Biochemical Characterization of the Human RAD51 Protein

II. ADENOSINE NUCLEOTIDE BINDING AND COMPETITION*

Received for publication, October 12, 2002, and in revised form, February 7, 2002
Published, JBC Papers in Press, February 11, 2002, DOI 10.1074/jbc.M109916200

Gregory Tombline, Kang-Sup Shim, and Richard Fishel‡

From the Genetics and Molecular Biology Program, Department of Microbiology and Immunology, Kimmel Cancer Center, Thomas Jefferson University, Philadelphia, Pennsylvania 19107

RecA mediated homologous recombination requires cooperative ATP binding and hydrolysis to assume and maintain an active, extended DNA-protein (nucleoprotein) filament. Human RAD51 protein (hRAD51) lacks the magnitude of ATP-induced cooperativity and catalytic efficiency displayed by RecA. Here, we examined hRAD51 binding and ATPase inhibition pattern by ADP and ATP/adenosine 5′-O-(thiotriphosphate) (ATPγS). hRAD51 fully saturates with ATP/ATPγS regardless of DNA cofactor (Km ∼ 5 µM; 1 ATP/h hRAD51). The binding of ADP to hRAD51 appeared bimodal. The first mode was identical to ATP/ATPγS binding (Kapp1 ∼ 3 µM; 1 ADP/h hRAD51), while a second mode occurred at elevated ADP concentrations (Kapp2 ≥ 125 µM; >1 ADP/h hRAD51). We could detect ADP → ATP exchange in the high affinity ADP binding mode (Kapp1) but not the low affinity binding mode (Kapp2). At low ATP concentrations (<0.3 mM), ADP and ATPγS competitively inhibit the hRAD51 ATPase (Kapp1 > Kapp2). However, at high ATP (>0.3 mM), the hRAD51 ATPase was stimulated by concentrations of ATPγS that were 20-fold above the Km for Ammonium sulfate plus spermidine decreased the affinity of hRAD51 for ADP substantially (~10-fold) and ATP modestly (~3-fold). Our results suggest that ATP binding is not rate-limiting but that the inability to sustain an active nucleoprotein filament probably restricts the hRAD51 ATPase.

Walker A/B nucleotide binding motifs are conserved in all members of the RecA family, which includes hRAD51 and several other human homologs (1, 2). RecA and its homologs belong to a larger more distantly related family of proteins that include myosin, dynein, the F0/F1-ATPase/synthase, adenylate kinase, the gene 4 helicase/primase, utilize cooperative interactions between neighboring protomers to similarly facilitate NTP hydrolysis (7–11). In the case of the F1-ATPase, ADP release appears limited and governed by the extent of cooperative ATP binding between α and β subunits (7, 9–11).

Cooperative ATP-induced conformational transitions are essential for RecA to assume and maintain an active nucleoprotein filament (12, 13). The first step in this process, ATP binding, results in conformational transitions that precede hydrolysis (14, 15). These transitions correlate with an extended nucleoprotein filament and appear to prevent microscopic reversibility during ATP hydrolysis (16). In addition, an actively hydrolyzing nucleoprotein filament appears to remain in an extended ATP-induced conformation (17). Moreover, ADP inhibition of the RecA ATPase displays hysteresis, suggesting that an actively hydrolyzing RecA nucleoprotein filament is not in continuous equilibrium (18). Taken together, these observations are consistent with the notion that cooperativity allows RecA to sustain a threshold of ATP-bound protomers within the nucleoprotein filament (12, 13). Cooperativity between ATP-bound protomers increases the efficiency of RecA mediated ATP hydrolysis, which in turn enhances the rate of recombinational strand exchange and allows the bypass of heterologous DNA (19–23).

DNA substantially stimulates the RecA ATPase (24, 25). DNA binding also correlates with an increased affinity of RecA for ATP and a decreased affinity of RecA for ADP (26, 27). Thus, it appears that DNA facilitates cooperative ATP hydrolysis in an actively hydrolyzing RecA nucleoprotein filament by maximizing its affinity for ATP while minimizing its affinity for ADP. In contrast, stimulation of the hRAD51 ATPase by DNA was minimal (28). The difference in the catalytic efficiency between the RecA and hRAD51 ATPases could indicate that hRAD51 does not efficiently mediate ATP-binding and/or ADP release in the context of an actively hydrolyzing nucleoprotein filament.

To determine the catalytic steps that distinguish RecA and hRAD51, we have examined the affinity of hRAD51 for ADP and ATP(ADPγS). In contrast to RecA, our results suggest that hRAD51 retains a high and equivalent intrinsic affinity for ADP and ATP(ADPγS) regardless of the DNA cofactor. In ad-

* This work was supported by National Research Service Award Grant 5-T32-CA09678 (to G. T.) and National Institutes of Health Grant CA66542 (to R. F.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence and reprint requests should be addressed: Kimmel Cancer Center, BLSB933, 233 S. 10th St., Rm. 933, Philadelphia, PA 19107. Tel.: 213-503-1346; Fax: 215-923-1098; E-mail: rfishel@hendrix.jci.tju.edu.

The abbreviations used are: hRAD51, human RAD51 protein; ATPase, ATP hydrolysis activity; ssDNA, single-stranded DNA; dsDNA, double-stranded DNA; nt, nucleotides; RFI and RFIII, replicative form I and III, respectively; ATPγS, adenosine 5′-O-(thiotriphosphate).
Fig. 1. ATPγS binding by hRAD51. A, hRAD51 (0.5 μM) was incubated with the indicated amount of ATPγS and, when added, 6 μM DNA (nt ssDNA or bp dsDNA) in 10 μl for 15 min at 37°C. Each reaction was then placed on ice for a minimum of 15 min or until filtered. Each reaction was then mixed with 4 ml of binding buffer and filtered through a nitrocellulose filter that had been presoaked with 4 ml of the same buffer. Each filter was subsequently washed with an additional 4 ml, allowed to air-dry, and scintillation-counted. B, kinetics of ATPγS binding by hRAD51. hRAD51 (2.0 μM) was incubated with 30 μM ATPγS and, when added, 18 μM DNA (nt of ssDNA or bp of dsDNA) in 10 μl for the indicated amount of time at 37°C and then directly filtered and processed as above without further incubation on ice. Each point represents the average of three experiments. B, the S.D. for each point shown is within the size of the symbols. ●, the addition of ssDNA; □, the addition of dsDNA (RFI); ○, reactions performed in the absence of DNA.

![Graph A](image)

![Graph B](image)

**Table I** Summary of nucleotide binding data for hRAD51

|        | ssDNA | dsDNA (RFI) | No DNA |
|--------|-------|-------------|--------|
| Kd_{ATP}\_γS (μM) | 3.5 ± 0.4 | 4.4 ± 0.5 | 3.5 ± 0.3 |
| K_{app1\_ADP} (μM) | 3.5 ± 1.0 | 3.1 ± 0.9 | 3.6 ± 1.2 |
| K_{app2\_ADP} (μM) | ~125–150 | ~125–150 | ~125–150 |
| ATPγS/hRAD51 | 1:1 | 1:1 | 1:1 |
| ADP/hRAD51 \_K_{app1} | >1:1 | >1:1 | >1:1 |
| ADP/hRAD51 \_K_{app2} (μM) with (NH4)_2SO_4 | 9.6 ± 1.3 | 13.5 ± 1.4 | ND* |

* These values were not determined.

dition, we have evaluated the ability of hRAD51 to release the ADP hydrolysis product and rebind ATP (ADP → ATP exchange) as well as the pattern of ATPase inhibition by ADP and ATPγS. Our results suggest that ATP binding does not limit the hRAD51 ATPase. Moreover, we find an enhanced ATPase activity at elevated ATP/ATPγS ratios. These results are consistent with the notion that hRAD51 protomers within the nucleoprotein filament may be unable to sustain a threshold ATP saturation necessary for cooperative ATP hydrolysis. This inability to assume and maintain an active nucleoprotein filament appears to provide at least a partial explanation for the distinct recombinational strand exchange activity and dramatically reduced ability to bypass heterologous DNA during strand exchange observed with purified RAD51 (29–32).

**Fig. 2. ADP binding by hRAD51.** hRAD51 (0.5 μM) was incubated with the indicated amount of ADP and, when added, 6 μM DNA (nt of ssDNA or bp of dsDNA) in 10 μl for 60 min at 37°C. For A and B, each reaction was then placed on ice for a minimum of 15 min or until filtered. Each reaction was then mixed with 4 ml of binding buffer and filtered through a nitrocellulose filter that had been presoaked with 4 ml of the same buffer. Each filter was subsequently washed with an additional 4 ml, allowed to air-dry, and scintillation-counted. C, 3.0 μM hRAD51 was incubated with 500 μM ADP and, when added, 18 μM DNA. These otherwise identical reactions were kept at 37°C until the indicated time and then directly filtered without further incubation on ice. Each point represents the average of three experiments, and S.D. is shown for each point or is within the size of the symbol. ●, the addition of ssDNA; □, the addition of dsDNA (RFI); ○, reactions performed in the absence of DNA.

**Experimental Procedures**

Materials—Chemicals were ultrapure grade (Amresco, Solon, OH) or of the highest grade available. ATP and ADP were purchased from Amersham Biosciences, and ATPγS was purchased from Roche Molecular Biochemicals and examined for purity by TLC. All radiochemicals were purchased from PerkinElmer Life Sciences/Applied Biosystems. hRAD51 was purified as previously described (28).

Nucleotide Binding—Binding was performed in 10-μl reactions in a buffer (20 mM HEPES (pH 7.5), 150 mM NaCl, 10% glycerol, 1 mM dithiothreitol, 2 mM magnesium acetate) containing 0.5 or 1.0 μM hRAD51, 6 μM nucleotides of DNA, if present, and the indicated amounts of ATPγS or ADP. Reactions were supplemented with either 4 μM [γ-32P]ATP or 2 μM [3H]ADP. Reactions were incubated at 37°C for 15 min for ATPγS binding or 60 min for ADP binding and then placed on ice for a minimum of 20 min or until filtered. 100 μl of ice-cold A buffer was added to the 10-μl reaction mix and mixed by pipetting once. This 110 μl was quickly removed and added to 4 ml of ice-cold A buffer and filtered through HAWP nitrocellulose filters (Millipore Corp.), which had been presoaked with 4 ml of A buffer. Each filter was washed with an additional 4 ml of A buffer, dried, and placed in 5 ml of
hRAD51 Nucleotide Binding/Exchange

Fig. 3. ATP binding by hRAD51 determined by UV-cross-linking analysis. hRAD51 (0.5 μM), 6 μM nt of ssDNA (when added) and 0.5 μM [α-32P]ATP were preincubated at room temperature for 20 min in 96-well polypropylene dishes. The plate was placed on ice, and hRAD51 was cross-linked to [α-32P]ATP for the indicated amount of time (A and B) by UV irradiation (254 nm). A, the [α-32P]ATP-hRAD51 complex was resolved by 10% SDS-PAGE, and the signal intensity was quantitated with a PhosphorImager (B). Unlabeled ATP (C) or ADP (D) was added as a competitor to determine an IC50 value (or relative Kd). Cross-linking for B and C was performed for 10 min. ○, cross-linking performed in the presence of ssDNA; □, reactions performed in the absence of DNA.

Scintiverse (Fisher) and allowed to soak overnight before counting. hRAD51 was omitted from control reactions that contained either the lowest or highest amount of ATP or ADP. After filtration, background binding was averaged and subtracted from the reactions that contained hRAD51, yielding an adjusted binding value (cpm). Specific activity of ATPS or ADP (cpm/mol; [3H]ATP or [35S]ATP) in each reaction was calculated by dividing the total counts in each reaction by the total amount of ATP or ADP. The amount of ATPS or ADP bound (mol) in each reaction was determined by dividing the adjusted binding value (cpm) by the specific activity of ATP or ADP in each reaction (cpm/mol ATP). In the absence of hRAD51, we observed no significant difference in the background binding at the lowest and highest ATP or ADP concentrations. The retention of hRAD51-ADP or hRAD51-ATP5S complexes was found to vary linearly within the range of 0.5–3 μM hRAD51 (data not shown). These observations indicate that the amount of hRAD51 used in these assays did not exceed the capacity of the HAWP filters. For nucleotide binding reactions containing additional salts, 0.5 μl of 2 M (NH4)2SO4 (pH 8.0) and/or 0.5 μl of 80 mM spermidine HCl were added to reactions with a final volume of 10 μl, yielding final concentrations of 100 mM and/or 4 mM, respectively. These reactions were subsequently processed in an identical manner as above. In all reactions, conditions were normalized for the DNA or hRAD51 storage buffers.

UV Cross-linking—These 10-μl reactions were identical to those used for binding and hydrolysis of ATP except that each reaction contained 0.5 μM [α-32P]ATP (30 Ci/mmol). If competitor was present, it was added in the initial mix before hRAD51 was added. Reactions were performed in a 96-well polypropylene microtiter plate (Nalge). After incubating at 25 °C for 15 min, the plate was placed on ice and irradiated at 254 nm in a Stratalinker (Stratagene) ~2 cm from the source. At the indicated time, or after 10 min for the competition reactions, 3 μl of 4× sample loading buffer was added (0.2 M Tris-HCl (pH 7.0), 8% SDS, 20% β-mercaptoethanol, 20% sucrose, 2 mg/ml bromophenol blue), the reactions were boiled, and 10% SDS-PAGE. The gels were dried, the lower portion containing free [α-32P]ATP was removed, and the top portion was quantitated with a PhosphorImager (Molecular Dynamics, Inc., Sunnyvale, CA).

ADP → ATP Exchange—ADP → ATP exchange was analyzed as previously described for the hMSH2/hMSH6 heterodimer (33) with minor modifications allowing adaptation to the hRAD51 system. Briefly, parallel ADP binding reactions (10 μl) were performed as described above using a saturating (20 μM) or supersaturating (200 μM) amount of ADP ([3H]ADP in A buffer for 60 min at 37 °C, at which point they were removed and kept at room temperature. Zero time points were taken at the beginning and end of a series (the latter to control for variation in the time required to perform the experiment) and were filtered as for nucleotide binding. To initiate ADP → ATP exchange, 10 μl containing A buffer supplemented with 5 mM ATP was added to the reaction. At the indicated time, the reaction was mixed with 4 ml of A buffer and filtered as in nucleotide binding. Effect of ADP and ATPγS on the hRAD51 ATPase—ATP hydrolysis was performed as previously described (28) with the exception that either 50 or 100 μM of ADP or ATPγS was included in each reaction prior to the addition of protein.

RESULTS

Nucleotide Binding—Our previous studies showed that the hRAD51 ATPase did not display the magnitude of ATP-induced cooperativity that is a hallmark of RecA (28). DNA enhanced the apparent affinity of hRAD51 for ATP (Km ~ 20 μM) in the presence of DNA compared with a Kd ~ 80–120 μM in the absence of DNA) but only enhanced the catalytic efficiency (kcat/Km) ~ 2–4-fold (28). In order to examine the fractional saturation of hRAD51 with ATP, we initially determined its binding affinity for ATPγS (Kd ~ 4 μM; Fig. 1, Table I). This binding affinity was independent of added DNA cofactor (Fig. 1A, Table I). The binding of ATPγS to hRAD51 does not appear cooperative, and hRAD51 becomes fully saturated with ATPγS...
The kinetics of ATP\(^3\) high affinity binding mode (Fig. 4A) compared with ATP\(\gamma\)S\(\gamma\)S (Fig. 1). In the initial mode, hRAD51 displayed equimolar saturation (1 ADP: 1 hRAD51) and exhibited an equivalent affinity for ADP (\(K_{app} \sim 3 \mu M\)) compared with ATP\(\gamma\)S (\(K_D \sim 4 \mu M\); Fig. 1). In the second mode, hRAD51 exhibited a lower affinity for ADP (\(K_{app} \geq 125 \mu M\)) and appeared supersaturated (>1 ADP: 1 hRAD51; Fig. 2B). The supersaturation of hRAD51 suggests that the \(K_{app}\) must \textit{a priori} be representative of multiple binding events and/or complex trapping of ADP. Saturation in the initial mode was identical to ATP binding (Fig. 2C; data not shown). In contrast, binding in the second mode occurred slowly and linearly with time and did not appear to saturate (Fig. 2C). DNA modestly enhanced the affinity of hRAD51 for ADP in the initial mode (Fig. 2A). DNA had a complex effect on the second mode of ADP binding by hRAD51 (Fig. 2C). While hRAD51 in the absence of DNA or in the presence of ssDNA continued to bind ADP over the course of 10 h, dsDNA appeared modestly competitive at time points that exceeded 1 h. Regardless, the lack of saturation by the second mode suggests that it is nonspecific. It is likely that this second mode may only be important for \textit{in vitro} studies. For example, during strand exchange assays, incubations may continue for 2 h in the presence of adenosine nucleotide.

To confirm the hRAD51 binding affinity for ADP, ATP, and ATP\(\gamma\)S, we employed photocross-linking experiments similar to those performed with RecA (34, 35). We found that ATP can be directly cross-linked to hRAD51, and this occurs linearly with time (Fig. 3, A and B). The photocross-linking of ATP displays a strict requirement for magnesium (data not shown). We determined the IC\(_{50}\) for the inhibition of ATP photocross-linking using unlabeled ATP (IC\(_{50} \sim 5 \mu M\); ATP; Fig. 3C). ADP similarly inhibited ATP photocross-linking (IC\(_{50} \sim 5 \mu M\); ATP; Fig. 3D). The presence of ssDNA did not alter the IC\(_{50}\) for ATP or ADP (Fig. 3, C and D). In addition, AMP appeared incapable of inhibiting ATP photocross-linking (data not shown). These latter results suggest that a \(\beta\)-phosphate is minimally required for competition of ATP photocross-linking to hRAD51. The correlation between the \(K_D\) or \(K_{app}\) for ATP\(\gamma\)S and ADP determined by filter binding with the IC\(_{50}\) for ATP and ADP competition determined by photocross-linking confirms the equivalent high affinity of hRAD51 for both ATP and ADP (\(K_D \sim 3–5 \mu M\)).

\textit{ADP \rightarrow ATPExchange}—An apparent lack of microscopic reversibility during RecA-mediated ATP hydrolysis is consistent with the idea that the exchange of ADP \rightarrow ATP is not rate-limiting. To examine ADP \rightarrow ATP exchange by hRAD51, we followed the release of ADP from hRAD51 preincubated with 20 \(\mu M\) ADP and from the low affinity ADP binding mode (\(K_{app} \sim 3 \mu M\)) using hRAD51 preincubated with 20 \(\mu M\) ADP. ADP \rightarrow ATP exchange appears slow (\(t_{1/2} \sim 30\) s) from the high affinity binding mode (Fig. 4A). Neither ssDNA nor dsDNA appeared to affect the rate of ADP \rightarrow ATP exchange by the high affinity binding mode. However, a significant fraction of ADP remained refractory to ADP \rightarrow ATP exchange in the presence of DNA. hRAD51 failed to exchange ADP \rightarrow ATP from the low affinity binding mode regardless of added DNA (Fig. 4B). These results suggest that the efficient exchange of ADP \rightarrow ATP may be rate-limiting for the hRAD51 ATPase. Moreover, ADP binding by the low affinity mode appears irreversible.

In the presence of ssDNA, ADP (50 \(\mu M\)) and ATP\(\gamma\)S (50 \(\mu M\)) appeared strictly competitive (\(K_{m(app)(50)} > K_m; V_{m(app)(50)} < V_{max}\); Fig. 5, A and B, and Table II). However, at high ATP concentration (>0.3 mM), the effect of ADP and ATP\(\gamma\)S on \(V_{max}\) displayed a mixed inhibition pattern that was dependent on the DNA cofactor. In the presence of ssDNA, low ADP (50 \(\mu M\)) appeared strictly competitive (\(K_{m(app)(50)} > K_m; V_{m(app)(50)} < V_{max}\); Fig. 5, A and B, and Table II). While a decrease in both the \(K_m\) and \(V_{max}\) would generally suggest uncompetitive inhibition, in this case the decreases were not proportional and therefore could not be easily classified. Comparison of the inhibition patterns between 50 and 100 \(\mu M\) ADP suggests that they may be related by noncompetitive inhibition (\(K_{m(app)(50)} > K_m; V_{m(app)(50)} < V_{max}\); Table II). These results are consistent with a model for hRAD51 ATP binding at multiple and/or nonspecific modes as suggested by the ADP binding studies (Fig. 2).

In the presence of dsDNA, ADP (50 \(\mu M\) and 100 \(\mu M\)) appeared to modestly stimulate the hRAD51 ATPase (\(V_{m(app)} \geq V_{max}\); Fig. 5, C and D, and Table II). Similarly, in the presence of ssDNA, ATP\(\gamma\)S (50 and 100 \(\mu M\)) also appeared to modestly stimulate the hRAD51 ATPase (\(V_{m(app)} \geq V_{max}\); Fig. 6, A and B). These meager stimulatory effects should be contrasted with the significant enhancement of the hRAD51 ATPase induced by...
ATPγS (50 and 100 μM) in the presence of dsDNA (V_{max}; Fig. 6, C and D, and Table II). These results are consistent with the conclusion that a sustained fraction of ATP-bound protomers within the hRAD51 dsDNA nucleoprotein filament may be capable of enhancing the ATPase activity.

The Effect of Ammonium Sulfate and Spermidine on the Binding of ATPγS and ADP to hRAD51—We found that ammonium sulfate reduced the apparent affinity (increased $K_m$) of hRAD51 for ATP 3–4-fold (28). To determine whether this effect was the result of a decreased binding affinity, we measured the binding constant ($K_D$) of hRAD51 for ATPγS under these conditions. Consistent with the increase in $K_m$, the $K_D$ for ATPγS binding also increased ~3-fold in the presence of ammonium sulfate and spermidine (Fig. 7A; Table I). In comparison, ammonium sulfate and spermidine increased the $K_{app}^D$ of hRAD51 for ADP ~10-fold. The de novo induction of a 3-fold difference between the binding affinity of hRAD51 for ADP and ATP begins to approach the 25-fold difference displayed by RecA (12, 27).

**DISCUSSION**

The hRAD51 ATPase displays a 50-fold reduced catalytic efficiency compared with the bacterial RecA ATPase (28). hRAD51 also lacks the magnitude of ATP-induced cooperativity displayed by RecA, suggesting that hRAD51 may be limited in its ability to overcome a rate-limiting step(s) in the ATPase cycle. In an effort to determine the rate-limiting step(s) of the hRAD51 ATPase, we have characterized hRAD51 binding to ATP (ATPγS) and ADP, the ability to release the ADP hydrolysis product (ADP $\rightarrow$ ATP exchange), and the effect of ADP and ATPγS on the hRAD51 ATPase.

We found that hRAD51 fully and rapidly saturates with ATPγS with high affinity ($K_D$ ~ 3–5 μM). This rapid binding and high affinity for ATP suggests that ATP binding is unlikely to be the rate-limiting step in the hRAD51 ATP hydrolytic cycle. The binding of ATPγS by hRAD51 is also independent of DNA. In comparison, DNA enhances the affinity of both RecA and yeast RAD51 for ATP (26, 36). These results are consistent
with the notion that hRAD51 displays an elevated intrinsic affinity for ATP compared with the bacterial and yeast RecA homologs.

The binding of ADP by hRAD51 is complex and bimodal. In the first mode, hRAD51 fully saturates with ADP at an apparently equivalent affinity compared with ATP/ATP\gamma S (K_{app1} \approx 3 μM). This baseline saturation continues until concentrations of ADP exceed 50 μM where an additional mode becomes apparent (K_{app2} \approx 125 μM). The second mode of ADP binding appears slow and increases linearly with time, suggesting that the low affinity interaction(s) requires the formation of additional hRAD51 structure/aggregate(s). While ADP exchange can be measured in the initial binding mode (K_{app1}), a large fraction of ADP remained bound in the presence of DNA. This suggests that reduced ADP exchange may limit the catalytic efficiency of the hRAD51 ATPase. We have also demonstrated that saturating ADP (K_{app1}) appears competitive for the hRAD51 ATPase. These results suggest that both ADP and ATP/ATP\gamma S utilize the same binding site in the initial binding mode. The second binding mode (K_{app2}) is refractory to ADP exchange and appears to display noncompetitive inhibition when compared with the initial binding mode (K_{app1}).

Taken together, these results suggest that ADP may interact and/or be trapped by hRAD51. Moreover, ADP → ATP exchange appears to depend on the extent of ADP saturation. These and additional studies presented in the following paper

**TABLE II**

Summary of ADP and ATP\gamma S inhibition data for hRAD51

| Condition                  | K_m or K_{app} | V_max   |
|----------------------------|----------------|---------|
| ssDNA ATPase               | 23 ± 3         | 0.211 ± 0.005 |
| ssDNA ATPase + 50 μM ADP   | 48 ± 2         | 0.260 ± 0.003 |
| ssDNA ATPase + 100 μM ADP  | 49 ± 10        | 0.146 ± 0.008 |
| ssDNA ATPase + 50 μM ATP\gamma S | 176 ± 25     | 0.293 ± 0.018 |
| ssDNA ATPase + 100 μM ATP\gamma S | 247 ± 26    | 0.295 ± 0.015 |
| dsDNA ATPase               | 27 ± 4         | 0.114 ± 0.003 |
| dsDNA ATPase + 50 μM ADP   | 57 ± 6         | 0.127 ± 0.005 |
| dsDNA ATPase + 100 μM ADP  | 146 ± 18       | 0.165 ± 0.008 |
| dsDNA ATPase + 50 μM ATP\gamma S | 780 ± 188    | 0.396 ± 0.068 |
| dsDNA ATPase + 100 μM ATP\gamma S | 860 ± 168   | 0.407 ± 0.058 |

**FIG. 6.** ATP\gamma S inhibition of hRAD51 ATPase activity. Each ATPase reaction contained 1.0 μM hRAD51, 6 μM (nt or bp) either ssDNA (A and B) or dsDNA (RFI) (C and D), and the indicated amount of ATP. Reactions were incubated at 37 °C for 1 h and processed by the Norit method. Either 50 μM ATP\gamma S (○) or 100 μM ATP\gamma S (□) was added as a competitor at the beginning of the reaction prior to the addition of hRAD51. Each point represents the average of three experiments. *, reactions performed in the absence of competitor. Data for A and C were fit to the Michaelis-Menten equation, whereas B and D were fit to a linear slope and extrapolated to the x axis. In B and D, insets indicate the points of intersection with x and y axes.
ATP were performed by incubating 0.5 μM hRAD51 with the indicated amount of ATP3S (A) or 1.0 μM hRAD51 with the indicated amount of ADP (B) and either 6 μM (nt) ssDNA (circles) or 6 μM (bp) dsDNA (squares). Each reaction was incubated at 37 °C for 20 min (ATP3S) or 1 h (ADP). Each reaction was then placed on ice for a minimum of 15 min or until filtered. Each reaction was washed with an additional 4 ml, allowed to air-dry, soaked overnight in 5.0 ml of Scintiverse mixture (Fisher), and scintillation-counted. Each point represents the average of three experiments.

(37) are consistent with the notion that elevated ADP induces an irreversible aggregate structure that may only be relevant to in vitro studies.

At elevated DNA concentrations, the Km for the hRAD51 steady-state ATPase approaches the Km for ATP3S binding (for Km analysis see Ref. 28). This contrasts with the RecA protein, where the S0.5 remains 10–20-fold higher than the Km at elevated DNA concentrations. A consistent difference between the Km and Km at elevated DNA concentrations is that hRAD51 may not form a cooperative ATP saturated nucleoprotein filament similar to RecA. Consistent with this notion, elevated levels of ATP3S that would normally inhibit the RecA ATPase (Vmax) (15) appear to stimulate the hRAD51 ATPase (Vmax). In this case, the slowly hydrolyzing ATP3S may artificially allow hRAD51 to mimic a nucleoprotein filament that maintains a minimal saturation with ATP/ATP3S. In the presence of ATP alone, hRAD51 appears unable to sustain a minimal number of ATP-bound protomers within the nucleoprotein filament. In addition, the equivalent binding affinity for ADP and ATP/ATP3S as well as the lack of ADP → ATP exchange probably contributes to a breakdown in the hRAD51 ATPase cycle that is ultimately manifest in a 50-fold reduction in catalytic efficiency compared with RecA.

Ammonium sulfate appears to induce an activated hRAD51 filament and thereby increases the efficiency of strand exchange (32, 38). Our studies suggest that ammonium sulfate allows hRAD51 to discriminate between ATP and ADP while decreasing its catalytic efficiency. Perhaps ammonium sulfate maintains a minimal number of ATP-bound protomers within the actively hydrolyzing hRAD51 nucleoprotein filament. Alternatively, although nonexclusively, ammonium sulfate may maintain a minimal number of protomers within the hRAD51 nucleoprotein filament in a pseudotransition state bound by ADP-SO42− (see Ref. 39 for precedent) (28). It is important to note that RAD51 displays a dramatically reduced ability to bypass heterologous DNA during strand exchange (29–32). Since bypass of heterologous DNA by RecA requires ATP hydrolysis (19–23), it is tempting to speculate that enhanced ATP hydrolysis by hRAD51 will be required to efficiently bypass heterologies and perform cellular recombination events (40).

Insight into the ATPase mechanism of RecA and hRAD51 may be gained by a comparison with the hexameric F1-ATPase. The F1-ATPase exhibits two modes of ATP hydrolysis that are dependent on ATP concentration: unisite mode (low ATP) and multisite mode (high ATP) (7, 9–11). ATP hydrolysis is inefficient in the unisite mode, because it lacks ATP-induced cooperativity, exhibits poor release of the ADP hydrolysis product (ADP → ATP exchange) and is reversible (microscopic reversibility). The multisite mode is at least 105-fold more efficient than the unisite mode, since multiple subunits are bound with ATP. This results in ATP-induced cooperativity, efficient release of the ADP hydrolysis product (ADP → ATP exchange), and corresponding absence of reversibility. RecA-promoted ATP hydrolysis appears to resemble the multisite mode of the F1-ATPase. In contrast, the hRAD51 ATPase appears to resemble the unisite mode of the F1-ATPase. A multisite mode implies an ordered sequence of ATP hydrolysis that is likely to drive efficient recombinational strand exchange (7, 41). In an extension of this analogy, hRAD51 protomers may more closely resemble the multisite mode in the presence of the modest amounts of ATP3S that enhance hydrolysis. These observations are consistent with the notion that a modicum of cooperative behavior between hRAD51 protomers may be induced by artificially altering the biochemical conditions. However, it is likely that a complete transition to the multisite mode by hRAD51 will require additional factors that enhance protomer cooperativity.

We consider three biochemical junctures at which additional factor(s) may increase the efficiency of ATP hydrolysis and consequent recombinational strand exchange by hRAD51. First, these factors may sustain ATP-bound hRAD51 protomers within a nucleoprotein filament. Second, they may provoke efficient and rapid exchange of ADP → ATP. Third, such factors may supply an accessory trans-catalytic “arginine finger,” which is predicted in RecA and appears to be missing in hRAD51 (42, 43). An essential trans-catalytic trans-arginine finger has been identified in the α-subunit of the F1-ATPase (Arg157) (44), the T7 gp41 hexameric helicase (Arg522) (8), and the pRSF1010 RepA hexameric helicase (Arg207) (45). Candidates for such factors would be one or several of the other known human RecA homologs.

Acknowledgments—We thank Pete Von Hippel, Christoph Schmutte, Chris Heinen, Samir Acharya, and Kristine Yoder for helpful discussions and careful review of the manuscript.

REFERENCES
1. Walker, J. E., Saraste, M., Runswick, M. J., and Gay, N. J. (1982) EMBO J. 1, 945–951
2. Thompson, L. H., and Schld, D. (1999) Biochimie (Paris) 81, 87–105
3. Vale, R. D. (1996) J. Cell Biol. 135, 291–302
4. Fishel, R. (1998) Genes Dev. 12, 2096–2101
5. Schellfre, K., Ahmadian, M. R., and Wittinghofer, A. (1998) Trends Biochem. Sci. 23, 257–262
6. Quilliam, L. A., Khosravi Far, R., Huff, S. Y., and Der, C. K. (1995) *Bioessays* 17, 395–404.
7. Boyer, P. D. (1997) *Annu. Rev. Biochem.* 66, 717–749.
8. Sawaya, M. R., Guo, S., Tabor, S., Richardson, C. C., and Ellenberger, T. (1999) *Cell* 99, 167–177.
9. Cross, R. L., Grubmeyer, C., and Penefsky, H. S. (1982) *J. Biol. Chem.* 257, 12101–12105.
10. Penefsky, H. S., and Cross, R. L. (1991) *Adv. Enzymol. Relat. Areas Mol. Biol.* 64, 173–214.
11. Kowalczykowski, S. C. (1991) *Annu. Rev. Biophys. Biophys. Chem.* 20, 539–575.
12. Roca, A. I., and Cox, M. M. (1997) *Prog. Nucleic Acid Res. Mol. Biol.* 56, 129–223.
13. Stole, E., and Bryant, F. R. (1997) *Biochemistry* 36, 3483–3490.
14. Paulus, B. F., and Bryant, F. R. (1997) *Biochemistry* 36, 7832–7838.
15. Cox, M. M., Soltis, D. A., Lehman, I. R., DeBrosse, C., and Benkovic, S. J. (1983) *J. Biol. Chem.* 258, 2586–2592.
16. Yu, X., and Epelman, E. H. (1992) *J. Mol. Biol.* 225, 193–216.
17. Lee, J. W., and Cox, M. M. (1990) *Biochemistry* 29, 7677–7683.
18. Rosselli, W., and Stasiak, A. (1991) *EMBO J.* 10, 4391–4396.
19. Kim, J. I., Cox, M. M., and Inman, R. B. (1992) *J. Biol. Chem.* 267, 16438–16443.
20. Shan, Q., Cox, M. M., and Inman, R. B. (1996) *J. Biol. Chem.* 271, 5712–5724.
21. MacFarland, K. J., Shan, Q., Inman, R. B., and Cox, M. M. (1997) *J. Biol. Chem.* 272, 17675–17685.
22. Weinstock, G. M., McEntee, K., and Lehman, I. R. (1981) *J. Biol. Chem.* 256, 8845–8849.
23. Gradia, S., Acharya, S., and Fishel, R. (1997) *Cell* 91, 995–1005.
24. Banks, G. R., and Sedgwick, S. G. (1986) *Biochemistry* 25, 5882–5889.
25. Kelley, J. A., and Knight, K. L. (1997) *J. Biol. Chem.* 272, 25778–25782.
26. Weinstock, G. M., McEntee, K., and Lehman, I. R. (1981) *J. Biol. Chem.* 256, 8850–8855.
27. Cotterill, S. M., Satterwait, A. C., and Fersht, A. R. (1995) *Biochemistry* 24, 4332–4337.
28. Holmes, V. F., Benjamin, K. R., Crisona, N. J., and Cozzarelli, N. R. (2001) *Nucleic Acids Res.* 29, 5052–5057.
29. Sung, P., and Robberson, D. L. (1995) *Cell* 82, 453–461.
30. Namsaraev, E. A., and Berg, P. (2000) *J. Biol. Chem.* 275, 3970–3976.
31. Yu, X., and Egelman, E. H. (1992) *J. Mol. Biol.* 225, 193–216.
32. Rice, K. P., Egger, A. L., Sung, P., and Cox, M. M. (2001) *J. Biol. Chem.* 276, 38570–38571.
33. Cotterill, S. M., Satterhwait, A. C., and Fersht, A. R. (1982) *Biochemistry* 21, 4332–4337.
34. Tombline, G., Heinen, C. D., Shim, K. S., and Fishel, R. (2002) *J. Biol. Chem.* 277, 14434–14442.
35. Menz, R. I., Walker, J. E., and Leslie, A. G. (2001) *Cell* 106, 331–341.
36. Moriison, C., Shinohara, A., Sonoda, E., Yamaguchi-Iwai, Y., Takata, M., Weichselbaum, R. R., and Takeda, S. (1999) *Mol. Cell. Biol.* 19, 6891–6897.
37. Niedenzu, T., Roleke, D., Bains, G., Scherzinger, E., and Saenger, W. (2001) *J. Biol. Chem.* 276, 4791–4800.
38. Sigurdsson, S., Trujillo, K., Song, B., Stratton, S., and Sung, P. (2001) *J. Biol. Chem.* 276, 8798–8806.
39. Horton, K., Voloshin, O. N., Kinal, H. H., Ma, N., Schaffer-Judge, C., and Camerini-Otero, R. D. (1999) *J. Mol. Biol.* 290, 599–610.