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Characterization of the ATPase Activity of the *Escherichia coli* RecG Protein Reveals that the Preferred Cofactor is Negatively Supercoiled DNA

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RecG is a member of the superfamily 2 helicase family. Its possible role *in vivo* is ATP hydrolysis driven regression of stalled replication forks. To gain mechanistic insight into how this is achieved, a coupled spectrophotometric assay was utilized to characterize the ATPase activity of RecG *in vitro*. The results demonstrate an overwhelming preference for negatively supercoiled DNA ((−)scDNA) as a cofactor for the hydrolysis of ATP. In the presence of (−)scDNA the catalytic efficiency of RecG and the processivity (as revealed through heparin trapping), were higher than on any other cofactor examined. The activity of RecG on (−)scDNA was not due to the presence of single-stranded regions functioning as loading sites for the enzyme as relaxed circular DNA treated with DNA gyrase, resulted in the highest levels of ATPase activity. Relaxation of (−)scDNA by a topoisomerase resulted in a 12-fold decrease in ATPase activity, comparable to that observed on both linear double-stranded (ds)DNA and (+)scDNA. In addition to the elevated activity in the presence of (−)scDNA, RecG also has high activity on model 4Y-substrates (i.e. chicken foot structures). This is due largely to the high apparent affinity of the enzyme for this DNA substrate, which is 46-fold higher than a 2Y-substrate (i.e. a three-way with two single-stranded (ss)DNA arms). Finally, the enzyme exhibited significant, but lower activity on ssDNA. This activity was enhanced by the *Escherichia coli* stranded DNA-binding protein (SSB) protein, which occurs through stabilizing of the binding of RecG to ssDNA. Stabilization is not afforded by the bacteriophage gene *32* protein, indicating a species specific, protein–protein interaction is involved. These results combine to provide significant insight into the manner and timing of the interaction of RecG with DNA at stalled replication forks.

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Keywords: RecG; DNA helicase; DNA repair; recombination; replication fork reversal

Introduction

DNA repair *via* genetic recombination is arguably one of the most important processes in the cell. This process involves extensive and diverse enzymatic functions on damaged DNA.[1–3] Critical enzymes involved in genetic recombination are the DNA helicases, proteins which harness the chemical free energy of ATP hydrolysis to catalyze the unwinding of double-stranded DNA (dsDNA).[4] Typically, DNA helicases such as the well characterized RecBCD enzyme provide single-stranded DNA (ssDNA) to facilitate repair processes. In contrast, there are several "DNA helicases" which act on unusual

Abbreviations used: dsDNA, double stranded DNA; Gp32, gene 32 protein; NADH, nicotinamide adenine dinucleotide; (-)scDNA, negatively supercoiled DNA; PEP, phosphoenol pyruvate; Poly (dT), polydeoxyribothymidilic acid; (+)scDNA, positively supercoiled DNA; ssDNA, single stranded DNA; SSB, single stranded DNA binding protein; SF2, Superfamily 2; Tet, tetracycline; WGT, wheat germ topoisomerase I.

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DNA structures such as Holliday junctions or stalled replication forks. These enzymes are unique motors that do not provide ssDNA *per se*, unless provided with the correct model substrate. Instead, they alter the structure of DNA through processing of recombination intermediates (e.g. RecQ),[5] branch migration of Holliday junctions (e.g. RuvAB)[6] or regression of stalled replication forks (e.g. RecG).[7]

Genetic evidence suggests that RecG is involved in recombinational repair pathways of both dsDNA breaks and ssDNA gaps.[1,8] Although it can act during branch migration to resolve Holliday junctions,[2,9,10] its primary role may be to regress or reverse stalled replication forks.[11,12] The activity of RecG overlaps that of RuvAB,[13] as both enzymes can act on stalled replication forks, and are able to create suitable substrates for RuvC cleavage.[7] However, studies with various mutants have shown that RecG acts independently through a distinct pathway.[2] This pathway allows for a non-cleavage resolution of the DNA damage, unlike the RuvAB pathway, minimizing the possibility of inappropriate recombination events.[14]

RecG is a 76 kDa protein that is a member of the superfamily 2 (SF2) helicase family of proteins.[15] The three-dimensional structure of RecG determined in the presence of a model fork substrate, showed that the protein consists of three structural domains.[16] The first makes extensive interactions with the DNA fork and contains a highly conserved Greek key motif, known as the "wedge" of the protein. This wedge domain confers substrate specificity and enhances the processivity of RecG on DNA.[17] Structural domains two and three contain the SF2 helicase motifs, which cluster about the ATP binding pocket of the enzyme. This positioning allows for conformational changes to be coupled to the binding of ATP and its subsequent hydrolysis and product release. These conformational changes presumably allow the enzyme to move along the DNA with the wedge domain contacting the DNA, allowing for the splitting of the nascent duplex regions into separate channels flanking the wedge. As neither of these channels are large enough to accommodate duplex DNA, translocation results in the prolonged splitting of the nascent strands, and consequently, the regression of stalled replication forks.[12,14,18]

To date, the majority of *in vitro* work to understand the biochemical mechanism of RecG has focused largely on the ability of the enzyme to act on forked or branched DNA structures. These experiments revealed that the enzyme functions as a monomer,[19] although *in vivo* data demonstrate that RecG mutants can interfere with the activity of the wild-type protein.[19] Although RecG is thought to act primarily on three and four-way DNA junctions,[9,10] it has also been shown to act upon R[20,21] and D-loops.[22] The flexibility of cofactors upon which this enzyme can act is indicative of its usefulness *in vivo*, implying that RecG may play a role in several genetic recombination and repair pathways. More recently, data from the Cox laboratory has shown that RecG has limited processivity, with the rate limiting step of the enzyme being rebinding of the dissociated RecG to the DNA substrate.[23] RecG does appear to function in a unidirectional manner when regressing forked DNA, in spite of an excess of protein coupled with extended reaction times, with the regression enhanced by stranded DNA-binding protein (SSB).[23]

To understand how this DNA motor functions it is necessary to determine the range of substrates or cofactors for optimal activity, and understand the efficiency of energy utilization by the enzyme. The multiple studies of RecG both *in vitro* and *in vivo* have shown many facets of its abilities.[12] A detailed biochemical examination of its hydrolysis of ATP, however, has yet to be performed. This is the focus of this work. We utilized a coupled spectrophotometric ATPase assay that has been used in the study of DNA helicases from superfamily 1 (RecBCD)[24] and translocases from superfamily 2 (*EcoR*124I).[25] We determined the catalytic efficiency of RecG in the presence of several DNA molecules under a variety of *in vitro* conditions.

The data presented are consistent with previous work,[10,19,26] and in addition, reveal several novel insights. First, the preferred unbranched DNA cofactor for ATPase activity is negatively supercoiled DNA. The activity on model 4Y-substrates is comparable to that on (−)supercoiled (sc)DNA. Second, the activity of RecG is lower on M13 ssDNA and is enhanced by the presence of the *Escherichia coli* SSB protein, which stabilizes the interaction of RecG with ssDNA. Third, the apparent affinity for 4Ysubstrates is higher than that for 3- and 2Y substrates. Fourth, heparin trapping experiments reveal insight into the translocation processivity of the enzyme and that it is highest on (−)scDNA followed by linear dsDNA and then M13 ssDNA. Collectively, these data provide insight into how RecG accesses the DNA at a stalled replication fork from which the replisome has already disassembled.

Results

A typical time course of RecG utilizing the coupled spectrophotometric assay displays a linear decay of signal

The hydrolysis of ATP by RecG in the presence of various DNA molecules under several assay conditions was monitored utilizing a coupled spectrophotometric assay. Typical time courses from this assay are shown in Figure 1, displaying the characteristic behavior of RecG both in the presence and absence of DNA. In the absence of DNA, no significant ATP hydrolysis is observed, with a rate of 0.2 μM/min. Thus as demonstrated previously, RecG is a DNA-dependent ATPase.[27] Furthermore, the absence of significant activity indicates that there are no contaminating DNA-independent ATPases present in the RecG preparations used here.

In contrast to the DNA-independent reaction, there is an 80 to 455-fold increase in the rate of

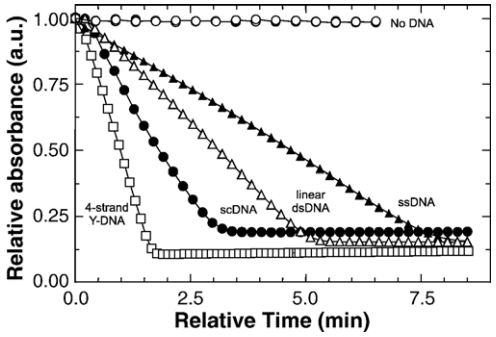

Figure 1. The time course for ATP hydrolysis by RecG is linear. Typical time courses of reactions are shown. Experiments were conducted at 37 °C as described in Materials and Methods and were initiated by the addition of RecG. For each assay except the four strand junction, the concentration of RecG was 100 nM and the concentration of ATP was 1 mM. For the four strand junction, DNA was present at 20 nM molecules and RecG was present at 2 nM. The optimal concentration of magnesium used was specific to each DNA cofactor, 4 mM for (−)scDNA, the 4Y substrate at 8 mM and 10 mM for linear dsDNA and ssDNA. The DNA cofactors were present at 15 μM nts, and yielded rates of 33 μM/min on M13 ssDNA (▲), 20 μM/min on linear dsDNA (△) and 57 μM/min on negatively supercoiled DNA, pPB67 preparation #1 (●) and 2.4 μM/min on the 4Y substrate (□). The no DNA control reaction produced a rate of 0.2 μM/min (○).

ATP hydrolysis in the presence of DNA (Figure 1). The calculated k_{cat} values of ATP hydrolysis for M13 mp18 ssDNA, linear duplex, (−)scDNA and a 4Ysubstrate are 330, 200, 570 and 1200 min^{-1}, respectively. Surprisingly, rate changes associated with the model junction were only occasionally observed, even though DNA strands were displaced from the junction as detected in gel assays (data not shown). In the time course shown, the rate of ATP hydrolysis increases moderately after approximately 30 s and remains constant for the remainder of the assay. The sharp differences observed in the rates of ATP hydrolysis demonstrate the DNA-dependent ATPase activity of RecG and further, that this enzyme displays a strong preference for the nature of the DNA cofactor. This is reflected in the apparent affinity for each DNA cofactor. These preferences are addressed in detail below. As appreciable DNA unwinding was not detected using supercoiled, relaxed or linear dsDNA, these are referred to as cofactors and the model 2-, 3- and 4Y DNA junctions will be referred to as substrates as DNA strands are displaced from them (data not shown).

RecG exhibits a complex dependence on magnesium ions

Several DNA helicases exhibit unusual behavior with respect to magnesium ion concentration, such as RecQ[28] and RecG itself. Previous studies of RecG[10,17,26] were done using model Holliday junction and stalled fork substrates which themselves are influenced by magnesium ion concentration[29–31] and could affect the resulting activity of RecG accordingly. These studies used DNA substrates with heterologous regions which themselves influence the optimal magnesium to ATP ratios for RecG. In spite of these complications, optimal conditions for DNA unwinding of these model substrates were found at 1 mM $MgCl_2$ and 2 mM ATP and that DNA unwinding was inhibited by free magnesium ions.[9]

To further characterize how the activity of RecG is influenced by the DNA molecule with which it interacts, magnesium titrations were performed utilizing six different DNA cofactors. These were poly(dT), pPB67 ssDNA, linear pPB67 dsDNA, relaxed circular dsDNA (pPB67), (−)scDNA#1 (pPB67) and the 4Y substrate. The concentrations of ATP, DNA and RecG were held constant and reactions were initiated by the addition of ATP or RecG following a 2–5 min pre-incubation time of all components at 37 °C. The change in order of addition did not affect the steady-state rate of ATP hydrolysis in the presence of all cofactors (data not shown).

The results are complex and can be summarized as follows. First, the hydrolysis of ATP in the presence of each DNA type demonstrates an optimal magnesium ion concentration (Figure 2). Second, the optimal magnesium concentration for each DNA is different, being observed at 4mM for (−)scDNA, 8 mM on relaxed circular DNA, 10 mM for linear dsDNA, ssDNA and the model, and at 17mM for poly(dT). Third, some cofactors exhibit a sharp optimum (scDNA, ssDNA and poly(dT)), whereas for the 4Y substrate, linear and relaxed circular dsDNA, the maximum in activity is observed over a broad range, as broad as 10 mM in magnesium ion concentration (Figure 2). The inhibition of ATPase activity at excess magnesium indicates that RecG is very sensitive to the concentration of free magnesium ions, similar to that observed for DNA unwinding[9]. The optimal magnesium ion concentrations obtained in the experiments described above were utilized throughout the remaining experiments for their respective DNA cofactors.

The apparent binding site size for RecG is dependent upon the DNA cofactor present

In order to determine the site size for DNA binding, the concentration of RecG was varied on each of the DNA cofactors and the hydrolysis of ATP measured. To minimize effects due to differences in apparent DNA affinity, the cofactors were used at concentrations at or above their respective $S_{0.5}^{DNA}$ or K_m^{DNA} values (this distinction is made clear in subsequent sections). Furthermore, ATP was present at 1 mM (well above that required to saturate the enzyme) and the magnesium ion concentration was used at the optimum for each cofactor (Figure 2). A site size in the presence of model junctions was not performed, since this presumably will provide the fraction of active protein, as these titrations will presumably saturate at ~1 RecG per junction as demonstrated.[19] Using the standard assay conditions, a linear time course of ATP hydrolysis was

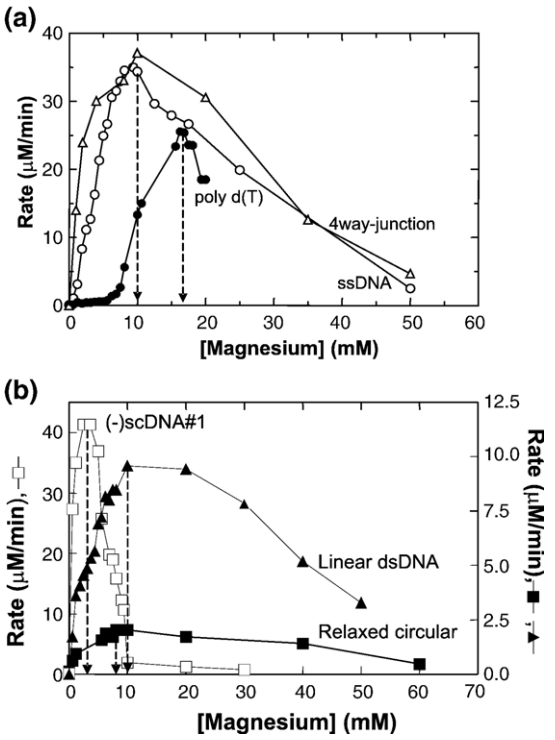

Figure 2. The rate of ATP hydrolysis by RecG shows a complex dependence on the concentration of magnesium ions. Magnesium acetate titrations were done using either single-stranded (a), or double-stranded DNA (b). Assays contained 100 nM RecG enzyme, 1 mM ATP and 15 μM nucleotides of each DNA cofactor. The cofactors used were poly(dT), average length 359 nts (●); ssDNA was pPB67 ssDNA 4,198 nts in length (○); (−)scDNA was pPB67 preparation #1 (□); linear was pPB67 preparation #1 linearized by restriction with HindIII (▲) and relaxed circular was pPB67 treated with WGT (■). The rates from linear portions of each time course were determined as in Figure 1 and these are graphed as a function of increasing magnesium ion concentration. The broken lines indicate the magnesium concentration producing maximum activity. The maximum activity of RecG was attained at a concentration of 10 mM and 16.5 mM Mg^{+2} for ssDNA and poly(dT), respectively. Maximal RecG activity was observed at 4, 8 and 10 mM magnesium acetate for scDNA, relaxed circular DNA and linear dsDNA, respectively.

observed in the presence of each cofactor, similar to those data presented in Figure 1. The resulting rates determined by straight line fitting to the steady-state regions of time courses were graphed as a function of RecG concentration (Figure 3) and displayed in Table 1.

The curves in Figure 3 demonstrate that on poly (dT), ssDNA and (−)scDNA, the rate of ATP hydrolysis paralleled the increase in RecG concentration, until a peak was reached. As the concentration of RecG was increased further, the rate of ATP hydrolysis declined. Presumably for circular cofactors, this occurs due to saturation of the lattice by RecG monomers restricting translocation of neighboring monomers and possibly, the associated turnover of ATP. Surprisingly, this phenomenon

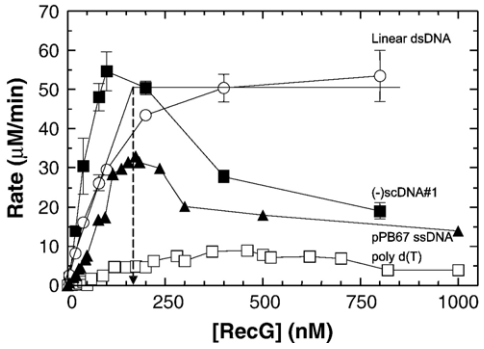

Figure 3. The site size of RecG is dependent on the DNA cofactor. ATPase assays were preformed as described in Materials and Methods and were initiated by the addition of RecG (concentrations as indicated) or ATP (1 mM, final). The concentration of magnesium acetate used was the optimal concentration for each cofactor as determined in Figure 2. The site size is only indicated for linear dsDNA as this was the only cofactor for which a plateau was achieved (the broken line). The cofactors used were poly(dT), (□);pPB67 ssDNA (▲); (−)scDNA #1 (■); linear dsDNA was pPB67 preparation #1 linearized by restriction with HindIII (○).

was also observed on poly(dT) although the reason for this is less clear. Thus we indicate an apparent site size for RecG on these cofactors, calculated from the peak in the observed ATPase rate. These site sizes are shown in Table 1 and range from 36 to 66 nt on ssDNA cofactors, to 229 bp on (−)scDNA. A lower site size of 87 bp was obtained on relaxed circular DNA. In contrast, on linear dsDNA the rate of ATP hydrolysis increased linearly and saturated at 165 nM RecG, corresponding to a site size of 182 base-pairs/RecG monomer (the broken line in Figure 3). Thus the site size for RecG is strongly influenced by the nature of the DNA cofactor.

Surprisingly, the ATPase activity of RecG is high in the presence of negatively scDNA

As RecG can act upon a wide variety of DNA substrates *in vivo*,[12] we examined the ability of the protein to hydrolyze ATP in the presence of various DNA cofactors *in vitro*. Here DNA titrations of each cofactor were done, the rates calculated as in Figure 1, and displayed graphically as a function of

Table 1. The site size[a] for RecG is dependent on the DNA cofactor

DNA cofactor	Site size (nt)	Site size (bp)
Poly(dT)	36	–
pPB67 ssDNA	66	–
Linear dsDNA	–	182
Relaxed circle	–	87
(−)scDNA#1	–	229

[a] The site size calculated from the optimum concentration of RecG utilized in each assay. For linear dsDNA, the protein was able to fully saturate the linear lattice. For poly(dT) and ssDNA, relaxed circular DNA and scDNA, the values of optimal protein concentration peak, and subsequently fall.

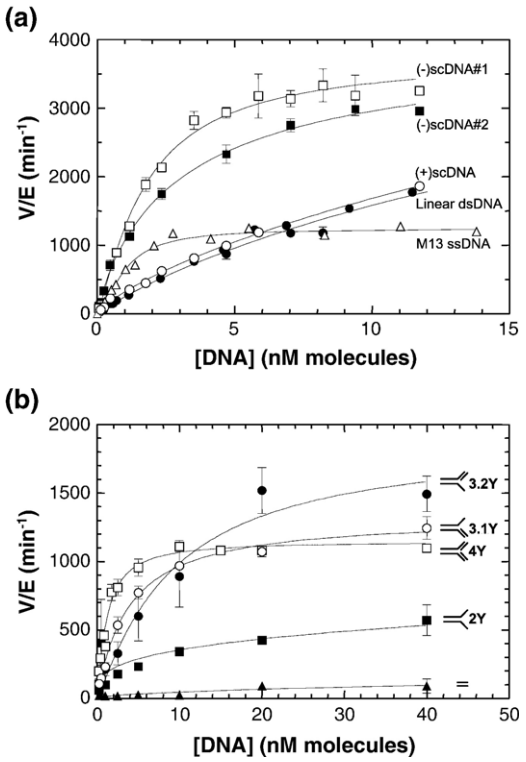

Figure 4. Negatively supercoiled DNA is the preferred cofactor for RecG. ATPase assays were performed as described in Materials and Methods. The assay for each cofactor was done at the optimal Mg^{2+} concentration for that cofactor as determined in Figure 2. Data were normalized to protein concentration to permit direct visual comparison of the data. In (a), RecG was added to initiate all reactions and was used at a concentration of 100 nM, while in (b) it was 2 nM. Time courses were analyzed as described for Figure 1 and the resulting rates are graphed as a function of DNA concentration. The rates were then plotted against the concentration of DNA in each respective reaction in order to find the kinetic parameters. (a) The titrations of five different DNA cofactors were done using RecG preparation #2. The (−)scDNA (□) was pPB67 preparation #1; the linear dsDNA (●) was pPB67 preparation #1 linearized with HindIII, ssDNA was M13 mp18 ssDNA (Δ) and (+)scDNA was pBR322 (○). Data were fit to the Hill equation,[64] $V = (V_{max}.[S]^n)/([S_{0.5}]^n + [S]^n)$ to determine whether cooperative binding is occurring. The resulting Hill coefficients obtained are as follows: M13 ssDNA 1.7(±0.2), linear dsDNA 0.9(±0.07), (−)scDNA#1 1.3(±0.1), (−)scDNA #2 0.9(±0.08) and for (+)scDNA 0.8(±0.05). (b) Titrations of model junction substrates using RecG preparation #1. The linear duplex substrate (▲) was 30 bp in length, corresponding to the internal oligonucleotides of each arm of the four strand junction.

increasing DNA concentration (Figure 4). In the data presented, different preparations of RecG were used. The primary difference between preparation 1 and subsequent preparations was a two- to fourfold increase in specific activity. Each preparation of RecG exhibited the same preference for DNA cofactor as indicated in Table 2 and Figure 4.

As RecG interacts with DNA junction substrates at a stoichiometry of 1 RecG per DNA molecule, we present the K_m^{app} or $S_{0.5}^{app}$ values for DNA in nM molecules. The resulting data from the titrations were fit to the Hill equation, with the various parameters allowed to float and be determined by the computer. The results demonstrate that the activity of RecG was highest on (−)scDNA with a k_{cat} value of 1207 to 1710 min^{-1} (Table 2 and Figure 4(a)). In contrast, k_{cat} decreased threefold to 559 min^{-1} when the cofactor was linear dsDNA, and 57-fold to 29 min^{-1} when the cofactor was relaxed circular DNA (Table 2). The rate of ATP hydrolysis on ssDNA was also reduced relative to (−)scDNA and was comparable to that observed on linear dsDNA or higher, depending on which ssDNA cofactor used (Table 2). The difference between M13 and pPB67 ssDNA may be attributed to secondary structure since the latter DNA was derived from pBluescript II, which has a large multiple cloning site. The catalytic efficiency of RecG was also the highest on negatively scDNA, where it was 17 to 33-fold higher than that on relaxed circular DNA. The preference of RecG for negatively scDNA is even more pronounced when positively scDNA was present, resulting in an 8 – 14-fold decrease in k_{cat}/K_m (Table 2). This is due in part to the 18 to 29-fold increase in the apparent K_m^{DNA}, although the K_m value reported for positively scDNA is not accurate as it was not possible to achieve 245 nM molecules of DNA i.e. five times K_m^{DNA} (data not shown).

As expected, the k_{cat} values observed in the presence of the Y substrates were high and comparable to that observed on negatively scDNA (Table 2 and Figure 4(b)). The catalytic efficiency of RecG was highest for the 4Y substrate (1091 min^{-1} nM^{-1}) and this was comparable to that observed in the presence of negatively scDNA (549 – 1069 min^{-1} nM^{-1}). Although the k_{cat} values in the presence of either of the 3Y substrates were slightly higher than that on the 4Y, the catalytic efficiency of RecG on these substrates was lower by a factor of 3. This is attributed to the 3.5 to 5.5-fold lower apparent affinity of RecG for each of these substrates. The K_m^{DNA} value increases further in the presence of the 2Y substrate (8 – 46-fold higher when the arms of the junction are both ssDNA) suggesting that duplex DNA in each arm of the model fork is important for full activity.

The calculated Hill coefficients for DNA binding of linear dsDNA, positively scDNA and junction substrates were all approximately 1 (Table 2 and Figure 4). Hill coefficients of 1.9 to 2.4 in the presence of (−)scDNA were observed for RecG preparation #1 and these values decreased to 0.95 to 1.3 in subsequent preparations. The reason for this difference is currently not clear. Positive cooperativity was observed for binding to ssDNA (i.e. Hill coefficients of 1.3 to 1.7 were observed). The Hill coefficient increased even further in the presence of poly(dT) to 2.1(±0.7). However, it should be noted that RecG behaves in an unusual manner on this cofactor and this is elaborated in a subsequent section.

The strong preference of RecG for (−)scDNA as a cofactor was surprising, even though RecG can

Table 2. DNA cofactor parameters

Cofactor	V_{max} (μM/min)	k_{cat} (min^{-1})	K_m^{app} or $S_{0.5}^{app}$ (nM)[a]	k_{cat}/K_m or $k_{cat}/S_{0.5}$ (min^{-1}/nM)	Hill coefficient
A. *RecG prep 1*					
Poly(dT)	7.3±0.3	73	1.2±0.03	61	2.1±0.7
M13 ssDNA	43±1.1	426	1.4±0.09	304	1.3±0.1
pPB67 ssDNA	71±4.5	1049	3.5±0.5	300	1.4±0.2
(−)scDNA #1[b]	171±5	1710	2.0±0.1	1069	1.9±0.2
(−)scDNA #2	121±4.0	1207	2.9±0.3	549	2.4±0.4
Relaxed circular	3±0.3	29	0.9±0.2	32	1.3±0.4
Linear dsDNA	66±5.5	559	5.3±1.2	106	0.9±0.1
4Y junction[c]	2.4±0.1	1200	1.1±0.1	1091	1.0±0.2
3.1Y junction[c]	3.3±0.3	1650	6.1±1.4	271	1.2±0.2
3.2Y junction[c]	2.7±0.2	1350	3.8±0.6	355	1.0±0.1
2Y junction[c]	1.9±0.1	950	51±11	19	1.1±0.2
B. *RecG preps 2, 3*					
Linear dsDNA	233±8	2330	7.5±4.5	311	0.98±0.16
(−)scDNA #1	372±24	3720	1.6±0.3	2325	1.3±0.2
(−)scDNA #2	387±28	3870	2.8±0.5	1382	0.95±0.07
(+)scDNA	802±223	3870	49±25	164	0.8±0.04
M13 ssDNA	125±4.0	1250	1.0±0.08	1275	1.7±0.2
M13+1 μM SSB	183±9.0	1830	2.8±0.3	654	2.7±0.6
M13+1:20 SSB	171±5.0	1710	1.6±0.1	1069	2.3±0.4

Assays were performed as described in Materials and Methods. The rate of ATP hydrolysis in the absence of DNA is 0.30 μM/min and the resulting k_{cat} is 3 min^{-1}. The K_m (or $S_{0.5}$) for DNA is reported in nM molecules.

[a] The K_m^{app} was used for all DNA cofactors except for poly(dT). Here the data were more precisely described utilizing the Hill equation; consequently the parameter $S_{0.5}^{app}$ is more precise.

[b] The numbers after each designation of scDNA, indicate the preparation number as explained in Materials and Methods. (−), negatively scDNA; (+), positively scDNA.

[c] These experiments contained 2 nM RecG, 8 mM MgOAc and 1 mM ATP. The 4Y-junction contains two dsDNA arms; 3.1Y has a single duplex arm and a gap in the leading strand; 3.2Y has a single duplex arm with a gap in the lagging strand; 2Y has two ssDNA arms (see Figure 4(b)).

drive fork regression when the DNA templates are negatively supercoiled.[32] Further, both D and R-loops are substrates for RecG as demonstrated previously.[20,21,33,34] Therefore, it is conceivable that this strong preference observed here could be an artifact due to the presence of contaminating ssDNA fragments invading the supercoiled DNA forming D-loops or residual RNA primers remaining from plasmid DNA replication initiation resulting in R-loops that could function as loading sites for RecG. To determine whether the preference of RecG is for the scDNA cofactor itself or the result of the above-mentioned loop structures, two different scDNA preparation techniques were used. The first preparation method used alkaline lysis followed by treatment with RNAase, a phenol/chloroform extraction and ethanol precipitation. Thereafter, the DNA was subjected to isopycnic centrifugation in two consecutive CsCl gradients. This DNA is designated as scDNA#1 (i.e. preparation #1; Table 2). Radioactive labeling of the resulting scDNA demonstrated a low level (<1%) of small nucleic acid fragments less than 200 bp in size. However, a band co-migrating with relaxed circular dsDNA was detected (and not observed in ethidium bromide-stained gels, and which was no longer present following restriction enzyme cleavage (data not shown). The alkaline lysis used in the isolation procedure could locally denature portions of the (−)scDNA, leading to extruded loops which could potentially function as additional loading sites for RecG onto otherwise supercoiled DNA. To eliminate these potential loading sites for RecG and to ascertain whether the enzyme does exhibit a preference for (−)scDNA, we utilized a plasmid DNA, purification protocol supplied by Ken Marians.[35] Here, cells were gently lysed using triton and lysozyme. The resulting lysate was treated as before. Thereafter, the DNA was subjected to two successive CsCl gradients, followed by a sucrose gradient in 1.0 M salt. This preparation yielded DNA that was greater than 95% supercoiled, contained no detectable contaminants (data not shown). The DNA from this preparation was designated as (−)scDNA#2. The k_{cat} on (−)scDNA #2 was 1207 min^{-1} and the catalytic efficiency of RecG on this cofactor was 549 min^{-1} nM^{-1}, approximately 1.9-fold lower than (−)scDNA #1 (Table 2). The catalytic efficiency of RecG preparations 2 and 3 were also lower on (−)scDNA#2 than on (−)scDNA#1, indicating that this is not protein preparation-dependent. Although the activity of RecG in the presence of this DNA cofactor is lower than that observed on (−)scDNA#1, the results from (−)scDNA#2 argue that RecG utilizes negatively scDNA as a cofactor for ATP hydrolysis.

RecG functions as an oligomer on poly(dT)

To determine whether the affinity of RecG for ATP is influenced by the nature of the DNA cofactor, ATP titrations were done using a fixed concentration of magnesium (the optimum for each cofactor as determined in Figure 2), and each DNA cofactor at concentrations in excess of the K_m^{DNA} or $S_{0.5}^{DNA}$ for

that cofactor (Table 2). Further, to eliminate the potential complications of elevated RecG concentrations, the concentration of RecG was fixed at 100 nM, below the amount of RecG expected to fully saturate the cofactor lattice (Figure 3). For reactions with the junction substrates, the RecG concentration was 2nM and DNA was 20 nM molecules.

For all DNA cofactors, with the exception of poly (dT), the hydrolysis of ATP by RecG can be accurately described using the Michaelis-Menten equation, consistent with the enzyme functioning as a monomer (Figure 5 and Table 3). Further, the data demonstrate that the affinity of RecG for ATP is essentially unaffected by the type of dsDNA cofactor present. However, K_m or $S_{0.5}$ for ATP are 1.7 to 5.8-fold higher in the presence of ssDNA than dsDNA cofactors.

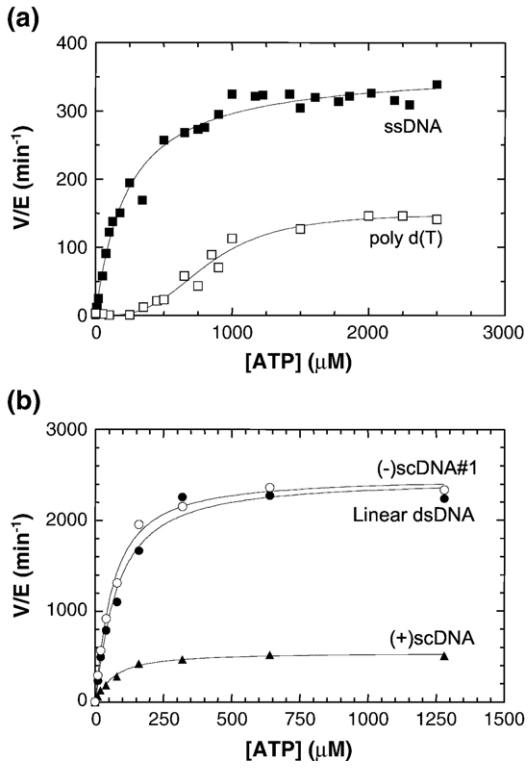

Figure 5. The Hill coefficient for ATP binding on poly (dT) is 3. ATPase assays followed the procedure described in Materials and Methods, with varying ATP concentration. RecG was held constant at 100 nM and the magnesium concentration was varied in order to produce optimal activity. Data were fit to the Hill equation,[64] $V = (V_{max}.[ATP]^n)/([S_{0.5}]^n + [ATP]^n)$ to determine whether cooperative binding is occurring. These results were then analyzed utilizing the Hill equation, in order to determine the kinetic parameters of the enzyme on the cofactors. (a) ssDNA cofactors: This analysis yielded a Hill coefficient of 3.2(±0.5) for poly(dT) (□) and 1(±0.1) for pPB67 ssDNA (■). (b) dsDNA cofactors: The Hill coefficients for linear dsDNA (●), (−)scDNA#1 (○) and (+)scDNA were 1.2(±0.1), 1.1(±0.2), and 1.2(±0.1), respectively. The data presented are from two to four experiments per cofactor with assays conducted on separate days. Data for (+)scDNA were from a single experiment.

As observed in DNA titrations, the k_{cat} for ATP hydrolysis on unbranched DNA molecules was found to be the highest on negatively scDNA, with values ranging from 720 min^{-1} to 845 min^{-1}. However, further indicating the heightened activity of RecG on scDNA, the catalytic efficiency of the enzyme was several-fold higher, ranging from twofold *versus* linear dsDNA to approximate 114-fold *versus* poly(dT). Consistent with the DNA titrations, relaxed circular DNA is a poor cofactor for the ATPase activity of RecG, as demonstrated by the low k_{cat} and poor catalytic efficiency of the enzyme (Table 3). Furthermore, and as observed in the DNA titrations, positively scDNA is also a poor cofactor for the ATPase activity of RecG. Again, it was not possible to attain DNA concentrations high enough to exceed the apparent K_m^{DNA} although these data suggest that the K_m^{ATP} is unaffected by the DNA cofactor.

Unexpectedly, the data from ATP titrations using poly(dT) as cofactor were not well approximated by the Michaelis-Menten equation (data not shown). Consequently, the data were fit to the Hill equation (Figure 5(a)), yielding a Hill coefficient for ATP binding of 3.2(±0.5) (Table 3). Therefore, for full ATPase activity to be attained, the enzyme functions as an oligomer, which is at least a trimer on this cofactor. In addition, the concentration of ATP required for full activity in the presence of either poly(dT) or ssDNA was 2 mM, approximately twofold higher than that for any other DNA cofactor or substrate. The affinity of the enzyme for ATP is also significantly reduced in the presence of poly(dT) as the $S_{0.5}$ for ATP (825(±46) μM), is six- to eightfold higher than the K_m^{ATP} observed on (−)scDNA. As expected from the above, the catalytic efficiency of RecG in the presence of poly(dT) is reduced 110-fold relative to (−)scDNA, indicating that poly(dT) is a poor cofactor for RecG ATPase activity.

Consistent with the DNA titrations, the results from ATP titrations in the presence of the junction DNA substrates demonstrate high activity for RecG. Here the k_{cat} for ATP hydrolysis was higher by almost twofold relative to that observed on (−)scDNA#1. In contrast to the DNA titrations (Table 2), the K_m^{ATP} is not significantly affected by the presence of duplex DNA present in the arms of the model junction substrates (Table 3).

RecG demonstrates a strong preference for negatively supercoiled DNA for maximal activity

To further elucidate the preference of RecG for a scDNA cofactor, ATPase assays were performed in the presence of wheat germ topoisomerase I (WGT), which removes both positive and negative supercoils from DNA.[36] Here, standard ATPase assay conditions were employed and initially, (−)scDNA#1 was the DNA cofactor. The control reaction done in the absence of WGT yielded a rate of 71 μM/min (open circles, Figure 6(a)). In sharp contrast, the addition of WGT to an ongoing reaction resulted in

Table 3. Parameters for ATP hydrolysis

DNA cofactor	V_{max} (μM/min)	k_{cat} (min^{-1})	K_m or $S_{0.5}$ (μM)[a]	k_{cat}/K_m or $k_{cat}/S_{0.5}$ ($min^{-1}/\mu M$)	Hill coefficient
A. *RecG prep 1*					
Poly(dT)	15±0.8	55	825±46	0.07	3.2±0.5
pPB67 ssDNA	36±1.5	234	235±20	0.9	1.0±0.1
(−)scDNA #1[b]	72±7.8	720	148±29	4.9	1.2±0.3
(−)scDNA #2	85±4.0	845	105±17	8.0	1.0±0.2
Relaxed circular	4±0.4	33	165±30	0.2	1.1±0.2
Linear dsDNA	43±5.6	430	149±33	2.9	1.0±0.2
4Y junction[c]	2.1±0.08	1050	74±9	14	1.3±0.2
3.1Y junction[c]	2.0±0.12	1000	101±25	10	1.0±0.2
3.2Y junction[c]	3.2±0.20	1600	72±14	22	1.2±0.2
2Y junction[c]	1.9±0.13	971	51±11	19	1.1±0.2
B. *RecG preps 2, 3*					
Linear dsDNA	247±7.5	2470	62±6	40	1.2±0.1
(−)scDNA #1	244±7.8	2440	70±11	35	1.1±0.2
(+)scDNA	54±1.9	540	66±7	8	1.2±0.1

[a] The Michealis-Menten equation was used to accurately describe data from ATP titrations done on all DNA cofactors except poly(dT). For poly(dT) the data were more precisely described utilizing the Hill equation; consequently the parameter $S_{0.5}^{ATP}$ is more precise. Initially, the Hill equation was used to fit data, hence the values for the Hill coefficient could be obtained.

[b] The numbers after each designation of scDNA, indicate the preparation number as explained in Materials and Methods. (−) negatively scDNA; (+) positively scDNA.

[c] These experiments contained 2 nM RecG, 8 mM MgOAc and 20 nM Y-junction DNA. The 4Y junction contains two dsDNA arms; 3.1Y has a single duplex arm and a gap in the leading strand; 3.2Y has a single duplex arm with a gap in the lagging strand; 2Y has two ssDNA arms (see Figure 4(b)).

an abrupt, 34-fold reduction in the rate of ATP hydrolysis to 2.6 μM/min (filled triangles, Figure 6(a)). An identical rate of ATP hydrolysis was observed using (−)scDNA pretreated with WGT prior to the addition of RecG (the relaxed, P trace, Figure 6(a)). In this case, the WGT was present and presumably still active in the reaction mix with RecG. It is conceivable that there is an unanticipated interaction between WGT and RecG resulting in a significant inhibition of the ATPase activity of RecG. To address this issue, scDNA was treated with WGT for 60 min at 37 °C, extracted once with PCI followed by an ether extraction and ethanol precipitated. The complete conversion of (−)scDNA to the relaxed form was verified using agarose gels stained with ethidium bromide (data not shown). The resulting relaxed, circular DNA was resuspended in TE buffer (10 mM Tris-acetate (pH 8.0), 1mM EDTA) and added to an ATPase assay. The rate of ATP hydrolysis in this reaction (Relaxed, A; Figure 6(a)) was identical to that observed using WGT-treated DNA with the topoisomerase still present. To further demonstrate that the presence of WGT does not adversely affect the ability of RecG to hydrolyze ATP, ATPase assays using linear dsDNA were performed in the presence or absence of WGT. Here, only a small reduction in the steady-state rate was observed, down from 55 to 47 μM/min (data not shown). In contrast, in parallel experiments done concurrently, the rate of ATP hydrolysis in the presence of (−)scDNA was 53 μM/min and decreased to 4 μM/min in the presence of WGT-relaxed DNA (data not shown). Thus the conversion of scDNA to relaxed DNA results in a poor cofactor for the ATPase activity of RecG.

It is conceivable that the scDNA could still possess some single-stranded loops that provide a loading site for RecG protein and that the WGT removes these as it relaxes the scDNA. Consequently, no ssDNA loading site is available for RecG, resulting in a low rate of ATP hydrolysis. To determine if any single-stranded regions were accessible, ATPase assays were done in the presence of P1 nuclease, which presumably would cleave scDNA molecules at these sites, resulting in their conversion to relaxed forms. The presence of P1, added either before or after RecG to reactions using (−)scDNA#1, produced rates of 365(±16) and 333(±9) μM/min, respectively (data not shown). Thus, the activity is not due to the presence of ssDNA loading sites present in the supercoiled DNA.

To determine whether it is in fact negatively supercoiled DNA for which RecG exhibits a preference, relaxed pBR322 DNA was treated with *E. coli* DNA gyrase for 60 min in the presence of ATP. This resulted in 60% to 75% of the relaxed circular DNA being converted to scDNA (as determined by agarose gel electrophoresis; data not shown). The DNA gyrase was removed by phenol/chloroform and ether extractions, followed by S-400 spin column chromatography. This DNA was used in ATPase assays with RecG and the data are shown in Figure 6(b). In the control reactions with either scDNA preparations 1 and 2, the *V/E* values were 1752(±94) and 1440(±60) min^{-1}, respectively. The addition of WGT to separate, ongoing reactions with each of these DNA cofactors, reduced *V/E* 40 and 17-fold to 45 min^{-1} and 85 min^{-1}, respectively. In the presence of relaxed pBR322 DNA a tenfold lower *V/E* value relative to (−)scDNA was observed and this was unaffected by the addition of WGT. In sharp contrast, when relaxed pBR322 DNA was converted to (−)scDNA by DNA gyrase a 12-fold increase in *V/E* was observed, slightly higher than

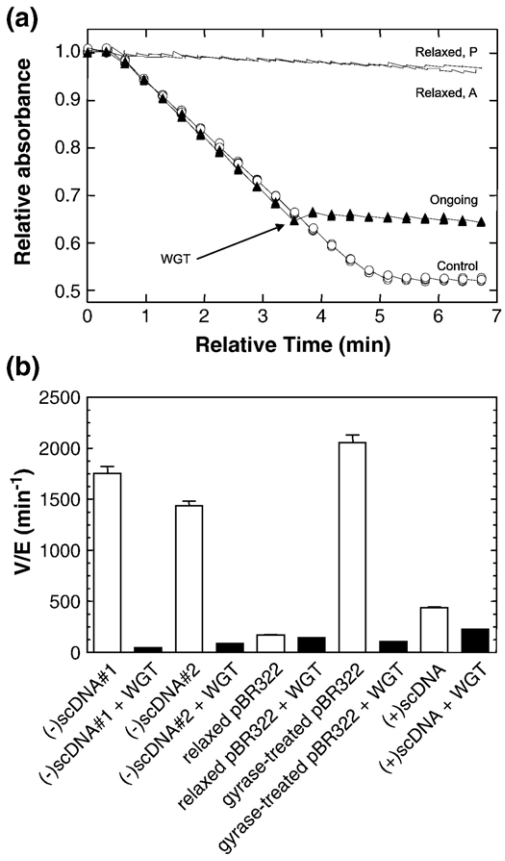

Figure 6. Removal of negative supercoils significantly reduces the ATPase activity of RecG, whereas addition of supercoiling restores ATPase activity. (a) Time courses for the ATPase activity of RecG on (−)scDNA done in the presence, absence or pre-treated with WGT are shown. ATPase assays were performed according to Materials and Methods with 100 nM RecG added to initiate reactions. The DNA cofactor utilized in all reactions was (−)scDNA#2. Where indicated, ten units of WGT were present in the reaction. The control reaction (○) contained (−)scDNA and the reaction rate was 50 μM/min; the ongoing reaction (▲) contained the same (−)scDNA and once a steady-state rate was achieved (54 μM/min), WGT was added as indicated by the arrow, producing a rate of 2.4 μM/min. The relaxed, A reaction (A=absent; continuous line; 2.7 μM/min) contained (−)scDNA pretreated with ten units of WGT for 60 min at 37 °C. The WGT was subsequently removed by PCI extraction followed by ethanol precipitation. The relaxed, P reaction (P=present; continuous line; 1.9 μM/min) contained (−)scDNA with WGT added prior to RecG for 10 min at 37 °C. (b) A comparison of the rates of ATP hydrolysis demonstrating the effects of DNA superhelicity on the hydrolysis of ATP by RecG is shown. White bars, reactions with RecG only; black bars, reactions in the presence of WGT (ten units) added to an ongoing reaction once a steady-state rate of ATP hydrolysis by RecG had been achieved. Error bars represent the average of at least two different experiments.

that with purified (−)scDNA (Figure 6(b)). Since this DNA should not contain R or D-loops, or alkali-extruded ssDNA regions, this demonstrates unequivocally that RecG utilizes scDNA as a cofactor for the hydrolysis of ATP. Addition of WGT to an ongoing reaction containing DNA gyrase-treated DNA, reduced the *V/E* value 19-fold, back to that observed for relaxed, circular DNA. Finally, the *V/E* in the presence of (+)scDNA (438(±12) min^{-1}) was 2.6-fold higher than that observed on relaxed circular pBR322 DNA (169(±12) min^{-1}) and addition of WGT had only a modest effect on the *V/E* value (that is, twofold reduction to 227 min^{-1}). The highest *V/E* value for (+)scDNA was still significantly lower than the values obtained in the presence of (-)scDNA. Therefore, these data demonstrate that RecG exhibits a strong preference for negatively supercoiled DNA.

SSB protein stabilizes the binding of RecG to ssDNA

In addition to DNA titrations, and to determine the relative affinity of RecG for the various cofactors, salt titrations were carried out under optimal conditions for the hydrolysis of ATP for each of the DNA cofactors. Once a steady-state rate of ATP hydrolysis was achieved, NaCl was added in small increments, resulting in a new steady-state rate. Once this was achieved, the process of NaCl additions was repeated until DNA-dependent ATPase activity of RecG ceased. The hydrolysis rate in each steady-state region was calculated and expressed as a percent of the steady-state rate in the absence of NaCl. These rates were subsequently graphed to determine the concentration of NaCl resulting in a 50% reduction in the rate of ATP hydrolysis, which corresponds to the salt-titration midpoint (STMP). The values for the STMP for the hydrolysis of ATP by RecG in the presence of each cofactor are presented in Table 4.

The results demonstrate that the STMPs on ssDNA are higher than those on dsDNA cofactors, by approximately twofold. As a collapsed or stalled replication fork (that is, the proposed *in vivo* substrate for RecG) may be partially single-stranded and bound by SSB, titrations were repeated in the presence of the *E. coli* SSB protein. For the two ssDNA cofactors examined, a twofold increase in the STMP was observed in the presence of SSB protein, suggesting that SSB stabilizes the binding of RecG to ssDNA. This stabilization is specific to SSB as the presence of the bacteriophage gene *32* protein (gp32) has no effect on the STMP on ssDNA cofactors (data not shown). As expected, the presence of SSB protein has no effect on the STMP on dsDNA cofactors (Table 4). Finally, the STMP on the 4Y substrate was two- to sevenfold higher than that observed on all other cofactors, consistent with the enzyme binding to this junction with high affinity (Table 2).

SSB influences the activity of RecG in the presence of ssDNA

In addition to increasing the STMP of RecG on ssDNA, it is conceivable that the presence of the *E. coli* SSB protein may affect other aspects of the

Table 4. The salt titration midpoint for the hydrolysis of ATP reveals that the binding of RecG to ssDNA is stabilized by SSB protein

	STMP (mM NaCl)		
Cofactor	− SSB	+ SSB	Fold change
Poly(dT)	38	ND	–
M13 mp 18 ssDNA	32	63	1.97
pPB67 ssDNA	50	82	1.64
Linear dsDNA	15	19	1.27
Relaxed circular	24	28	1.17
(−)scDNA #1	15	18	1.20
4Y junction[a]	102	120[c]	1.18

Assays were performed as described in Materials and Methods.
[a] The concentration of junction used was 50 nM and SSB was present at 1 μM.

behavior of RecG on ssDNA. To determine whether SSB affects the hydrolysis of ATP by RecG in the presence of ssDNA, ATPase assays were carried out in the presence or absence of sub-stoichiometric amounts of SSB relative to ssDNA. In these assays, SSB was added to M13 mp18 ssDNA prior to the addition of RecG. Two separate sets of titrations were performed: a DNA titration and an ATP titration. In the DNA titration, two ratios of SSB to ssDNA were used. In the first, the concentration of SSB was held constant (i.e. varying the ratio of SSB to DNA) while in the second the ratio of SSB to DNA was held constant at 1:20.

The results show that the presence of SSB protein resulted in a radical change in the kinetic parameters for RecG in the presence of ssDNA (Figure 7). The resulting curves from these DNA titrations become increasingly sigmoid-shaped as evidenced by the increase in the Hill coefficient for DNA binding from 1.7(±0.2) in the absence of SSB to 2.3 to 7.7(±0.8) in the presence of SSB protein (Figure 7). Simultaneously, the $S_{0.5}^{app}$ for DNA increased slightly in the presence of SSB from 1.0(±0.08) to 1.6(±0.1) nM molecules and the V_{max} increased 1.4-fold (Table 2). Furthermore, the ATP titrations using the two different SSB to DNA ratios, revealed that the V_{max} for ATP hydrolysis by RecG increased approximately twofold in the presence of SSB (data not shown); no change in the K_m^{ATP} was observed. This alteration of kinetic behavior with respect to DNA binding is specific to SSB, as the presence of gp32 has no effect (data not shown). Identical experiments were carried out using linear dsDNA. As for the STMP experiments, the presence of sub-stoichiometric amounts of SSB protein had no effect on the kinetic parameters for RecG, as the K_m^{app} and k_{cat} values remained unaltered (data not shown). Thus the *E. coli* SSB protein has pronounced affects on the interaction of RecG protein with ssDNA.

Heparin trapping reveals that the translocation processivity of RecG is the highest on (–)scDNA

In order to determine the relative processivity of RecG on the DNA cofactors studied here, heparin was used as a protein trap to capture RecG molecules that dissociate from DNA, as described for superfamily I DNA helicases[37,38] and dsDNA translocases.[25] Heparin is similar to DNA as both molecules are highly charged linear polymers that behave as polyelectrolytes.[39] However, in contrast to DNA, it does not stimulate the ATPase activity of RecG (data not shown).

First, heparin titrations were done to determine the appropriate concentration of heparin to trap RecG molecules that dissociate from, and not compete them off the DNA. The lowest concentration that inhibits ATP hydrolysis by RecG when added prior to the enzyme should correspond to the amount of heparin that can be used as a trap. These titrations revealed that the lowest concentration (final) of heparin that inhibited the hydrolysis of ATP by RecG was 1 μg/μl for supercoiled and linear dsDNA, and 10 μg/μl for M13 mp 18 ssDNA (data not shown). The tenfold higher amount of heparin required to inhibit RecG on ssDNA suggests that RecG has a higher binding affinity for single strand DNA cofactors in comparison with duplex cofactors, consistent with the STMP data (Table 4). These heparin concentrations were utilized in the experiments presented below.

In heparin trapping experiments, ATPase assays were performed utilizing rapid data collection mode with the spectrophotometer (i.e. one data point was collected every 1.2 s) to visualize the anticipated transition from the DNA on- to the DNA off-state of RecG, correlating with the heparin-trapped form of

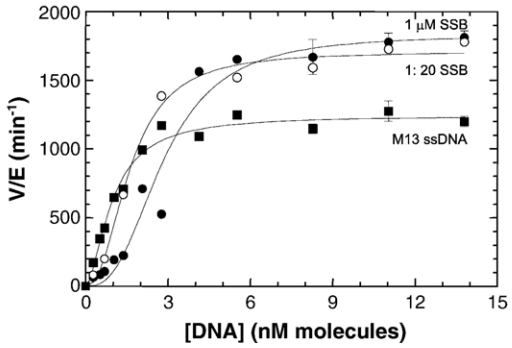

Figure 7. SSB protein stabilizes the interaction of RecG with ssDNA. Reactions were conducted as described in Materials and Methods, utilizing 100 nM RecG, M13 mp18 ssDNA at the concentrations indicated, and the optimum magnesium ion concentration as determined in Figure 2. The M13 ssDNA contained only DNA and RecG; the 1 μM SSB reactions contained a fixed concentration of SSB protein (i.e. 1 μM) while in the 1:20 reactions, the ratio of SSB to DNA was held constant (i.e. both SSB and DNA were varied). Reactions were initiated by addition of RecG, following a 4 min incubation of the reaction mixture at 37 °C. Rates were calculated from linear steady-state portions of time courses as in Figure 1 and are presented as *V/E* to permit visual comparison to the data in Figure 4. The resulting data were fit to the Hill equation[64] to yield apparent $S_{0.5}$, V_{max} and values for n respectively of: 0.98(±0.08), 1,245(±34) and 1.7(±0.2) (ssDNA only); 1.6(±0.1), 1711(±52) and 2.3(±0.4) (1:20) and 2.8(±0.3), 1834(±90) and 2.7(±0.6) (1 μM SSB).

the enzyme. Assays were initiated using DNA (either ssDNA, linear dsDNA or (−)scDNA#1) following a pre-incubation of all other components including RecG. Once a steady-state rate of ATP hydrolysis had been achieved, the pre-determined amount of heparin was added to the assay. If RecG exhibits low processivity, it was anticipated that ATP hydrolysis would rapidly cease following the addition of heparin. In contrast, if processivity is higher, then ATP hydrolysis should continue for some period of time following the addition of heparin. Consequently, the amplitude in the change in absorbance following the addition of heparin should correlate with the processivity of the enzyme on a DNA cofactor. This follows if the enzyme couples the hydrolysis of ATP to translocation on each of the DNA cofactors used as demonstrated for the model 3Y substrate.[40]

The results, presented in Figure 8, show that the cessation of the hydrolysis of ATP with M13 ssDNA as a cofactor occurs rapidly after addition of heparin. There is a brief period (3.6 s) during which ATP is hydrolyzed, followed by an abrupt transition to a state where there is a low rate of ATP hydrolysis. This low rate is similar to that observed in the absence of DNA. In contrast, significantly more ATP hydrolysis by RecG is observed on both dsDNA cofactors following the addition of heparin. For linear dsDNA, the absorbance signal decreases linearly for 14.4 s and then abruptly ceases. During this period of 14.4 s, ATP hydrolysis occurs at a rate of 33 μM/min; thereafter the rate is reduced to the DNA independent rate. In contrast, in the presence of scDNA, the signal is unaffected for 1.4 s and then decays exponentially over approximately 30 s, at which point ATP hydrolysis rate is reduced to the DNA-independent rate.

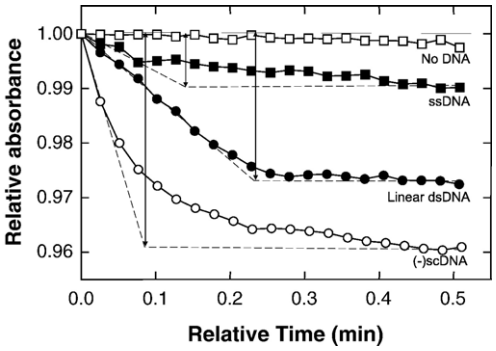

Figure 8. Heparin trapping demonstrates that the processivity of RecG is highest on negatively scDNA. Reactions were conducted as described in Materials and Methods, utilizing 100 nM RecG, DNA at a concentration in excess of K_m^{app} DNA for each cofactor, and the optimum magnesium ion concentration for each DNA cofactor as determined in Figure 2. Reactions were initiated by addition of RecG, following a 4 min incubation of the reaction mixture at 37 °C. After a steady-state rate was achieved, heparin was added to the reaction. The amount of heparin added was the minimal concentration needed to fully stop the ongoing reaction for each individual DNA cofactor. The reaction rates prior to heparin addition are as follows, 29 μM/min for ssDNA, 66 μM/min for linear dsDNA and 103 μM/min (−)scDNA. The post-addition rates are 1(±0.1) μM/min for all cofactors. The rate of RecG ATP hydrolysis in the absence of DNA for 100 nM RecG is 0.56 μM/min. To permit direct comparison, the reaction times were normalized to the time point at which heparin was added (t=0) and the absorbance readings were normalized to the absorbance point directly prior to the addition of heparin. The arrows indicate the amplitude of the absorbance change in NADH that was used to calculate the amount of ATP hydrolyzed by RecG molecules bound to DNA.

As RecG translocates 3 bp per ATP molecule hydrolyzed,[40] the amount of ATP hydrolyzed following the addition of heparin should provide an estimate of the average processivity of RecG on each DNA cofactor. This follows, since the ATPase activity of RecG is DNA-dependent, and thus the amount of ATP hydrolyzed will correspond to those RecG molecules bound to the DNA at the time of heparin addition. If all of the RecG present is bound to DNA at the time of heparin addition, then the amount of ATP hydrolyzed per RecG molecule can be calculated. Thereafter, using the value of 3 bp/ATP, the distance translocated prior to dissociation can be estimated. On (−)scDNA, the distance translocated is 736 bp, on linear dsDNA it decreases to 556 bp and on ssDNA it decreases further to 224 nt. However, since the value of 3 bp translocated per ATP was obtained in the presence of a 3Y substrate, these distances may not be the same on the different cofactors used here. Consequently, the processivity values were corrected using the catalytic efficiency values obtained in the presence of each DNA cofactor using RecG preparation #1 (Table 2). This resulted in a threefold increase for (−)scDNA to 2315 bp, a threefold decrease to 166 bp for linear dsDNA and 0.86-fold decrease for ssDNA to 192 nt.

Discussion

The primary conclusion of this work is that the preferred unbranched, DNA cofactor for the ATPase activity of RecG is negatively supercoiled DNA. The activity of the enzyme on 4Y substrates is comparable to that observed on (−)scDNA. Second the binding of RecG to ssDNA is stabilized by the *E. coli* SSB protein as indicated by an increase in the STMP and V_{max} values for ATP hydrolysis. These findings have implications for the timing of DNA access by RecG at stalled replication forks and consequently the enzyme's role in replication fork reversal.

The preferred DNA cofactor for RecG was shown to be (−)scDNA through a variety of means. It was, however, critical to demonstrate that this preference is due to the superhelical nature of the DNA and not due to regions that are partially single-stranded and which may function as high-affinity binding and loading sites for the enzyme.[20,21] Therefore, we went to great lengths to produce (−)scDNA lacking detectable contaminants (as described in Materials and Methods). Using this negatively scDNA as cofactor, (−)scDNA#2, RecG

exhibited significant ATPase activity, an extremely high catalytic efficiency more so than on any other type of cofactor, and comparable to that on the 4Y substrate (Table 2). As the presence of P1 nuclease did not affect the ATPase activity of RecG on (−)scDNA, the observed activity was not the result of single-stranded regions (not detected using 5′end-labeling,) functioning as loading sites for RecG. Therefore, we conclude that RecG utilizes negatively supercoiled DNA as a cofactor for the hydrolysis of ATP.

The ability of RecG to utilize (−)scDNA as a co-factor for ATP hydrolysis was further demonstrated in experiments using relaxed circular DNA which is a poor cofactor for RecG. When relaxed circular DNA was treated with DNA gyrase, the change in the ATPase activity of RecG was profound, increasing 12-fold from 169 min^{-1} to 2058 min^{-1} (Figure 6(b)). Treatment of all (−)scDNA cofactors including DNA gyrase-treated pBR322 with WGT, resulted in a 17 to 40-fold decrease in the rate of ATP hydrolysis by RecG demonstrating that a covalently closed DNA molecule is not the optimal cofactor (Figure 6(b)). This reduction in ATPase rate was not due to some fortuitous interaction between RecG and WGT as (i) the rate of ATP hydrolysis was similar in the presence or absence of WGT on linear dsDNA and (ii), removal of WGT from relaxed DNA produced identical ATP hydrolysis rates to that observed in ongoing reactions with (−)scDNA to which WGT was added (Figure 6(a)). To determine whether the preference was for negatively or positively scDNA or potentially both, the catalytic properties of RecG in the presence of these DNA cofactors were compared. The catalytic efficiency of RecG was 8–14-fold lower in the presence of (+)scDNA than in the presence of (−)scDNA (Table 2); in fact the (+)scDNA titrations were almost super-imposable with that of linear dsDNA (Figure 4(a)). The lower catalytic efficiency in the presence of (+)scDNA is due to a 22-fold lower affinity for positively supercoiled relative to (−)scDNA. Therefore, the preferred cofactor for ATP hydrolysis by RecG is negatively supercoiled DNA.

The data presented herein also demonstrate that RecG has a high affinity for ssDNA as an approximately twofold higher STMP was observed for ssDNA relative to dsDNA cofactors (Table 4). Further, a tenfold higher level of heparin was required to trap RecG in the presence of ssDNA *versus* dsDNA cofactors. The high affinity of RecG for M13 ssDNA does not, however, translate into a better cofactor for ATP hydrolysis as the catalytic efficiency of the enzyme is lower by 1.8 to 5.8-fold relative to (−)scDNA (Table 2). The binding of RecG to M13 ssDNA was stabilized by the presence of the *E. coli* SSB protein, a stabilization that was not afforded by the bacteriophage gene *32* protein. This is consistent with a species-specific protein–protein interaction between RecG and SSB that is currently under investigation. Further, this interaction may be a common feature for coordinating the proteins that interact with stalled forks as demonstrated for the PriA–SSB interaction.[41] This interplay may be complex, as suggested by work demonstrating the concerted actions of both PriA and RecG in stabilizing stalled replication forks.[11,42]

As expected and consistent with previous work, RecG exhibits significant activity on the 3Y and 4Y substrates used here (Figure 2). The preference for these DNA molecules as cofactors for the hydrolysis of ATP parallels the large body of unwinding data obtained by the Lloyd laboratory.[9,12] That is, RecG exhibits a preference for a 4Y substrate (i.e. a Y-junction with two two dsDNA arms) relative to those with gaps in either the leading or lagging strands (substrates 3.1 and 3.2Y; Tables I2, 3 and Figure 4(b)). As the K_m^{ATP} value is unaffected by the DNA molecule present, the difference in ATPase activity observed for these model junctions is due to the difference in apparent affinity of RecG for these DNAs. The affinity varied from 1.1 nM molecules for the 4Y substrate to 51 nM for the 2Y (i.e. the three-way junction with two single-stranded arms; Table 2 and Figure 4(b)). The differences in activity are not due to the presence of contaminating single-stranded oligonucleotides present in the reaction sequestering RecG since the presence of SSB did not significantly affect the rate of ATP hydrolysis for each of the Y substrates used (data not shown). Therefore, RecG exhibits a strong preference for duplex DNA in each arm of the model Y-substrate, i.e. a chickenfoot structure.

RecG is a DNA motor protein that translocates 3 bp for each ATP molecule hydrolyzed in the presence of a model 3Y substrate.[40] Previous work suggested that the processivity of RecG on branched DNA structures was low, in the range of 20–30 base-pairs.[23] As we have demonstrated here that RecG exhibits significant activity on a variety of DNA cofactors, we wanted to measure the processivity of translocation of the enzyme on each cofactor. To achieve this, we used heparin trapping to bind RecG molecules that dissociate from the DNA during translocation. The results show that the estimates of processivity were 2315 bp in the presence of (−)scDNA, 166 bp for linear dsDNA and 192 nt for ssDNA. The low value for processivity on linear dsDNA is consistent with previous work.[23] We emphasize that these values are estimates and experiments are in progress to more precisely determine the processivity of the enzyme using both ensemble[43] and single molecule approaches.[44] Furthermore, the values obtained here are higher than those suggested using branched DNA structures. This arises for two reasons. First, measurements of processivity on branched DNA employed substrates that contained regions of heterology which inhibit the DNA helicase activity of RecG,[9] and subsequently may result in limited processivity. Second, we do not observe DNA helicase activity on unbranched DNA molecules, thus the processivity values obtained here are primarily for dsDNA translocation (i.e. in the absence of fork regression).

The different levels of ATPase activity observed in the presence of diverse DNA cofactors and substrates, provides insight into the timing and means by which RecG can access and process DNA at a

replication fork. The *E. coli* chromosome has been shown to exist in fluid, topological domains approximately 10 kb in size.[45] During DNA replication, these domains alter position as the fork moves through the chromosome, with a pre-replicated domain ahead of the replication machinery, a replicating domain in the immediate proximity of the replisome and a replicated domain in the replisome's wake (Figure 9).[46] Consequently, DNA in the pre and post-replicated domains will be (−) supercoiled due to the actions of DNA gyrase and/or topoisomerase IV, while DNA within the replicating domain would be (+) supercoiled. In addition, the (+) supercoils immediately in front of the fork can equilibrate across the fork to create (+) precatenanes.[47] Thus, during DNA replication, DNA immediately flanking the replisome on both sides would be (+) supercoiled. The data presented in Tables 2, 3 and Figures 4, 5, 6 demonstrate that (+) supercoiled DNA is a poor cofactor for RecG (i.e. the affinity for (+)scDNA is low). Consequently, RecG most likely will not bind to DNA within replicating domains, i.e. to an active and fully functional DNA replication fork. This makes sense since there really is no obvious need for RecG to be associated with a functional replication fork (Figure 9(b), center panel).

In contrast, should the fork encounter a translocation block resulting in replisome stalling leading to protein disassembly the situation would be quite different (Figure 9(b), right panel). Once the replisome disassembles, the (+) superhelical strain which had built up would be released, driving the formation of a chickenfoot structure as demonstrated previously.[48] Not surprisingly, RecG demonstrates a high apparent affinity for 4Y substrates relative to linear dsDNA and 3Y substrates (Table 2). Thus, the nascent, extruded chicken foot structure would be an excellent loading site for RecG (Figure 9(b), upper scenario). If, however, replisome disassembly leads to a structure with a gap in either the leading or lagging strands, the ssDNA within the gap would most likely be bound by SSB first (Figure 9(b), lower scenario). This, however, does not

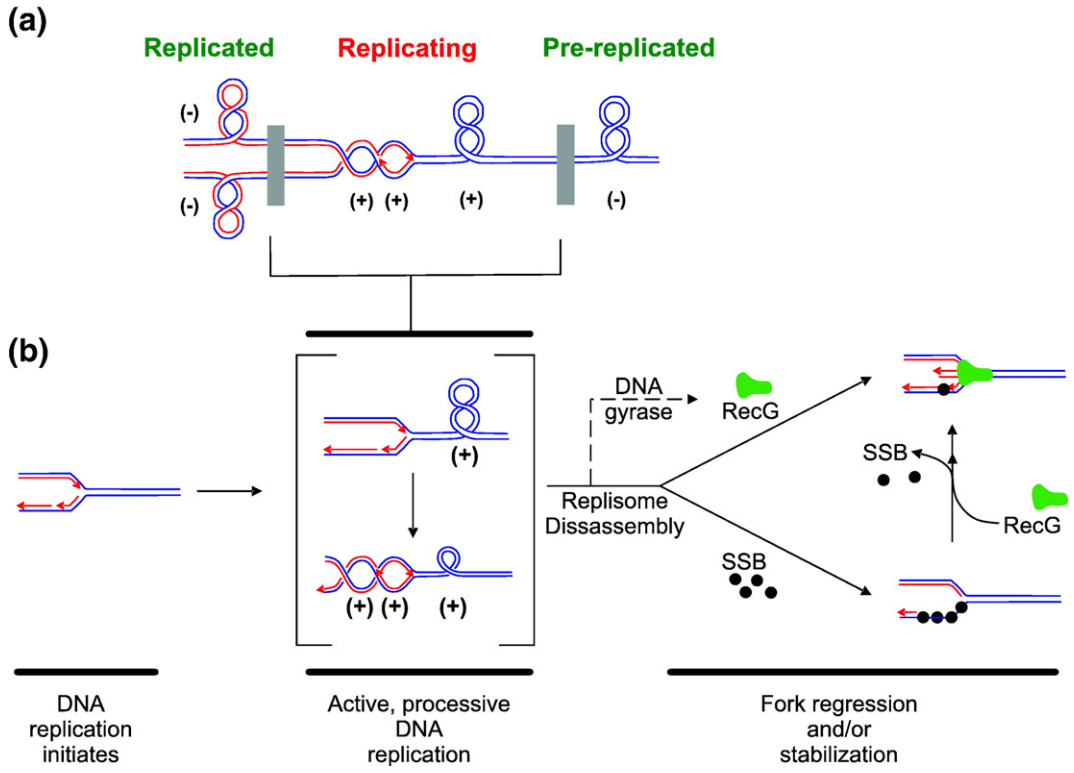

Figure 9. The DNA conformation at a stalled fork determines access of RecG to the DNA. (a) A model of the topological domains of a segment of the *E. coli* chromosome is shown. This Figure is adapted from references.[46,65] Parental DNA is colored blue and nascent daughter DNA is colored red with arrowheads indicating 3′ ends. Domain barriers are indicated by the grey boxes. The three stages of the chromosome are indicated immediately above and the DNA topology is indicated by (−) and (+). Each domain is labeled in color: replicating (red) = RecG exhibits low DNA affinity and low activity; replicated and pre-replicated (green) = RecG exhibits high DNA affinity and high catalytic efficiency. (b) A schematic showing replicating DNA with the fork encountering a block, leading to replisome disassembly (adapted from Posto *et al.*[46]). Once the replisome disassembles, DNA is released and is free to extrude the chicken foot to which RecG (colored green) binds with high affinity (top scenario). The broken line in the upper scenario indicates a potential alternate path if DNA gyrase were to act first. RecG could then bind to the (−)scDNA that may have regressed or, assist regression similar to that observed for (+)scDNA.[48] Alternatively, if a gap for example in the lagging strand occurs it will be rapidly bound by SSB (grey spheres). RecG then binds, possibly displacing SSB, but together they can co-exist to reverse the fork and/or stabilize it. Similar results might be expected if the gap were present in the leading strand, since RecG acts on either gapped substrate with equal efficiency.

constitute a problem for RecG since the data in Figure 7 demonstrate that RecG and SSB can coexist on the DNA, with SSB stabilizing the binding of RecG to ssDNA. Further, preliminary data indicate that RecG can displace SSB from ssDNA (S.S. & P.B., unpublished observations). In addition, RecG is catalytically efficient on model forks with either leading or lagging strand gaps (Tables 2 and 3). Thus in addition to nascent chicken foot structures, RecG can act on structures where one of the arms is single stranded with SSB protein possibly providing a stabilizing effect.

The strong preference of RecG for (−)scDNA represents a possible conundrum as the *E. coli* chromosome is itself (−)supercoiled.[49–52] However, the concentration of RecG has been estimated to be less than ten copies per cell,[53] so the action of RecG at the global level of the chromosome should not be a significant problem, since the chromosome is organized into on average, 460 topological domains.[45] Furthermore, the processivity of RecG in the presence of (−)scDNA estimated here, is at its maximum 2315 bp, well below the average size of a topological domain of the chromosome (i.e. ~10 kb;[45]). So even if RecG were continuously acting on (−)scDNA, the potentially deleterious effects of its action would be limited to 2% of the topological domains and furthermore, be constrained to within an individual domain.

However, as (−)scDNA is the preferred cofactor for RecG, it must have a function in the role of the enzyme. In the scenario depicted in Figure 9(b), if for example, DNA gyrase acts immediately before the replisome disassembles (i.e. within the 5 to 7 min functional half-life of the replication proteins at a fork[54]), then the DNA immediately ahead of the fork will be negatively instead of positively supercoiled. As RecG binds to (−)scDNA with high affinity, we propose that this would be a loading site for RecG immediately ahead of the stalled replication fork. Following binding, the enzyme could translocate to the fork and regress it. As (−)scDNA has the ability to extrude a chickenfoot structure similar to that observed for (+)scDNA,[55] and as RecG can drive fork regression when DNA is (−)supercoiled,[32] these properties would combine to provide an efficient fork regression reaction. The processivity of RecG on (−)scDNA measured here dictates that the enzyme would regress the fork on average at least 2315 bp.

In summary, the data presented here suggest that the timing of RecG access to the DNA at stalled forks is determined by the DNA topology; that is, it must be first converted to (−)scDNA for RecG to function efficiently. Once this has occurred, the types of structures RecG can act on are dictated by the affinity of the enzyme for the DNA available, with a preference for a fork with two duplex arms. Gapped DNA molecules would not be refractory to regression by RecG as the enzyme is catalytically efficient on these substrates and SSB protein stabilizes the binding of RecG to ssDNA. The combination of the above-mentioned properties, provide further evidence for the ability of RecG to act at stalled replication forks to drive their regression leading to subsequent repair.

Materials and Methods

Chemicals

Phosphoenol pyruvate (PEP), FTE nicotinamide adenine dinucleotide (NADH), heparin, pyruvate kinase, lactate dehydrogenase and the ssDNA cellulose resin were obtained from Sigma Chemical Company. ATP, DEAE Sepharose Fast Flow resin and the 16/10 heparin FF column were obtained from Amersham Pharmacia Biotech. The hydroxyapitite Bio-Gel® resin was from Bio-Rad Laboratories. Dithiothreitol (DTT) was purchased from Acros Organics. BSA was purchased from New England Biolabs. Wheat germ topoisomerase 1 (WGT) was from Promega. *E. coli* DNA gyrase, relaxed, circular pBR322 DNA and positively supercoiled DNA were purchased from John Innes Enterprises (Norwich, UK). Oligonucleotides used to construct the junction substrates were purchased from Integrated DNA Technologies, Coralville, IA.

Reagents

All solutions were prepared using Barnstead Nanopure water. Stock solutions of Phosphoenol pyruvate (PEP) were prepared in 0.5 M Tris–acetate (pH 7.5). ATP was dissolved as a concentrated stock in 0.5 M Tris–HCl (pH 7.5), with the concentration determined spectrophotometrically using an extinction coefficient of 1.54×10^5 M^{-1} cm^{-1}. NADH was dissolved in 10 mM Tris–acetate (pH 7.5), concentration determined using an extinction coefficient of 6250 M^{-1} cm^{-1}, and stored in small aliquots at −80 °C. Dithiothreitol (DTT) was dissolved as a 1M stock in Nanopure water and stored at −80 °C. Stocks of heparin were made up in 50 mM Tris–acetate (pH 7.5) and stored in small aliquots at −80 °C.

Strains and plasmids

The RecG protein over expressing strain, GC1269, was a gift from Dr Robert Lloyd (Institute of Genetics, University of Nottingham). GC1269 was derived by transforming strain B834 (DE3) *plysS* with pGS772.[27] Supercoiled DNA was isolated from a strain harboring plasmid pPB67 derived from pBluescript II SK$^-$ (Stratagene) by the insertion of a fragment containing the *RAD52* gene from *Saccharomyces cerevisiae* to generate a plasmid of 4263 bp. This plasmid, previously designated as pSKPB10, contains no intentional, sequence specific elements and was generated to make a high copy number plasmid similar in size to pBR322.[56] Strain MV 1190 containing the plasmid p323-21a which over-expresses bacteriophage gene *32* protein was provided by Nancy Nossal (NIH).

DNA cofactors

Negatively supercoiled DNA (scDNA; pPB67) was purified using two different procedures. The first method utilized alkaline lysis followed by successive isopycnic centrifugation in CsCl gradients.[57] This method included RNAase treatment of the cleared cell lysate, followed by extraction with phenol/chloroform/isoamyl alcohol (PCI; 25:24:1 by volume), and ethanol precipitation. The second method of (−)scDNA purification was provided by Dr Ken Marians (Memorial Sloan Kettering[35]). Here cells were lysed using lysozyme and Triton X-100. The cleared cell lysate was treated with RNAase, followed by PCI

extraction. The aqueous phase was subsequently ethanol precipitated, the resulting pellet dried, resuspended in TE and subjected to two successive CsCl gradients. The band corresponding to (−)scDNA was isolated, extracted with organic solvents and subjected to centrifugation in a sucrose gradient containing 1.0 M NaCl. For all preparations of DNA, the concentration of DNA was determined spectrophotometrically using an extinction coefficient of 6500 M^{-1} cm^{-1} (nucleotides).

Linear dsDNA was produced by subjecting pPB67 to cleavage by HindIII and heat inactivating the enzyme at 70 °C for 20 min. Following inactivation, an equal volume of PCI was added to the DNA, followed by vortexing, and centrifugation at 9610*g* for 5 min. Subsequently, the aqueous layer was removed and treated with an equal volume of TE (10 mM Tris (pH8.0), 1mM EDTA) saturated ether. DNA was subsequently ethanol precipitated, dried and resuspended in 1×TE buffer (pH 8.0). The concentration of DNA was determined spectrophotometrically using an extinction coefficient of 6500 M^{-1} cm^{-1} (nucleotides).

Poly(dT) (average length 359 nt) was purchased from Amersham Biosciences, dissolved in 1X TE and stored in small aliquots at −80 °C. The concentration was determined spectrophotometrically using an extinction coefficient of 8520 M^{-1} cm^{-1} (nucleotides).

pPB67 ssDNA was prepared by infecting the strain harbouring pPB67 with VCSM13 helper phage at a multiplicity of infection of 10 as described in the Bluescript® II Exo/Mung DNA sequencing system instruction manual (Stratagene). Infected cells were selected by adding kanamycin to a final concentration of 70 μg/ml. Following overnight growth at 37 °C, the cells were removed by centrifugation and the supernatant containing phage particles was processed as described.[58] The concentration of DNA was determined spectrophotometrically using an extinction coefficient of 8 780 M^{-1} cm^{-1} (nucleotides). Purified DNA was stored in small aliquots at −80 °C.

M13 mp18 ssDNA was prepared by infecting *E. coli* strain KK2186 Δ*recA1398 srl*::Tn*10* (tet) at a multiplicity of infection of 10, and incubating overnight at 37 °C with shaking. Cells were removed by centrifugation, and the phage precipitated using PEG-NaCl precipitation. Following centrifugation, the resuspended phage pellet was then purified through a CsCl gradient, and the DNA was extracted through phenol/chloroform extraction.[58] The concentration of DNA was determined spectrophotometrically utilizing an extinction coefficient of 8780 M^{-1} cm^{-1} (nucleotides). The purified DNA was stored in small aliquots at −80 °C.

Negatively supercoiled pBR322 was produced using DNA gyrase and assay conditions exactly as described in the Gyrase Assay Kit (John Innes Enterprises, Norwich, UK). Reactions were terminated by the addition of EDTA (10 mM final), followed by a 15 min incubation at 37 °C. Reactions were subsequently extracted once with PCI, once with ether and passed through S-400 spin columns pre-equilibrated in 1×TE. To determine the level of supercoiling following DNA gyrase treatment, small aliquots of reactions were subjected to agarose gel electrophoresis, in the absence of ethidium bromide. Under standard conditions, 60% to 75% of the relaxed DNA was converted into negatively supercoiled DNA (data not shown).

Positively supercoiled pBR322 was purchased from John Innes Enterprises (Norwich, UK). It was produced by treating relaxed circular pBR322 DNA with an excess of DNA gyrase.

Model fork-DNA substrates consisting of a homologous core of 12 bp flanked by heterologous duplex arms of 19–25 bp was constructed by annealing gel-purified oligonucleotides. The substrate design was similar to that used previously for three and four-strand substrates.[10] The junction point has the ability to branch migrate within the homologous core, whereas the heterologous arms prevent spontaneous resolution of the junction DNA. This design was used as RecG protein can unwind heterologous arms less than 30 bp.[9] Since each substrate mimics a fork, we designate a model fork with two duplex arms as Y4; a model fork with a gap in the leading strand as Y3-1 and a gap in the lagging strand as Y3-2 and finally a model fork with two single-stranded arms as Y2.

Junction DNA was prepared by annealing four oligonucleotides in various combinations: PB170 (5′-CTAGAGACGCTGCCGAATTCTGGCTTGGATCTGAT GCTGTCTAGAGGCCTCCACTATGAAATCGCTGCA-3′), PB171 (5′-GCGATTTCA TAGTGGAGGCCTCTAGACAGCA-3′), PB172 (5′-TGCTGTCTAGAGACTATCGA TCTATGAGCTCTGCAGC-3′) and PB173 (5′–CCGGGCTGCAGAGCTCATAGA TCGATAGTCTCTAGACAGCATCAGATCCAAGCCAGAATTCGGCAGCGTCT-3′). Each oligonucleotide was purified using denaturing polyacrylamide gels containing 6 M urea. Purified oligonucleotides (1 μM each in molecule) were annealed in a total volume of 50 μl containing incubation at 100 °C for 5 min, followed by cooling to room temperature overnight. The extent of annealing was verified by non-denaturing PAGE using 5′ end-labeled oligonucleotides annealed under identical conditions (data not shown). Junctions were added directly to ATPase assays without further purification. As the annealing reactions contained 10 mM magnesium acetate, the concentration of magnesium ions in each assay was adjusted accordingly.

Proteins

RecG protein

Large scale growth (10 l) of the RecG over expressing strain, GC1269, was performed in a Microgen Fermentor (New Brunswick Scientific Co. Inc). RecG protein was purified according to a method described[27] with the following modifications. The order of chromatography columns used was: DEAE Sepharose® Fast Flow equilibrated with buffer A (20 mM Tris–HCl (pH 8.5), 1 mM EDTA, 0.5 mM DTT, 10% (v/v) glycerol); heparin FF equilibrated with buffer A (pH 7.5); hydroxyapitite (HAP) equilibrated with buffer P (10 mM potassium phosphate (pH 6.8), 0.5 mM DTT, 150 mM KCl, 10% glycerol) and ssDNA-cellulose equilibrated with buffer A (pH 7.5). Throughout the purification, the presence of RecG protein was determined by SDS–PAGE. The final yield of RecG protein contained no contaminating bands as determined on staining of SDS–PAGE gels with Coomassie blue (data not shown). Further, no contaminating single-stranded or double-stranded DNA nucleases were detected using radioactive nuclease assays (data not shown). The protein concentration was determined spectrophotometrically using an extinction coefficient of 49,500 M^{-1} cm^{-1}.[59]

Purification of SSB protein

E. coli single stranded DNA-binding protein (SSB) was purified from strain K12ΔH1Δtrp as described.[60] The concentration of purified SSB protein was determined at 280 nm using ε=30,000 M^{-1} cm^{-1}. The site size of SSB

protein was determined to be ten nucleotides per monomer by monitoring the quenching of the intrinsic fluorescence of SSB that occurs on binding to ssDNA, as described.[61]

Bacteriophage gene 32 protein

(gp32) was over expressed and purified as described.[62] The concentration of purified gp32 was determined at 280 nm using $\varepsilon = 37{,}000\ M^{-1}\ cm^{-1}$.[63] The site size of gp32 was determined to be seven nucleotides per monomer by monitoring the quenching of the intrinsic fluorescence of gp32 that occurs on binding to ssDNA, as described.[63]

ATP hydrolysis assay

Hydrolysis of ATP was monitored using a coupled spectrophotometric assay as described.[25] The conversion of ATP to ADP and P_i is linked to the oxidation of NADH to NAD^+, and is monitored as a decrease in absorbance at 340nm. Assays were performed at 37 °C for 60 min and monitored using a Varian, Cary-50 Bio UV-visible spectrophotometer. The standard reaction buffer contained 20 mM Tris–acetate (pH 7.5), 1 mM DTT, 0.3 mM NADH, 5 mM PEP, 20 units/ml pyruvate kinase, 20 units/ml lactate dehydrogenase, 1 mM ATP, 10 mM magnesium acetate, DNA (either single-stranded or double-stranded;10 μM nt of poly(dT) or ssDNA, 5 μM bp of supercoiled or linear dsDNA; or as indicated in Figure legends) and 100 nM RecG. Where present in reactions containing linear duplex or M13 ssDNA, SSB was used at a ratio of 1 monomer per 20 nucleotides, unless otherwise indicated. Wheat germ topoisomerase was used at a concentration of 0.05 unit/μl (final concentration). In experiments using the model junction substrates, magnesium ion concentration was at 8 mM for the ATP and DNA titrations and SSB was present at 1 μM and RecG was present at either 2 nM or 10 nM. The rate of ATP hydrolysis was calculated by multiplying the slope of a tangent drawn to linear portions of the time course by 159. Reactions were initiated with either ATP, RecG protein or DNA so as to keep the volume of addition small. In a typical reaction, close to 200 data points were used to draw a linear fit to the data.

In salt titration experiments, ATPase assays were carried out under standard conditions. When the steady -tate was achieved as indicated by a linear region in the time course, NaCl was added in initially 20 μM increments until ATPase activity ceased on the specific DNA cofactor present in the experiment. The total volume used to calculate final concentration of NaCl was adjusted after each addition in order to correct for the additions themselves. A line of best fit was drawn for data points between each addition to obtain the ATP hydrolysis rate after each salt increment.

Heparin trapping

These experiments were performed in one of three different ways: (1) heparin was added to an ongoing ATPase assay in which a steady-state rate of DNA-dependent ATP hydrolysis was achieved; (2) heparin was present in the initial reaction mixture prior to the addition of RecG; and (3) the reaction was initiated with a mixture of ATP and heparin, following an incubation of all components including DNA and RecG. These three procedures were used in concert to determine the minimum concentration of heparin required to rap RecG but not strip it off the DNA cofactor. The concentrations of heparin used as an effective trap for RecG were 10 μg/μl for M13 mp18 ssDNA and 1 μg/μl for linear dsDNA and (−)scDNA, respectively.

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