated to the right of the panel. Where indicated, 0.09 μM SsoSSB was pre-incubated with the oligonucleotide prior to addition of SsoRal3 and/or SsoRadA. Lane 1 represents reactions that did not contain protein and were incubated for 30 min, serving as a control. (c–f) Quantitation of D-loop formation. All conditions are shown relative to the invasion product

generated after 1 min by saturating SsoRadA, which was arbitrarily set at 1. Error bars represent the standard deviation of a minimum of 3 separate experiments. The concentration of protein in each time course is indicated below the corresponding group of bars. (c) SsoRadA or SsoRal3 alone or with SsoSSB at various concentrations; (d) SsoRadA and SsoRal3 were added to the reaction simultaneously; (e) SsoRadA was added to the reaction prior to addition of SsoRal3; (f) SsoRal3 was added to the reaction prior to addition of SsoRadA.

Fig. 8.

Model for the involvement of SsoRal3 in stabilization of the SsoRadA presynaptic filament during strand invasion. (a) Tetrameric SsoSSB displacement is necessary to allow SsoRadA binding (b) SsoRadA presynaptic filament formation and strand invasion in the absence of SsoRal3. The dynamic character of the presynaptic filament results in slow formation of an active presynaptic filament, delaying strand invasion. (c) SsoRadA presynaptic filament formation and strand invasion in the presence of SsoRal3. SsoRal3 is stored in an inactive state on dsDNA in the absence of ATP. ATP addition permits SsoRal3 binding to ssDNA, stabilizing the presynaptic filament and enhancing strand invasion.

Table 1

Effect of order of protein addition on ATP hydrolysis by SsoRal3 and SsoRadA at 70 or 80 °C

Temperature (°C)	Protein concentration	Assay initiating component	Velocity (μmol ATP hydrolyzed/minute)	Yield (μmol ATP hydrolyzed)
70	Sat. SsoRal3	SsoRal3	2.4 ± 0.6	69.1 ± 10.8
	Sat. SsoRadA	SsoRadA	0.8 ± 0.2	22.1 ± 4.8
	Subsat.	SsoRal3	1.5 ± 0.2	40.1 ± 5.0
	SsoRal3			
	Subsat.	SsoRadA	0.5 ± 0.2	14.1 ± 3.5
	SsoRadA			
	Sat. SsoRal3	SsoRadA	1.4 ± 0.2	38.7 ± 4.0
	Sat. SsoRadA	SsoRal3	0.4 ± 0.2	10.8 ± 5.3
		φ174 ssDNA	1.6 ± 0.4	43.1 ± 8.1
	Subsat.	SsoRadA	0.2 ± 0.0	17.3 ± 1.5
	SsoRal3	SsoRal3	0.9 ± 0.2	19.3 ± 3.8
	Subsat.	φ174ssDNA	0.9 ± 0.3	21.0 ± 6.2
	SsoRadA			
	Sat. SsoRal3	SsoRadA	1.3 ± 0.1	39.9 ± 1.9
	Subsat.	SsoRal3	0.5 ± 0.2	18.1 ± 4.7
	SsoRadA	φ174ssDNA	0.9 ± 0.3	23.7 ± 6.5
	Subsat.	SsoRadA	0.6 ± 0.1	16.0 ± 6.8
	SsoRal3	SsoRal3	0.3 ± 0.1	13.3 ± 1.6
	Sat. SsoRadA	φ174ssDNA	0.8 ± 0.4	21.1 ± 9.0
80	Sat. SsoRal3	SsoRal3	2.2 ± 0.5	19.7 ± 4.0
	Sat. SsoRadA	SsoRadA	1.2 ± 0.2	11.1 ± 2.0
	Subsat.	SsoRal3	0.4 ± 0.1	7.1 ± 1.6
	SsoRal3			
	Subsat.	SsoRadA	0.9 ± 0.2	8.6 ± 1.5
	SsoRadA			
	Sat. SsoRal3	SsoRadA	0.9 ± 0.4	11.2 ± 3.9
	Sat. SsoRadA	SsoRal3	0.9 ± 0.2	9.7 ± 1.6
		φ174ssDNA	1.0 ± 0.2	9.4 ± 1.5
	Subsat.	SsoRadA	0.6 ± 0.1	8.7 ± 3.9
	SsoRal3	SsoRal3	0.3 ± 0.2	5.5 ± 3.2
	Subsat.	φ174ssDNA	3.2 ± 0.3	34.2 ± 3.5
	SsoRadA			
	Sat. SsoRal3	SsoRadA	0.3 ± 0.1	6.2 ± 2.6
	Subsat.	SsoRal3	0.8 ± 0.1	9.7 ± 1.0
	SsoRadA	φ174ssDNA	0.5 ± 0.3	5.0 ± 1.8
	Subsat.	SsoRadA	0.6 ± 0.1	7.9 ± 1.2
	SsoRal3	SsoRal3	0.4 ± 0.1	9.0 ± 5.3
	Sat. SsoRadA	φ174 ssDNA	0.7 ± 0.2	10.0 ± 1.8

Reaction velocity was measured by the slope of the line in the early linear portion of the graph of substrate hydrolysis versus time. In reactions in which SsoRadA and SsoRal3 were not added simultaneously, velocity was calculated after addition of the second protein. Yield was calculated at the completion of a 30 minute reaction.

Table 2

Effect of order of protein addition on ATP hydrolysis by SsoRal3 and SsoRadA at 70 or 80 °C on SsoSSB coated ssDNA

Temperature (°C)	Protein concentration	Assay initiating component	Velocity (μmol ATP hydrolyzed/minute)	Yield (μmol ATP hydrolyzed)
70	Sat. SsoRal3	SsoRal3	1.3 ± 0.2	36.5 ± 4.3
	Sat. SsoRadA	SsoRadA	0.2 ± 0.0	3.9 ± 0.6
	Subsat. SsoRal3	SsoRal3	0.5 ± 0.2	13.2 ± 2.1
	Subsat. SsoRadA	SsoRadA	0.2 ± 0.1	2.3 ± 0.7
	Sat. SsoRal3	SsoRadA	0.9 ± 0.1	18.4 ± 3.9
	Sat. SsoRadA	SsoRal3	0.5 ± 0.1	10.2 ± 1.3
		φ174 ssDNA	0.7 ± 0.2	15.2 ± 3.7
	Subsat. SsoRal3	SsoRadA	0.5 ± 0.1	13.2 ± 3.3
	Subsat. SsoRadA	SsoRal3	0.5 ± 0.1	10.0 ± 1.5
		φ174 ssDNA	0.6 ± 0.0	18.3 ± 3.4
	Sat. SsoRal3	SsoRadA	1.0 ± 0.1	22.2 ± 1.1
	Subsat. SsoRadA	SsoRal3	0.7 ± 0.2	11.8 ± 2.6
		φ174 ssDNA	1.1 ± 0.2	25.9 ± 4.5
	Subsat. SsoRal3	SsoRadA	1.0 ± 0.1	19.6 ± 2.2
	Sat. SsoRadA	SsoRal3	0.3 ± 0.1	10.6 ± 1.7
		φ174 ssDNA	0.6 ± 0.0	13.1 ± 2.4
80	Sat. SsoRal3	SsoRal3	0.3 ± 0.1	5.6 ± 2.3
	Sat. SsoRadA	SsoRadA	0.8 ± 0.2	12.5 ± 1.0
	Subsat. SsoRal3	SsoRal3	0.8 ± 0.2	10.1 ± 2.4
	Subsat. SsoRadA	SsoRadA	0.4 ± 0.1	8.8 ± 2.7
	Sat. SsoRal3	SsoRadA	0.0 ± 0.0	6.6 ± 2.0
	Sat. SsoRadA	SsoRal3	0.9 ± 0.2	8.7 ± 4.1
		φ174 ssDNA	0.5 ± 0.2	6.4 ± 1.5
	Subsat. SsoRal3	SsoRadA	0.2 ± 0.0	4.6 ± 1.1
	Subsat. SsoRadA	SsoRal3	0.4 ± 0.1	7.6 ± 1.7
		φ174 ssDNA	0.3 ± 0.1	5.8 ± 2.4
	Sat. SsoRal3	SsoRadA	0.0 ± 0.0	9.1 ± 2.8
	Subsat. SsoRadA	SsoRal3	1.0 ± 0.2	10.3 ± 2.1
		φ174 ssDNA	1.3 ± 0.5	8.6 ± 2.9
	Subsat. SsoRal3	SsoRadA	0.1 ± 0.0	5.8 ± 0.9
	Sat. SsoRadA	SsoRal3	1.0 ± 0.0	10.5 ± 1.8
		φ174 ssDNA	0.6 ± 0.1	6.9 ± 2.1

Reaction velocity was measured by the slope of the line in the early linear portion of the graph of substrate hydrolysis versus time. In reactions in which SsoRadA and SsoRal3 were not added simultaneously, velocity was calculated after addition of the second protein. Yield was calculated at the completion of a 30 minute reaction.