Interaction of Rad51 with ATP and Mg^{2+} Induces a Conformational Change in Rad51†

Eugeni A. Namsaraev and Paul Berg*

Department of Biochemistry, Beckman Center for Molecular and Genetic Medicine, School of Medicine, Stanford University, Stanford, California 94305

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ABSTRACT: The presumptive first step in the Rad51-promoted formation of joint molecules is binding of the protein to ssDNA in the presence of ATP and Mg^{2+}. In this paper, we report that Rad51's ability to bind DNA is rapidly inactivated when incubated at 30−37 °C but is stabilized by the presence of ATP and Mg^{2+}. Although unable to promote binding to DNA, ATP-γ-S also prevents inactivation of Rad51 at 37 °C. AMP-P-N-P lacks this property, while ADP protects partially but only at 5−10 times higher concentrations than ATP. These observations correlate with the dissociation constant of those nucleotides for Rad51 determined by equilibrium dialysis. Rad51 binds ATP and ATP-γ-S with a 1:1 stoichiometry and K_ds of 21 and 19 μM, respectively. The presence of DNA significantly increases the affinity of Rad51 for ATP, while DNA has a smaller effect on the affinity of ATP-γ-S. Competition binding studies show that ADP and AMP-P-N-P bind with a 5- and 55-fold lower affinity, respectively, than ATP. The CD spectrum of Rad51 with negative double minima at around 210 and 222 nm is characteristic of an α-helical protein. Upon binding ATP and Mg^{2+}, the CD spectrum is altered in the regions 194−208 and 208−235 nm, changes that are indicative of a more structured state; this change does not occur with Rad51 that has been inactivated at 37 °C. We surmise that the active conformation is more resistant to inactivation at elevated temperature. Our data suggest that one of the roles of ATP and Mg^{2+} in Rad51-mediated strand exchange is to induce the proper protein structure for binding the two DNA substrates.

The *Saccharomyces cerevisiae* Rad51 protein, like RecA, whose sequence it resembles (*1*−*3*), catalyzes the formation of joint molecules and the fully strand-exchanged product from circular single-stranded DNA (ssDNA)[1] and linear double-stranded DNA (dsDNA). Both reactions require ATP, Mg^{2+}, and a single-stranded DNA binding protein, the yeast replicative protein A (RPA) being most efficient for the reaction catalyzed by Rad51 (*4*−*7*).

Recently, we reported that Rad51 binds ssDNA and dsDNA only in the presence of ATP and that neither ADP nor the nonhydrolyzable ATP analogues, ATP-γ-S and AMP-P-N-P, can substitute (*8*). ATP promotes activation of Rad51 binding to DNA, while ADP promotes dissociation (*8*). Despite the similarity of Rad51 and RecA in their requirement for ATP, the two proteins differ in their response to ATP-γ-S. RecA forms a more stable complex with DNA in the presence of ATP-γ-S than with ATP (*9*−*11*). By contrast, ATP-γ-S fails to promote efficient binding of Rad51 to DNA but causes dissociation of the protein from DNA (*8*).

To understand the role of ATP in Rad51-promoted joint molecule formation and complete strand exchange, we have investigated how ATP binding affects the protein. The first clue that ATP binding to Rad51 may alter its structure was the finding that the interaction of Rad51 with ATP and Mg^{2+} protects the protein from inactivation at 37 and 30 °C. Unexpectedly, ATP-γ-S, which is unable to promote Rad51 binding to DNA, is as effective as ATP in protecting Rad51 against temperature inactivation. By contrast, ADP protects Rad51 poorly and only at 5 to 10 times the concentration at which ATP and ATP-γ-S are effective, while AMP-P-N-P is completely inactive.

To determine if the response to the different nucleotides is related to their affinity for Rad51, we measured their binding constants (K_d) by equilibrium dialysis. ATP and ATP-γ-S in the presence of Mg^{2+} have nearly the same binding constants for Rad51, and both K_ds are lowered in the presence of single-stranded DNA although the affinity of ATP-γ-S is less affected by DNA than is ATP. ADP and AMP-P-N-P bind Rad51 about 5 and 55 times respectively more weakly than ATP.

A more direct indication that the interaction of Rad51 with ATP influences its structure was obtained by measurement of the circular dichroism in the presence and absence of ATP and Mg^{2+}. Changes in the CD spectra indicate that the binding of ATP and Mg^{2+} induces an alteration of Rad51's secondary structure. We surmise that the effect of ATP−

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* Author to whom correspondence should be addressed at Beckman Center, B062, Stanford University School of Medicine, Stanford, CA 94305-5425. Phone: (650) 723-6170. Fax: (650) 725-4951. E-mail: pberg@cmgm.stanford.edu.

[1] Abbreviations: RPA, yeast single-stranded DNA binding protein and replication protein A; ssDNA, single-stranded DNA; dsDNA, double-stranded DNA; ϵDNA, 1, N^6-etheno-adenosine and 3, N^4-etheno-cytidine ssDNA; Mes, 2-[*N*-morpholino]ethanesulfonic acid; ATP-γ-S, adenosine 5′-*O*-thiotriphosphate; AMP-P-N-P, adenylyl-imidodiphosphate.

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Mg^{2+} in stabilizing Rad51 at elevated temperature and promoting a structural alteration evidenced by the altered CD spectrum reflects the ability of ATP−Mg^{2+} to promote the proper conformation required for Rad51's catalytic activity.

EXPERIMENTAL PROCEDURES

Proteins, DNA, and Buffers. Yeast Rad51 protein was purified from insect cells as described previously (*6*). The concentration of Rad51 was determined using an extinction coefficient (determined from its amino acid composition) of 1.26×10^4 at 280 nm. RPA was expressed in *Escherichia coli* using the plasmid pJM126 (provided by S. Brill and B. Stillman) and was purified as described (*12*). Linear DNA was prepared by digestion of pBluscriptSK(+) dsDNA with the *Pst*I restriction endonuclease. Single-and double-stranded DNA and ϵDNA were prepared as described (*13*, *14*). The concentration of DNA is expressed as nucleotide equivalents. Poly(dT), ATP, and ADP were obtained from Pharmacia Biotech Inc., and ATP-γ-S and AMP-P-N-P from Boehringer Mannheim. [α^{32}P]ATP and [^{35}S]ATP-γ-S were purchased from NEN Life Science Products. Unless otherwise noted, the standard reaction buffer used in the binding experiments contained 40 mM K-Mes (pH 6.5), 4 mM $MgCl_2$, 1 mM DTT, and 5% glycerol. Where indicated, Rad51 was inactivated by incubation without ATP in the standard buffer at 37 °C for 9 min. The buffer used in the circular dichroism measurements contained 5 mM KH_2PO_4 (pH 7.2), 4 mM $MgCl_2$, and 5% glycerol. For some experiments, ATP was added to 0.1 mM, and in others Mg^{2+} was removed and EDTA added to 0.1 mM.

Strand Exchange. Strand exchange was measured with the agarose gel assay described previously (*6*). To assess the effect of ATP, 5.6 μM Rad51 was incubated for 8 min in the presence of 2 mM $MgCl_2$ with or without 0.5 mM ATP; then 20 μM of ϕX 174 ssDNA, 0.7 μM yeast RPA, and 0.5 mM ATP were added (where ATP had been omitted). After an additional 10 min, the reaction was started by the addition of 20 μM linear ϕX 174 dsDNA and 12 mM $MgCl_2$ (final concentrations).

DNA Binding Methods. The conditions and procedures used to assay binding of Rad51 to ϵDNA and dsDNA were performed as described (*8*). The stability of Rad51 at 37 °C was determined by measuring Rad51 binding to ϵDNA (*8*) following the addition of the reaction components in different sequences. After the final addition, the binding reaction contained the standard buffer, 4 mM $MgCl_2$, 100 μM nucleotide, 5 μM ϵDNA, and 1 μM Rad51. To assess the extent of inactivation, Rad51 (2 μM) was preincubated at 30 or 37 °C with or without nucleotides in the presence of 4 mM $MgCl_2$. Its binding to dsDNA was measured by nuclease protection after adding ATP and ^{3}H-labeled linear dsDNA to 1 mM and 10 μM, respectively (*8*).

Equilibrium Dialysis. Dialysis was carried out in cylindrical 100-μL volume dialysis chambers (Hoefer) fitted with membranes (MWCO 25 kDa). Rad51 (4 and 8 μM) was dialyzed to equilibrium with various concentrations of [α^{32}P]-ATP (20−120 μM) or [^{35}S]ATP-γ-S (15−60 μM). To assess the effect of ssDNA on ATP and ATP-γ-S binding, dialysis was carried out with reaction mixtures containing [α^{32}P]ATP (2−10 μM), 2 μM Rad51, and 20 μM Poly(dT) or [^{35}S]ATP-γ-S (12−30 μM), 4 μM Rad51, and 40 μM Poly(dT). Binding of ATP or ATP-γ-S to Rad51 in the presence of linear dsDNA was carried out in a reaction mixture containing [α^{32}P]ATP (15−40 μM), 2 μM Rad51, and 20 μM dsDNA or [^{35}S]ATP-γ-S (20−80 μM), 4 μM Rad51, and 40 μM dsDNA. To determine the association constants for ADP and AMP-P-N-P, the binding of [^{35}S]ATP-γ-S (60 μM) to 6 μM Rad51 was measured in the presence of ADP (0−600 μM) or AMP-P-N-P (0−6000 μM). All reactions were carried out in standard buffer with the indicated amounts of Rad51 on one side of the dialysis membrane and the nucleotides and DNA on both sides of the membrane. Where ATP was used, an ATP-regenerating system consisting of 10 u/mL creatine kinase and 1 mM creatine phosphate was added to both sides of the dialysis membrane. The dialysis chamber was rotated at 22 °C at a speed of 20 rpm, and equilibrium was reached within 5 h. The nucleotides and nucleotide analogues were quantified by liquid scintillation counting. In those instances where Rad51 was inactivated during the dialysis, we sought to minimize the error in calculating the Scatchard θ value (number of moles of nucleotide bound per mole of Rad51) by measuring the residual ability of Rad51 to bind to DNA after 5 h incubation in the presence of different amounts of nucleotides. The Scatchard plot and the derived dissociation constants were obtained only at those nucleotide concentrations at which 85−100% of Rad51 remained active after 5 h at 22 °C.

Circular Dichroism (CD) Spectroscopy. CD spectra were recorded on an Aviv 62DS CD spectropolarimeter using a cuvette with a path length of 0.1 cm using a spectral bandwidth of 0.2 nm and a time constant of 1 s. Measurements were made at 20 °C at a Rad51 concentration of 2.9 μM. Each of the recorded spectra was the average of six scans, each experiment being repeated several times with essentially the same results. Temperature-dependent changes in the CD spectra were monitored at 198 and 226 nm. The mean signal value was measured for 600 s over the temperature range 0−80 °C in a built-in temperature controlling unit in steps of 2 °C. CD signals (in millidegrees) were converted to mean residue ellipticity (in degree squared centimeters per decimole) after subtracting the blank signal for the cuvette and buffer. The equation $[\theta] = (100 \times \theta)/(l \times c)$ was used, where θ is the observed ellipticity in degrees, c is the molar residue concentration of protein, and l is the path length of the cuvette in centimeters. In the calculation of molar residue ellipticity, an average residue weight of 107.4 was used.

RESULTS

Joint Molecule Formation and Strand Exchange by Rad51 Is Inactivated at 37 °C in the Absence of ATP. In our previous studies of Rad51-promoted strand exchange (*6*), Rad51 was preincubated at 37 °C with $MgCl_2$, ATP, and ssDNA, then RPA was added, and 10 min later the reaction was started by the addition of dsDNA. Under these conditions, joint molecules between circular ssDNA and the overhanging end of the linear dsDNA's complementary strand were formed, and over time these were converted to nicked circular dsDNA (*6*). However, if ATP is omitted from the preincubation with Rad51 but is added back along with the other components of the reaction, neither joint molecules nor completely strand-exchanged product were

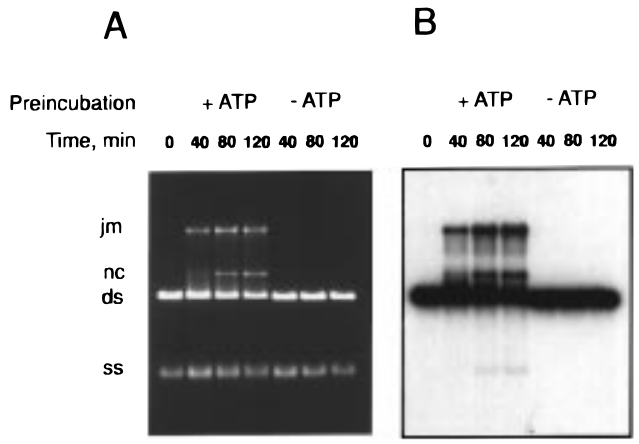

FIGURE 1: Inactivation of Rad51's ability to form joint molecules and promote strand exchange. Reaction mixtures containing 5.3 μM Rad51 without or with ATP (0.5 mM) were incubated for 8 min at 37 °C. ATP was added, where it had been omitted, along with 20 μM circular ϕX 174 ssDNA, 0.7 μM RPA, and 20 μM linear ϕX 174 dsDNA; the reaction was incubated at 37 °C for the indicated times, and the products of the reaction were analyzed as described in Experimental Procedures". (A) Ethidium bromide-stained reaction products. (B) Reactions in whih ^{32}P-labeled linear dsDNA was used as a substrate. The products were detected by autoradiography. Labels are as follow: ss, circular ssDNA; ds, linear dsDNA; jm, joint molecules; nc, nicked circular dsDNA.

formed (Figure 1). The same result was obtained even with the increased sensitivity of ^{32}P-labeled DNA substrates (Figure 1B). These data indicate that even a brief incubation of Rad51 at 37 °C without ATP inactivates its ability to catalyze strand exchange.

The failure to produce joint molecules suggests that the inactivation occurs at an early step of the reaction, namely, the ability of Rad51 to interact with ATP, ssDNA, or dsDNA. We therefore determined which of the activities associated with Rad51 is inactivated by incubation at 37 °C and the role of ATP in preventing that inactivation.

The Capacity of Rad51 To Bind to DNA Is Inactivated at 37 °C in the Absence of ATP. In initial experiments, the ability of ATP to prevent the loss of Rad51 binding to DNA at 37 °C was detected by varying the order in which the components were added to the reaction. Addition of Rad51 to a reaction containing ATP, Mg^{2+}, and ϵDNA resulted in an increase of fluorescence from 1.8 to about 5.0, indicative of protein binding to ϵDNA (Figure 2A). If Rad51 is preincubated with ATP and Mg^{2+} before the addition of ϵDNA, the increase in fluorescence was the same (Figure 2B). However, if ATP was added following incubation of Rad51 with ϵDNA the increase in fluorescence was marginal, some of the increase being due to the intrinsic fluorescence of the protein preparation (Figure 2C). Figure 2D shows that the addition of Rad51 to a reaction in which the Rad51 had been inactivated restored the binding, indicating that the reactants are fully responsive to active Rad51. If Mg^{2+} is omitted from the preincubation, binding was appreciably impaired and was not reversed by the subsequent addition of Mg^{2+} (data not shown). These experiments (Figure 2A−D) describe the binding of Rad51 to ϵDNA, but the same requirements exist for Rad51 binding to dsDNA. Thus, Rad51 binding to dsDNA was severely impaired if the protein was incubated at 37 °C in the absence of ATP before the addition of dsDNA (Figure 2E). These data show that the ability of Rad51 to bind to DNA is inactivated relatively rapidly at 37 °C. However, inactivation is prevented by the addition of ATP.

Figure 3 describes the ability of Rad51 to bind dsDNA following incubation at 37 or 30 °C in the presence or absence of ATP. At 37 °C, Rad51 loses its ability to bind to dsDNA completely within 10 min; at 30 °C the rate of inactivation was slower, but complete inactivation occurred between 40 and 60 min. ATP increased the stability of Rad51 at both temperatures: at 30 °C, protection by ATP was complete even after 60 min, while at 37 °C the half-life

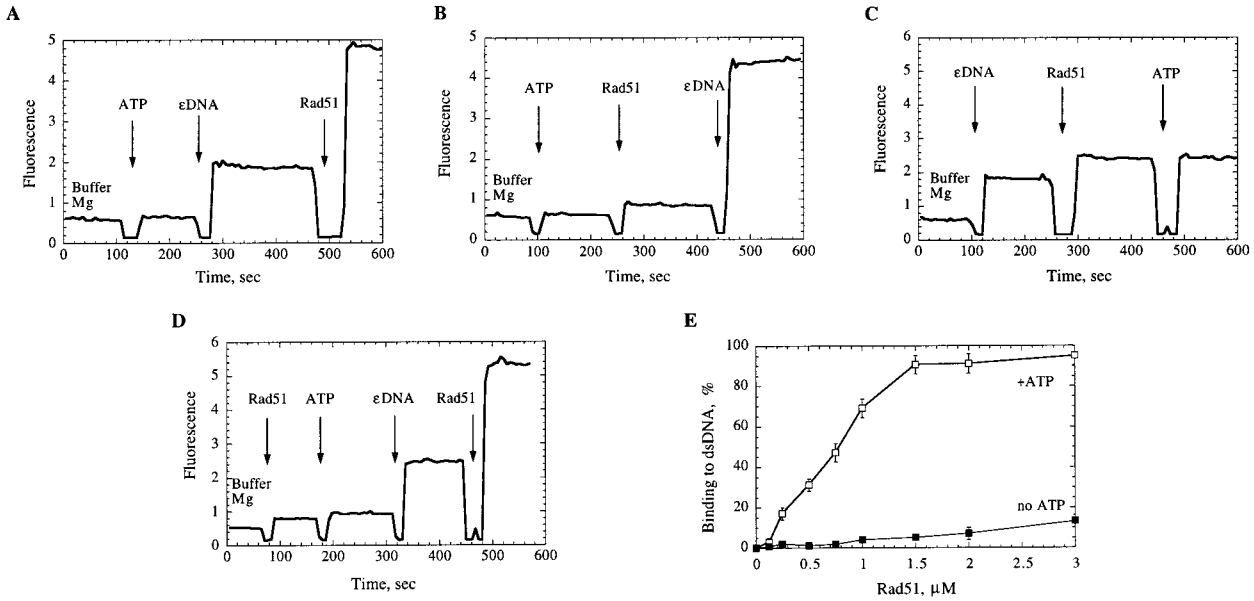

FIGURE 2: Effect of the order of ATP addition on the binding of Rad51 to DNA at 37 °C. In experiments presented in panels A, B, C, and D, the reaction mixture contained 0.1 mM ATP, 5 μM ϵDNA, 1 μM Rad51, and 4 mM $MgCl_2$. Fluorescence was measured after each addition until the emission had reached a steady value. The arrows indicate the times of the additions. (E) Binding to dsDNA; different concentrations of Rad51 were incubated at 37 °C for 9 min in the presence of 4 mM $MgCl_2$ with or without 0.5 mM ATP. The reaction was started by the addition of 10 μM ^{3}H-labeled linear dsDNA and 0.5 mM ATP where it had been omitted. Binding was detected by the nuclease protection assay (*8*).

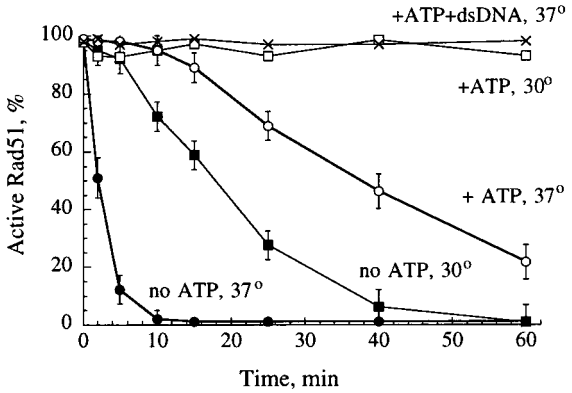

FIGURE 3: Effect of ATP on the stability of Rad51. The kinetics of inactivation of Rad51 was measured as described in Figure 2, except that 2 μM Rad51 was incubated with or without 0.5 mM ATP at either 30 or 37 °C for the indicated times. The amount of the protein's dsDNA binding activity (*8*) remaining at each time was measured in the standard buffer containing 10 μM ^{3}H-labeled dsDNA and 0.5 mM ATP at the same temperature used for the preincubation. The enhanced stability of Rad51 at 37 °C in the presence of dsDNA and ATP is also shown.

of DNA binding was extended from 2 min to about 40 min in the presence of ATP. With both ATP and dsDNA, Rad51 was completely stable for at least 60 min, suggesting that DNA enhances the stabilizing effect of ATP (Figure 3).

ATP-γ-S Prevents Temperature Inactivation of Rad51. Neither ATP-γ-S, AMP-P-N-P, nor ADP promotes binding of Rad51 to DNA (*8*). Moreover, ATP-γ-S but not AMP-P-N-P or ADP causes dissociation of a preformed Rad51-ϵDNA complex (*8*). These findings suggest that ATP-γ-S can bind to Rad51 but do not support binding to DNA. It was, therefore, of interest to determine if ATP-γ-S can prevent inactivation of Rad51 at 37 °C. As noted in Figure 2, the ability of Rad51 to bind to ϵDNA is lost at 37 °C and is not restored if ATP is added subsequently. But incubation with ATP-γ-S appears to prevent inactivation of Rad51 at 37 °C because the subsequent addition of ATP is able to partially restore Rad51 binding to ϵDNA (Figure 4A). We surmise that ATP-γ-S binds to and protects Rad51 and that ATP is able to displace ATP-γ-S from its association with Rad51, thereby restoring the ability to bind to DNA.

If we assume that it is bound nucleotide that protects the protein from inactivation, then the efficiency of protection should depend on the affinity of the nucleotide for Rad51 and, therefore, on the concentration of the nucleotide. Accordingly, we measured the ability of different concentrations of ATP, ADP, AMP-P-N-P, and ATP-γ-S to protect Rad51 following incubation at 37 °C (Figure 4B). AMP-P-N-P fails to protect Rad51 at all concentrations tested; however, ADP, while comparatively ineffective at 100 μM, does prevent inactivation as its concentration is increased. As expected, inactivation of Rad51 is effectively suppressed by ATP and somewhat more effectively by ATP-γ-S.

Binding of ATP to Rad51. The binding of ATP and its analogues to Rad51 was measured directly by equilibrium dialysis. With increasing ATP concentrations the amount of bound ATP increased, reaching a maximum at 80 μM, indicating saturation binding behavior (Figure 5A). Low or no ATP binding was observed with Rad51 that had been inactivated by incubation at 37 °C, indicating that inactivation of Rad51 by this means causes a loss in the ability to interact with ATP (Figure 5A).

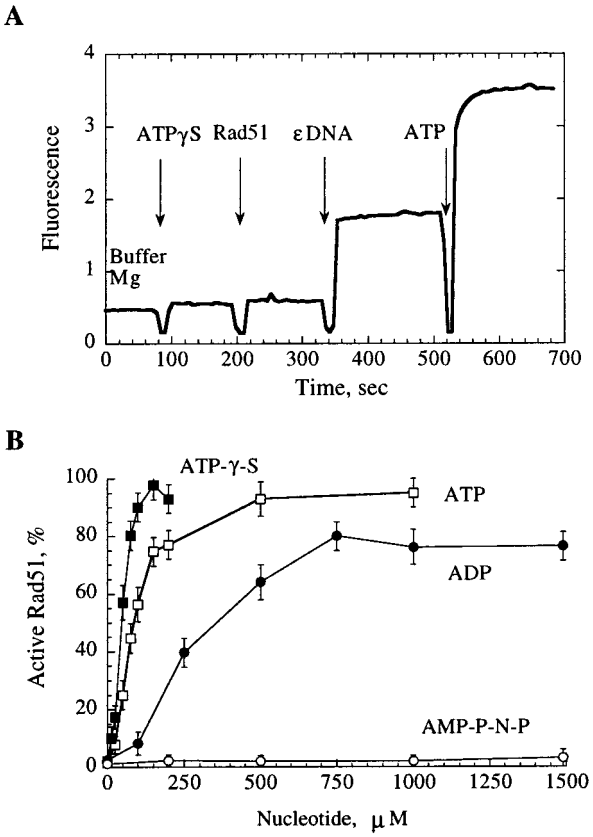

FIGURE 4: Protection of Rad51 against inactivation at 37 °C by ATP-γ-S. (A) Reactions were carried out as described in Figure 2A, except that the concentration of ATP was 0.2 mM and that of ATP-γ-S was 0.1 mM. The arrows indicate the times of additions. (B) Effect of concentration of ATP, ADP, ATP-γ-S, and AMP-P-N-P on the stability of Rad51 at 37 °C was determined as described in Figure 2E. Thus, 2 μM Rad51 was incubated in the presence of different concentrations of ATP, ADP, ATP-γ-S, or AMP-P-N-P at 37 °C for 9 min, at which time ATP (1.25 mM) and ^{3}H-labeled dsDNA (10 mM) were added for an additional 10 min and the remaining DNA binding activity was assayed by nuclease protection (*8*).

Table 1: Parameters of Nucleotide Binding to Rad51

	K_d[a] (μM)		
nucleotide	no DNA	with ssDNA[b]	with dsDNA[c]
ATP[e]	21 ± 5.5[d]	2.3 ± 0.3	12.9 ± 1.1
ADP[f]	88.9 ± 10.7	ND[h]	ND
ATP-γ-S[g]	19.1 ± 5.5	7.0 + 0.9	27.8 ± 4.6
AMP-P-N-P[f]	1100 ± 100	ND	ND

[a] The value of K_d was determined by equilibrium dialysis at 22 °C (See also Figures 5B and 6. [b] Poly(dT) (20 μM) was added to dialysis buffer. [c] Linear dsDNA (20 μM) was added to dialysis buffer. [d] Errors calculated by curve fitting procedure. [e] [α^{32}P]ATP was used to determine K_d. [f] K_ds for ADP and AMP-P-N-P were determined from competition experiments using [^{35}S]ATP-γ-S. [g] [^{35}S]ATP-γ-S was used. [h] Not determined.

The ATP binding data were replotted in a Scatchard plot, and the binding constants are summarized in Table 1. Figure 5B shows that the intercept extrapolates to 1, indicating that 1 mol of ATP is bound per mol of Rad51. The dissociation constant for ATP is close to the value of 15−27 μM reported for ATP binding to RecA (*15*, *16*). However, the value of 21 ± 5.5 is considerably higher than the optimal amount of ATP for binding Rad51 to DNA (1−3 μM) (*8*). It is possible that the affinity of Rad51 for ATP is enhanced when it is

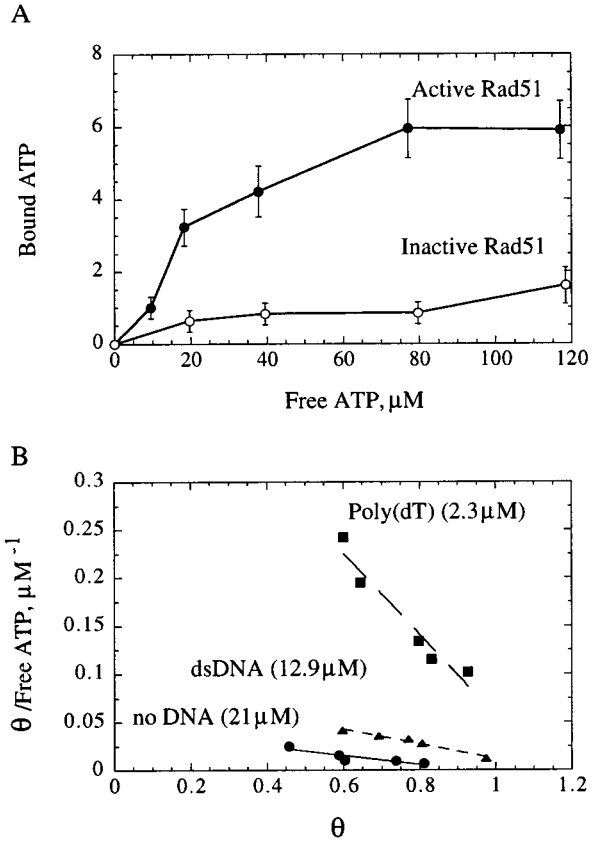

FIGURE 5: Binding of ATP to Rad51 determined by equilibrium dialysis. The reactions were performed in standard reaction buffer containing 4 mM $MgCl_2$ with 8 μM Rad51 on one side of the dialysis membrane and varying amounts of [α^{32}P]ATP in the two chambers. (A) The amount of labeled nucleotide associated with the protein at equilibrium is plotted on the ordinate. Rad51 was inactivated by incubation for 9 min at 37 °C in the absence of ATP. (B) The calculated amounts of bound and free ATP were analyzed using Scatchard plots. Measurements of the binding of ATP to Rad51 in the presence of ssDNA were performed in a reaction mixture containing 2 μM Rad51, 20 μM Poly(dT), and [α^{32}P]ATP (2−10 μM); with dsDNA, the reaction contained 2 μM Rad51, 20 μM linear dsDNA, and [α^{32}P]ATP (15−40 μM. θ represents the number of moles of ATP bound per mole of Rad51.

bound to DNA, as is the case for RecA (*16*). Consequently, equilibrium dialysis measurements of ATP binding were performed in the presence of ssDNA or linear dsDNA. The K_d values obtained from the Scatchard plot in Figure 5B are 2.3 ± 0.3 and 12.9 ± 1.1 μM in the presence of ssDNA and dsDNA, respectively (Table 1). The affinity of ATP for Rad51 is about 10 times greater in the presence of ssDNA than in its absence.

Binding of ATP-γ-S and Other Analogues of ATP. Figure 6A shows Scatchard plots for the binding of ATP-γ-S to Rad51 in the absence and in the presence of DNA, and the K_d values are summarized in Table 1. The dissociation constant for ATP-γ-S is 19.1 ± 5.5 in the absence of DNA and 7.0 ± 0.9 and 27.8 ± 4.6 μM in the presence of ssDNA or dsDNA, respectively. Thus, in the absence of DNA, ATP-γ-S binds to Rad51 with virtually the same affinity as ATP. DNA also increases the affinity of ATP-γ-S for Rad51, but less so than for ATP. The lesser effect of DNA on the affinity of ATP-γ-S for Rad51 may be due to the poorer binding of DNA in the presence of ATP-γ-S (*8*).

Binding of ADP and AMP-P-N-P was determined by their ability to compete with the binding of [^{35}S]ATP-γ-S. Table

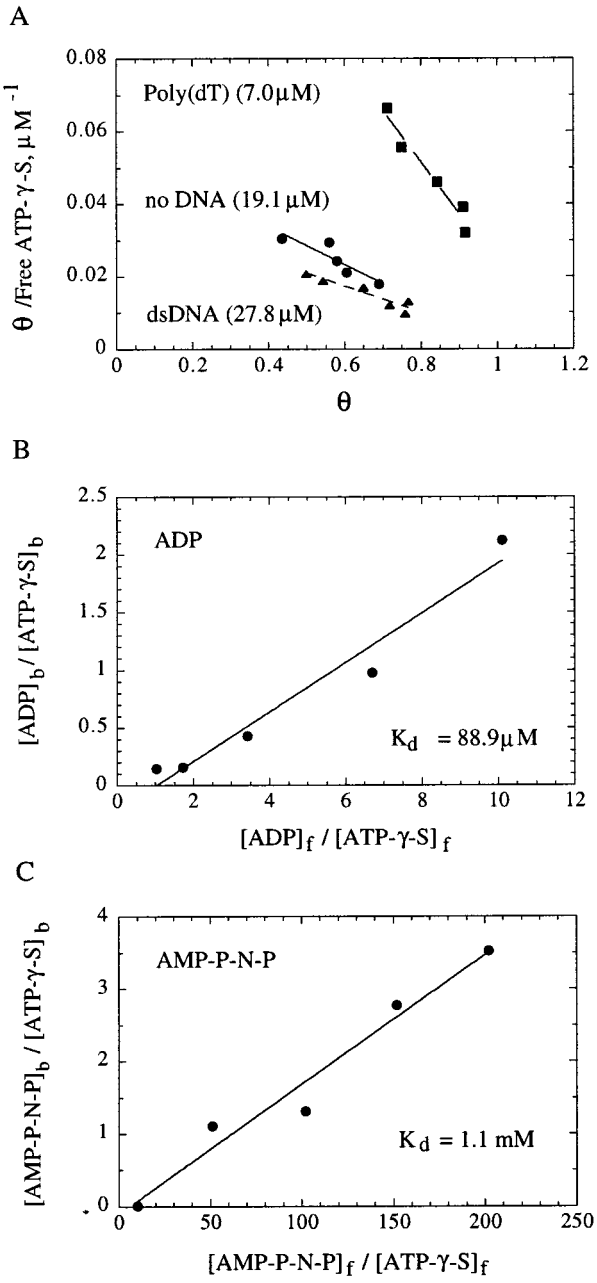

FIGURE 6: Binding of ATP-γ-S and ATP analogues to Rad51. (A) Binding of ATP-γ-S in the absence of DNA was determined by equilibrium dialysis with 4 μM Rad51 and [^{35}S]ATP-γ-S (15−60 μM) in the standard reaction buffer containing 4 mM $MgCl_2$. To determine the effect of ssDNA and dsDNA, the dialysis was performed in reaction mixtures containing 4 μM Rad51, 40 μM Poly(dT), and [^{35}S]ATP-γ-S (12−30 μM) or 4 μM Rad51, 40 μM dsDNA, and [^{35}S]ATP-γ-S (20−80 μM), respectively. The Scatchard plot was derived as described in Figure 5. (B) Binding of ADP or (C) AMP-P-N-P to Rad51 was determined by competition with the binding of ATP-γ-S. Increasing amounts of ADP (0−600 μM) or AMP-P-N-P (0−6 mM) were dialyzed to equilibrium with 6 μM Rad51 and [^{35}S]ATP-γ-S (60 μM). The data were plotted as the ratios of bound ADP/ATP-γ-S or bound AMP-P-N-P/ATP-γ-S [ ]$_b$ versus the same ratios of free nucleotides [ ]$_f$.

1 and Figure 6B, C show that the K_d for ADP is 88.9 ± 10.7 μM and for AMP-P-N-P, 1.1 ± 0.1 mM. Thus, ATP-γ-S has a nearly 5-fold higher affinity than ADP for Rad51 and about 55-fold greater affinity than for AMP-P-N-P. These data are consistent with the finding that higher concentrations of ADP protect Rad51 from inactivation at 37 °C while

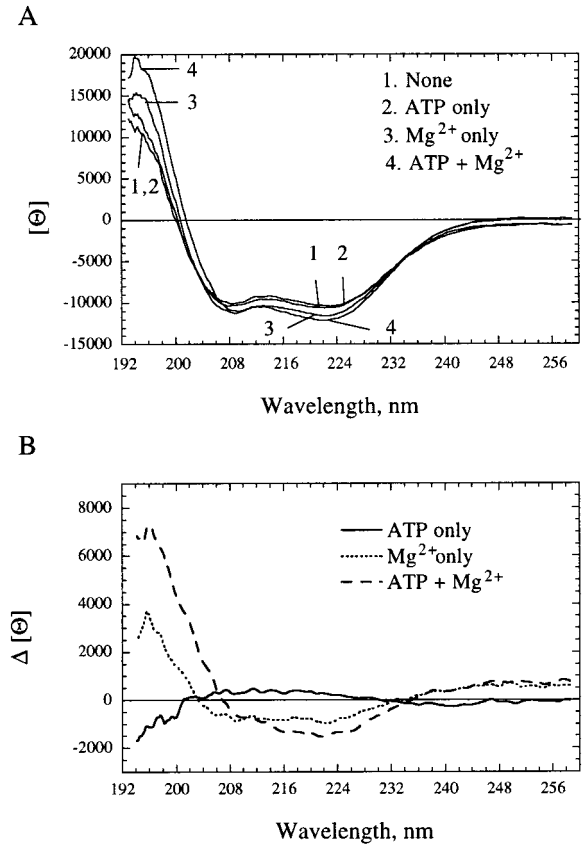

FIGURE 7: CD spectra of Rad51. Circular dichroism spectra of Rad51 were recorded at 20 °C. (A) Measurements of the CD spectrum were performed in buffer containing 2.9 μM Rad51. (B) To determine the effect of Mg^{2+} and ATP on the structure of Rad51, the scan made in the absence of Mg^{2+} and ATP was subtracted from the scan made in the presence of 0.1 mM ATP alone (—) or in the presence of 4 mM $MgCl_2$ alone (···) or in the presence of 0.1 mM ATP and 4 mM $MgCl_2$ (---). The calculated residue ellipticity (deg cm^2 $dmol^{-1}$) [Θ] is plotted.

AMP-P-N-P appears to be completely inactive in this regard (Figure 4B).

Circular Dichroism of Rad51. The CD spectrum of Rad51 at 20 °C with or without ATP and Mg^{2+} displays negative double minima at 208 and 222 nm and positive ellipticities at 193−198 nm, features that are characteristic of an α -helical protein (Figure 7A); to emphasize the spectral changes that are induced by the presence of ATP and Mg^{2+}, we show the difference spectra in Figure 7B. With ATP alone the spectrum differs only marginally from that obtained in the absence of ATP (Figure 7, compare spectra 1 and 2 and Figure 7B), suggesting that ATP does not interact with Rad51 in the absence of Mg^{2+}. With Mg^{2+} alone; however, the ellipticity of the protein increased in the region 194−208 nm and decreased between 208 and 236 nm. In the presence of both ATP and Mg^{2+}, the changes in ellipticity in these spectral regions are even more pronounced. These data suggest that binding of Mg^{2+} alone but even more so by ATP and Mg^{2+} induces a structural change in the protein. The nature and extent of these changes are not ascertainable directly, but the more pronounced lowering of the ellipticity between 208 and 236 nm is consistent with an increase in α-helical domains, while the increased ellipticity at about 196 nm is thought to indicate an increase in β-structured domains (*17*). Taken together, the spectral changes suggest that Rad51 is more structured with ATP and Mg^{2+} bound.

We considered the possibility that the change in the CD spectrum of Rad51 in the presence of ATP and Mg^{2+} reflects a change in the state of Rad51 polymerization rather than a change in the secondary structure of the Rad51 monomer. To test that possibility, gel filtration studies of Rad51 were carried out. Under the conditions of our assay (2.9−3.6 μM Rad51 in CD buffer), the apparent molecular mass based on its mobility in a Superose 6 (Pharmacia) column was 250−300 kDa, which is equivalent to the mass of a Rad51 hexamer. The chromatographic profile was unaffected by the addition of Mg^{2+} and ATP under conditions that activate DNA binding and produce a change in the CD spectrum.

ATP and Mg^{2+} Stabilize Rad51 Secondary Structure. We presume that the protection of Rad51 against inactivation at elevated temperatures by the binding of ATP and Mg^{2+} results from a change or stabilization of its structure. That presumption was tested by comparing the CD spectrum of Rad51 that had been inactivated at 37 °C for 9 min with the spectrum of the protein that had been incubated under the same conditions in the presence of ATP and Mg^{2+}. The same comparison was done with Rad51 incubated with and without ATP and Mg^{2+} at 20 °C. At 20 °C, the CD spectrum of the protein incubated with and without ATP and Mg^{2+} differed only marginally (Figure 8A). This is not surprising because the protein is not substantially inactivated at 20 °C in the absence of ATP and Mg^{2+}. However, the CD spectrum of the protein incubated in the absence of ATP and Mg^{2+} at 37 °C, where the protein is completely inactivated (see Figure 3), was distinctly different from that of the fully protected protein in the spectral range of 194−208 nm and 208−236 nm (Figure 8A). These measurements indicate that inactivation is accompanied by a significant change in secondary structure.

The transition from active to inactive protein was also followed by measuring the ellipticity values at 198 nm (Figure 8B) and 226 nm (Figure 8C) of active and inactive Rad51 as a function of temperature. Note that the ellipticity values of the active and inactive forms at the two wavelengths are distinctly different at temperatures up to about 30 °C. As the temperature rises between 30 and 40 °C the active form undergoes a sharp decrease in ellipticity; above 40 °C the values for the two forms remain the same. Thus, the structural change in Rad51 induced at elevated temperature occurs concomitantly with its inactivation. The addition of ATP and Mg^{2+} to Rad51 induces both a structural change and the ability to bind DNA. It seems simplest to assume that the stabilization and activation of the protein have the same structural basis.

The experiments described in Figures 7 and 8 were also performed with ATP-γ-S in place of ATP with virtually identical results, but the data are not included in the respective figures for the sake of simplicity. Because ATP-γ-S is unable to promote binding of either ssDNA or dsDNA (*8*) and, therefore, fails to support strand exchange, we infer that there are subtle differences in the structure of Rad51 when it is bound with ATP and Mg^{2+} compared to ATP-γ-S and Mg^{2+}.

DISCUSSION

Our experiments demonstrate that Rad51's ability to bind ATP, or more properly an ATP−Mg^{2+} complex, ssDNA,

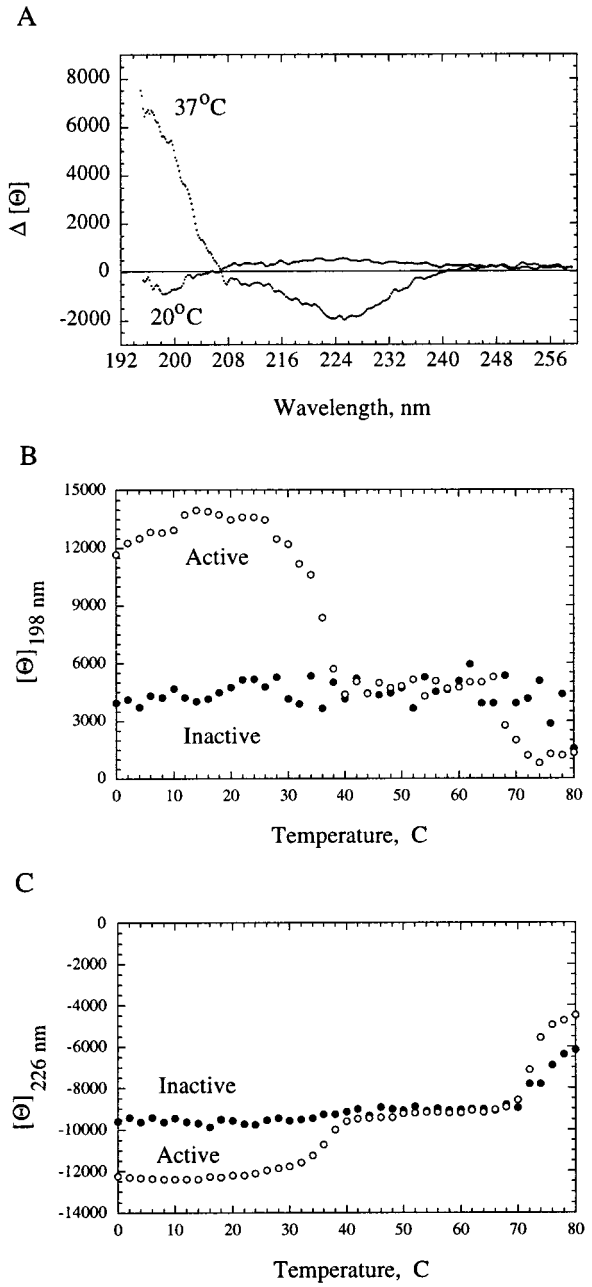

FIGURE 8: Inactivation causes an irreversible change in the secondary structure of Rad51. (A) Effect of inactivation on the structure of Rad51. Rad51 (2.9 μM) was preincubated in the presence or absence of ATP and Mg^{2+} (0.1 mM ATP, 4 mM $MgCl_2$) for 9 min at 20 or 37 °C. ATP and Mg^{2+} were added in CD buffer where they had been omitted. The CD spectrum of each mixture was determined, and the difference spectrum of the samples incubated with and without ATP and Mg^{2+} were plotted. (B and C) The change in ellipticity of active and inactive Rad51 as a function of increasing temperature. The temperature of the cuvette was increased by 2 °C steps from 0 to 80 °C. Rad51 was inactivated by preincubation at 37 °C for 9 min in the absence of ATP and Mg^{2+}, and then both were added in CD buffer where they had been omitted. The ellipticity was measured at the two wavelengths: (B) 198 nm and (*C*) 226 nm. [Θ], residue ellipticity (deg cm^2 $dmol^{-1}$).

and dsDNA, and to promote both joint molecule formation and strand exchange are rapidly lost when the protein is incubated at 37 °C and somewhat less rapidly at 30 °C. However, each of these activities is protected when ATP and Mg^{2+} are present during exposure of the protein to these temperatures. Although ATP-γ-S is unable to promote DNA binding (*8*), it does protect Rad51 against inactivation at 37 °C. By contrast, neither ADP nor AMP-P-N-P is able to protect the protein at elevated temperature. These properties correlate with the relative binding constants. Thus, ATP and ATP-γ-S bind to Rad51 with a molar stoichiometry of about 1 and K_ds of 21 and 19 μM, respectively. ADP and AMP-P-N-P, however, bind poorly with K_ds of 89 and 1100 μM, respectively. The K_d for ATP binding is lowered to 2.3 μM and for ATP-γ-S to 7 μM in the presence of poly(dT).

We presumed from these data that binding ATP−Mg^{2+} stabilizes and activates Rad51 by inducing a structural change in the protein. Measurements of the Rad51 CD spectrum under a variety of conditions are consistent with this view. Thus, the CD spectrum of Rad51 displays negative double minima at around 210 and 222 nm and positive ellipticities at 193−198 nm, characteristics displayed by proteins with α-helical regions. The addition of ATP and Mg^{2+} alters the CD spectrum, increasing the ellipticity values between 194 and 204 nm and decreasing them between 208 and 226 nm. The CD spectrum of inactive Rad51 differs distinctively from that of the active form and fails to change upon the addition of ATP and Mg^{2+}. Furthermore, the ellipticity value of active Rad51 at 198 and 226 nm undergoes a marked transition as the temperature is raised from 30 to 40 °C, attaining the value of inactive protein at 40 °C and above. Although the precise changes in the structure cannot be deduced from CD measurements alone, it is likely that the changes reflect alterations in the structure when it is activated by ATP−Mg^{2+} and when it is inactivated at elevated temperatures.

A puzzling feature of our experiments is the behavior of ATP-γ-S. ATP- γ-S mimics ATP in its binding affinity for Rad51, and in its ability to protect the protein against inactivation at 37 °C and to induce the same changes in the CD spectrum shown in Figures 7 and 8 (data not shown). Nevertheless, in our hands, ATP-γ-S is unable to promote strand exchange or even to initiate joint molecule formation (our unpublished observations), although such an activity has been reported previously (*18*); parenthetically, the inactivity of ATP-γ-S in strand exchange is not surprising in view of the fact that it is unable to promote Rad51 binding to either ssDNA or dsDNA (*8*). Although the structural alterations produced by ATP-γ-S and ATP appear to be indistinguishable by the measure of protein stabilization and CD spectral changes, these properties fail to distinguish between the functional and inactive form of the protein.

At this point, it is informative to compare Rad51 and RecA with respect to the consequence of binding nucleotides and Mg^{2+}. ATP binds to Rad51 with a stoichiometry of 1.0 and a K_d of 21 μM in the absence of DNA and 2.3 μM in the presence of Poly(dT); the comparable binding constants for ATP binding to RecA are K_ds of 15−24 and 2.5 μM in the absence or presence of ssDNA, respectively (*15*, *16*). However, Rad51 and RecA differ strikingly in their binding affinities with ATP-γ-S. Thus, the K_d for binding ATP-γ-S with RecA is less than 0.3 μM (*16*), and when ATP-γ-S is bound the protein forms a very stable complex with ssDNA (*9*, *10*, *16*). At present, we cannot explain why ATP-γ-S fails to activate Rad51 binding to DNA even though its affinity for the protein is as high as that of ATP.

A common feature of most nucleoside triphosphatases is that binding of the nucleoside triphosphate activates and changes the structural state of the protein. It has been shown

that the size of the RecA oligomer depends on the presence and concentration of ATP and Mg^{2+} (*19*), and that the structure of the monomer (*20*), the helical pitch, and cross-sectional parameters of the RecA filament change on the addition of ATP and Mg^{2+} (*21*, *22*). The CD spectrum of Rad51, like that of RecA, has negative double maxima between 210 and 220 nm, suggestive of α-helical domains (*23*). Furthermore, Rad51 appears to undergo a structural change when bound with ATP and Mg^{2+}. That structural change, signaled by the altered CD spectrum between 194 and 208 and between 208 and 235 nm, suggests that Rad51 acquires a more structured state, very likely one able to bind DNA. In that connection, nucleotide cofactors were recently shown to modulate the structure of the *Xenopus laevis* Rad51 filament (*24*).

The structural analysis of the α-subunits, of two different G proteins, $G_{i\alpha1}$ and $G_{t\alpha}$, implicated specific arginine and glutamine residues for guanosine triphosphatase activity: arginine 178 and glutamine 204 in the $G_{i\alpha1}$ subunit, and arginine 174 and glutamine 200 in $G_{t\alpha}$ (*25*, *26*). Glutamine has been proposed to stabilize the "trigonal-bipyramidal" transition state and orient the hydrolytic water molecule, while arginine is believed to stabilize the negative charge on the oxygen atoms of the "penta coordinate phosphate intermediate" (*25*). Significantly, glutamine 194 and arginine 196 have also been implicated in the ATP′ase activity of RecA. Voloshin et al. (*27*) have shown that a 20-amino acid peptide corresponding to RecA residues 193−212 has DNA binding activity and can even promote joint molecule formation between single- and double-stranded DNA. In this model, arginine 196 interacts with DNA and the γ-phosphate of ATP and is essential for its hydrolysis. Moreover, RecA proteins with mutations in glutamine 194 exhibit characteristics similar to Rad51. They bind ATP-γ-S as efficiently as ATP, but ATP-γ-S induces dissociation of mutant RecAs from DNA (*28*). Interestingly, an alignment of the amino acid sequences of RecA and Rad51 shows that the glutamine is conserved but that Rad51 lacks the nearby arginine. This could account for the lower ATP′ase activity of Rad51 than RecA. We surmise that glutamine alone is sufficient to promote ATP binding and the ensuing structural change in Rad51 even without efficient hydrolysis of ATP's terminal phosphate.

If ATP hydrolysis is not essential for RecA binding to DNA and even for formation of joint molecules, what role does it have in strand exchange? It has been suggested that ATP hydrolysis is necessary to drive the unidirectional branch migration of the RecA-mediated strand exchange (*29*, *30*). Perhaps the bidirectional branch migration we have observed in Rad51-promoted strand exchange (*6*) is related to Rad51's low ATP′ase activity. There is, however, a preference for Rad51-driven branch migration to occur in the 5′ to 3′ direction on the single-strand circular DNA (unpublished observation). We are currently investigating if this preference is a consequence of the preferential polymerization of Rad51 in the 5′ to 3′ direction on the displaced strand. Branch migration in the other direction—where joint molecules are formed using a complementary 5′ end and the displaced strand has the 3′ end—may be promoted by a less efficient, nonoriented, binding of RPA or SSB to the displaced strand.

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