Like RecA, Saccharomyces cerevisiae Rad51p promotes strand exchange between circular single-stranded DNA (ssDNA) and linear double-stranded DNA (dsDNA). We have investigated several parameters characteristic of the interaction of Rad51p with ssDNA and dsDNA, particularly the effects of the nucleotide cofactors ATP and ADP and the analogs adenosine 5'-O-(thiotriphosphate) (ATPγS) and adenylyl-imidodiphosphate (AMP-PNP). Rad51p binding to both 1-N⁶-ethenoadenosine and 3-N⁴-ethenoctydine ssDNA (eDNA) and dsDNA requires the presence of Mg²⁺ and ATP; no binding occurs in the presence of ADP, AMP-PNP, or ATPγS. Binding of Rad51p to dsDNA also requires ATP; ADP is ineffective, whereas ATPγS and AMP-PNP are considerably less able to promote binding and only at elevated concentrations of Rad51p. ATP binding, not ATP hydrolysis, is required for Rad51p binding to DNA. The Kₜ values for ATP for promoting binding of Rad51p to ssDNA and dsDNA are 1 and 3 μM, respectively. Rad51p binding occurs with a stoichiometry of one monomer of Rad51p per ~6.3 nucleotides of eDNA and ~3.3 base pairs of dsDNA. Once formed, Rad51p:ssDNA complexes are stable so long as sufficient ATP levels are maintained. ATP hydrolysis causes dissociation of Rad51p from DNA. Moreover, the preformed complex is stable in the presence of a 10-fold excess of ADP or AMP-PNP over ATP. ATPγS, however, in the same -fold excess over ATP causes dissociation of the Rad51p:ssDNA complex.

The Saccharomyces cerevisiae RAD51 gene is a member of the RAD52 epistasis group, that is required for efficient DNA double strand break repair and genetic recombination (1). The amino acid sequence of RAD51 strongly resembles that of the Escherichia coli RecA protein (2–4), and like RecA, Rad51p catalyzes joint molecule formation and strand exchange between circular ssDNA and linear dsDNA (5–8). The reaction promoted by Rad51p also requires Mg²⁺, ATP, and a single-stranded DNA-binding protein, yeast replication protein A, being the most efficient (6–8). However, Rad51p-promoted strand exchange has certain striking differences compared with RecA. For example, RecA has the unique ability to pair homologous ssDNA and dsDNA to form three-stranded joint molecules, which are then resolved into heteroduplex DNA; this pairing can occur even between DNAs without ends (9–11). In the reaction promoted by Rad51p, joint molecules are formed only between ssDNA and linear dsDNA having either 3' or 5'-overhanging complementary ends; joint molecules are not formed with linear DNA having blunt or recessed complementary ends (8). As a consequence of being able to use either a 3'- or 5'-overhanging complementary strand to initiate joint molecule formation, the direction of strand exchange depends on which end initiates the reaction (8).

The interactions of RecA with ssDNA and dsDNA are important determinants for strand exchange (12–14). To understand the molecular mechanism of Rad51p-promoted strand exchange, we have examined the interactions between Rad51p and single- and double-stranded DNA, particularly the role of nucleotides and salt in these interactions. Rad51p binding to ssDNA was followed by measuring the increased fluorescence of chemically modified 1-N⁶-ethenoadenosine and 3-N⁴-ethenoctydine ssDNA (etheno-DNA, eDNA) that accompanies protein binding (13, 15); binding to dsDNA was followed by protection of the DNA against DNase I degradation (14) and, in some cases, by nitrocellulose filter binding (16). Our data indicate that ATP and Mg²⁺ are essential for Rad51p binding to both kinds of DNA and that ADP, adenosine 5'-O-thiotriphosphate (ATPγS), and adenylyl imidodiphosphate (AMP-PNP) do not satisfy this requirement. The ATP analog ATPγS, which is unable to promote Rad51p binding to DNA, nevertheless causes dissociation of the Rad51p:eDNA complex. The results of dissociation of the Rad51p:eDNA complex at different temperatures indicate that ATP binding, not ATP hydrolysis, is required for Rad51p binding to DNA.

**EXPERIMENTAL PROCEDURES**

**Proteins, Buffers, and Reagents**—Yeast Rad51p was expressed in and purified from insect cells as described previously (9). The concentration of Rad51p was determined using an extinction coefficient (determined from amino acid composition) of 1.26 × 10⁵ M⁻¹ cm⁻¹ at 280 nm. Unless otherwise noted, the standard reaction buffer used in all experiments contained 40 mM K-Mes (pH 6.5), 4 mM MgCl₂, 1 mM dithiothreitol, and 5% glycerol. In experiments testing pH dependence, K-Mes was replaced by 25 mM Tris-HCl (pH 7.5 or 8.5). ATP and ADP were obtained from Pharmacia Biotech Inc., and ATPγS and AMP-PNP were from Boehringer Mannheim. All reactions were carried out at 30 and 37 °C.

**DNA**—The concentrations of the DNA substrates are expressed as nucleotide equivalents. Etheno-M13 DNA (eDNA) was prepared as described (17), and the concentration was determined using an extinction coefficient of 7000 at 260 nm (18). Uniform ⁵⁷⁴H-labeled pBluescript SK− DNA was prepared with SssI methylase and 5'-methyl-⁵⁷⁴Hadenosyl-L-methionine. Linear DNA was prepared by digestion of ⁵⁷⁴H-labeled pBluescript SK−+ dsDNA with PstI restriction endonuclease.

**ssDNA Binding Assay**—The binding of Rad51p to ssDNA was monitored by measuring the change in fluorescence of eDNA at 410 nm following excitation at 305 nm using an AMINCO-Bowman lumines-
cence spectrometer. Sample volumes were normally 0.6 ml, and additions did not exceed 40 µl. The titrant was either Rad51p in “forward” titrations or concentrated sodium chloride solution in the “salt-back” titration. When ATP, ADP, ATPγS, and AMP-PNP were included in the titrations, their concentrations were 100 µm each. Following the addition of each titrant, the fluorescence was measured until the value reached a stable plateau. In experiments measuring the stability of the Rad51p-DNA complex, the complex was formed in the presence of 100 µM ATP, and the change in fluorescence was monitored after the addition of 1000 µM ADP, ATPγS, or AMP-PNP. Measurements were performed at a constant temperature, maintained by a circulating water bath. Corrections for fluorescence of the protein were made from the values obtained after adding Rad51p in the absence of DNA. The fluorescence values observed in these control titrations were subtracted from the measured values made in the presence of DNA.

Nuclease Protection Assay—^3H-Labeled linear dsDNA (10 µm) and variable amounts of Rad51p were incubated in the standard reaction buffer containing 0.5 mM ATP, ADP, ATPγS, or AMP-PNP at 30 °C for 10 min. Aliquots (20 µl) of the reaction mixtures were incubated with 1 µl (12 units) of pancreatic DNase I (Boehringer Mannheim) under standard conditions for 1 min. The reaction was stopped by adding 10 µl of a solution containing 2 mg/ml salmon sperm DNA (heat-denatured) and 0.375 mM EDTA followed by 0.9 ml of ice-cold 10% trichloroacetic acid. The samples remained on ice for 30 min and then were filtered through Whatman GF/C filter discs. The filters were washed twice with 1 ml of 10% trichloroacetic acid followed by 2 ml of 95% ethanol. The filters were dried and assayed for radioactivity in a liquid scintillation counter. For each experiment, complete protection was equivalent to the value obtained without DNase I digestion. Complete digestion was equivalent to the value obtained after DNase I digestion when Rad51p was omitted from the binding reaction. The degree of protection was obtained by dividing the amount of acid-precipitable ^3H for each sample by the value obtained without DNase I digestion after the background (the value without addition of Rad51p) was subtracted from both. This background was ~10% of the value for 100% protection.

Nitrocellulose Filter Binding Assay—Rad51p binding to DNA was also measured by the protein-dependent retention of DNA on nitrocellulose filters (20). 1 ml of 10% trichloroacetic acid followed by 2 ml of 95% ethanol. The titrant was either Rad51p in “forward” titrations, their concentrations were 100 µm each. Following the addition of each titrant, the fluorescence was measured until the value reached a stable plateau. In experiments measuring the stability of the Rad51p-DNA complex, the complex was formed in the presence of 100 µM ATP, and the change in fluorescence was monitored after the addition of 1000 µM ADP, ATPγS, or AMP-PNP. Measurements were performed at a constant temperature, maintained by a circulating water bath. Corrections for fluorescence of the protein were made from the values obtained after adding Rad51p in the absence of DNA. The fluorescence values observed in these control titrations were subtracted from the measured values made in the presence of DNA.

Effect of ATP and Mg^{2+} on Rad51p Binding to DNA—The ATP dependence of Rad51p binding to ssDNA was determined by measuring the effect of ATP. The fluorescent DNA in the standard reaction buffer containing 5 mM ATP, 1 mM Rad51p, and varying levels of ATP with an ATP-regenerating system. The effect of ATP concentration on the binding of Rad51p to dsDNA was studied with the nuclease protection assay using the same reaction conditions except that the mixture contained 10 µM ^3H-labeled linear dsDNA and 2 µM Rad51p.

Measurements of the effect of Mg^{2+} on Rad51p binding to ssDNA were performed by varying the concentration of MgCl2 in the presence of 25, 100, 750, or 3000 µM ATP. The effect of Mg^{2+} on Rad51p binding to dsDNA was determined by varying the MgCl2 concentration in the presence of 750 µM ATP, and binding was measured by the nitrocellulose filter binding assay. All reactions were carried out at 30 °C.

ATP Hydrolysis Assay—ATPase activity was measured as described. Aliquots (20 µl) of the reaction mixtures were incubated with 1 µM Rad51p and 5 mM ^3H-labeled RNA in a 90-µl reaction mixture containing 2.5 mM ATP, 10 units/ml pyruvate kinase, 10 units/ml lactate dehydrogenase, 0.3 mM phosphoenolpyruvate, and 100 µM NADH in the standard reaction buffer. Oxidation of NADH was measured as a decrease in absorbance at 340 nm.

RESULTS

Binding of Rad51p to ssDNA—Rad51p binding to ssDNA was followed by measuring the increase in fluorescence associated with binding of the protein to chemically modified ssDNA (eDNA) (21). The increase in fluorescence is presumed to be a consequence of stretching the DNA and unstacking the bases in the Rad51p-eDNA complex (13, 15).

Rad51p bound to eDNA only when both ATP and Mg^{2+} were present (Fig. 1). Omitting ATP or replacing it with ADP or AMP-PNP eliminated the binding. ATPγS did not support Rad51p binding to eDNA at 37 °C; but did so, somewhat, at higher Rad51p concentrations at 30 °C. There was no significant difference in the quantitative or qualitative characteris-

![Figure 1](#)
Rad51p-mediated protection of the dsDNA against DNase I action in the presence of ATP reached a limiting value of 3.3 base pairs/Rad51p. Unlike RecA, which binds to dsDNA most effectively at low pH (14, 16, 24, 25), Rad51p bound dsDNA with the same efficiency at pH 6.5–8.5 in the presence of ATP (data not shown). Here, too, as in the case of ssDNA, we estimate an association constant for Rad51p binding to dsDNA of $8 \times 10^5$ M$^{-2}$, which is close to the value for Rad51p binding to ssDNA.

Effect of ATP and Mg$^{2+}$ Concentration on Binding of Rad51p to DNA—Fig. 3 demonstrates the dependence of Rad51p binding to DNA on ATP concentration. The apparent $K_a$ value for ATP for Rad51p binding to εDNA is $1 \mu M$, and that for binding to dsDNA is $2 \mu M$. However, because binding to ssDNA and dsDNA was measured in the presence of 1 and 2 $\mu M$ Rad51p, respectively, these values may reflect the stoichiometry of binding of Rad51p and ATP rather than a $K_a$, which could be considerably different.

The concentration of Mg$^{2+}$ required for promoting Rad51p binding to εDNA varied with the amount of ATP in the reaction (Fig. 4). With 25, 100, or 750 $\mu M$ ATP, maximal Rad51p binding occurred with 1–2 mM Mg$^{2+}$. With 750 $\mu M$ ATP, however, there was little or no effect of Mg$^{2+}$ until its concentration reached nearly 1 mM. This shows that ATP alone is ineffective in supporting Rad51p binding to εDNA and suggests that an ATP-Mg$^{2+}$ complex is the preferred substrate for promoting binding. In the presence of 5 mM ATP, Mg$^{2+}$ had little effect on Rad51p binding to εDNA until most or all of the ATP was titrated. This further emphasizes the point that ATP alone is ineffective and may even interfere with activation by the ATP-Mg$^{2+}$ complex. The binding of Rad51p to dsDNA showed the same dependence on the relative amounts of ATP and Mg$^{2+}$ (data not shown). The data indicate that a substantial excess of Mg$^{2+}$ over ATP is needed to form the active species of the ATP-Mg$^{2+}$ complex, although we cannot discount the possibility that Mg$^{2+}$ is also contributing to the binding in other ways, e.g., reacting with Rad51p or the DNA substrate.
Stability of the Rad51p-eDNA Complex in the Presence of Different Nucleotides—Once formed in the presence of 100 μM ATP and maintained in this medium, the Rad51p-eDNA complex was stable for an extended period of time (Fig. 5A). However, if the complex was formed with 2.5 μM ATP, its half-life was ~12–13 min at 30 °C. This is most likely due to loss of ATP by hydrolysis and not to the accumulation of ADP because even a 10-fold excess of ADP over ATP did not inhibit the binding (Fig. 5A). Similarly, AMP-PNP, which was also unable to support complex formation (Fig. 1), also failed to destabilize the Rad51p-eDNA complex formed with ATP (Fig. 5B).

By contrast, the Rad51p-eDNA complex was rapidly dissociated in the presence of ATPγS (Fig. 5B), even though ATPγS itself was unable to support Rad51p binding to eDNA (Fig. 1A). We attribute the dissociation of the complex to the ability of ATPγS to compete with ATP for binding to Rad51p and the inability of the Rad51p-ATPγS complex to bind eDNA.

The inability of ADP, ATPγS, or AMP-PNP to promote Rad51p binding to DNA suggests that ATP hydrolysis might be necessary for DNA binding. To test this supposition, we measured both Rad51p binding to eDNA (Fig. 6A) and the rate of ATP hydrolysis (Fig. 6B) at 30, 20, and 10 °C. At 30 °C, dissociation of the rapidly formed Rad51p-eDNA complex occurred concomitantly with ATP hydrolysis and was 70% complete after 1000 s. At 20 °C, Rad51p bound to eDNA with about the same efficiency, but dissociation of the complex occurred at one-third the rate, mirroring the slower rate of ATP hydrolysis. At 10 °C, Rad51p associated with eDNA to an even greater extent and as rapidly as at 30 °C; however, in the absence of appreciable ATP hydrolysis, the complex was relatively stable.

These results suggest that Rad51p requires association with ATP, but not hydrolysis, for DNA binding and that hydrolysis of the bound ATP causes dissociation of Rad51p from DNA.

Discussion

The strong sequence similarity between S. cerevisiae Rad51p and bacterial RecA proteins suggested early on that they perform similar or related functions in recombination (2–4). Indeed, both proteins promote strand exchange between a linear duplex DNA and a homologous circular single-stranded DNA to form a double-stranded nicked circle and a linear single strand, although the mechanistic details of their actions differ somewhat (6–8).
Rad51p Binding to DNA

We are especially interested in understanding the Rad51p-promoted reaction in greater detail, particularly the nucleotide-dependent binding of Rad51p to the two kinds of DNA substrates. To this end, we examined the binding of Rad51p to a modified single-stranded DNA, etheno-DNA (eDNA), which has been used previously to characterize RecA binding to ssDNA (13). Protein binding to eDNA is readily monitored by an appreciable increase in the fluorescence of the DNA. Our results show that Rad51p binding to eDNA is strongly dependent on the presence of ATP and Mg\(^{2+}\). At 37 °C, ADP, ATP\(\gamma\)S, and AMP-PNP cannot substitute for ATP in promoting Rad51p binding to eDNA; however, at 30 °C, in the presence of high concentrations of Rad51p, ATP\(\gamma\)S supports a low level of binding. By contrast, RecA binds to ssDNA without ATP, but ATP induces a transition to a high affinity binding form (13). The nonhydrolyzable analogs ATP\(\gamma\)S and AMP-PNP also increase the affinity of RecA for DNA, but the RecA-DNA complex is more stable in the presence of these nucleotides (15).

Under optimal conditions, the stoichiometry of Rad51p association with eDNA indicates a binding site size of 6.3 nucleotides/Rad51p and a K\(_a\) (binding affinity) of 9 × 10\(^5\) M\(^{-1}\) based on the value at half-saturated binding. This binding site size is different from the previously reported value of 3.6 nucleotides determined from studies of the strand exchange reaction (7, 8). The difference in the binding stoichiometry can be explained if the Rad51p monomer has two binding sites, each of which can bind −3 nucleotides/DNA strand. A similar difference in binding stoichiometry exists when measuring the binding stoichiometry of RecA and eDNA and the binding site size at which the DNA-dependent ATPase is maximal; these were 7.0 and 3.1 bases/RecA, respectively (17).

The greatest difference between RecA and Rad51p has been found in their ability to interact with dsDNA. RecA binds very weakly to dsDNA at pH 7.5, and the efficiency is increased substantially at low pH or with ATP\(\gamma\)S (14, 16, 24, 25). Rad51p, however, binds to dsDNA equally well in the presence of ATP and Mg\(^{2+}\) over the pH range of 6.5–8.5. ATP\(\gamma\)S and AMP-PNP are able to activate binding somewhat, but only at higher concentrations of Rad51p, whereas ADP is only weakly active. The binding site size estimated from the titration curve is −3.3 base pairs/Rad51p, with a binding affinity (K\(_a\)) of 8 × 10\(^5\) M\(^{-1}\); these values are about the same as those for RecA binding to dsDNA (14).

Binding of Rad51p to eDNA is maximal at −5 μM ATP and half-maximal at 1 μM; for binding to dsDNA, the values are about two times higher, respectively. However, because binding to ssDNA and dsDNA was measured in the presence of 1 and 2 μM Rad51p, respectively, the K\(_a\) value for ATP binding could be considerably lower. The optimal amount of Mg\(^{2+}\) is influenced by the amount of ATP; at 25–750 μM ATP, 2 mM Mg\(^{2+}\) is optimal, but with excess ATP (3 mM), maximal binding requires 4–6 mM Mg\(^{2+}\). This suggests that the active species for promoting Rad51p binding to ssDNA and dsDNA is a specific ATP-Mg\(^{2+}\) complex, which requires for its formation a large excess of Mg\(^{2+}\) over ATP. Quite possibly, however, Mg\(^{2+}\) has an additional role in the binding reaction, perhaps by its association with the DNA ligand or by action on the protein itself.

At NaCl concentrations >0.1 M, the binding of Rad51p to eDNA is inhibited. The degree of inhibition increases with increasing salt concentration until complete inhibition occurs at 0.5 M. We have noted, but not investigated further, that other anions, e.g. phosphate and sulfate, are far more inhibitory than the chloride anion. Once formed at optimal levels of salt, the Rad51p-eDNA complex is readily dissociated as the salt concentration is increased. But at any particular salt concentration, the complex is considerably more stable than would be expected from the effect of that salt concentration on complex formation.

Once formed, the Rad51p-eDNA complex is stable so long as ATP and Mg\(^{2+}\) are maintained. Removal of either causes the complex to dissociate. Addition of ADP or AMP-PNP at levels 10 times higher than the ATP level does not affect the half-life of the complex. However, the addition of ATP\(\gamma\)S to a preformed Rad51p-eDNA complex at a concentration 10 times that of the ATP concentration causes a relatively rapid dissociation of the complex. We surmise that ATP\(\gamma\)S competes with ATP for binding to Rad51p, but because the Rad51p-ATP\(\gamma\)S complex is unable to bind to eDNA, dissociation of the Rad51p-eDNA complex ensues. The results of dissociation of the Rad51p-eDNA complex formed in the presence of 2.5 μM ATP show that ATP binding, not ATP hydrolysis, is required for Rad51p binding to DNA. ATP hydrolysis serves to recycle Rad51p off the DNA. The difference in the ability of Rad51p to bind DNA in the presence of ATP and ATP\(\gamma\)S suggests that only the interaction with ATP induces the structural change needed to bind to DNA. An ATP-induced structural transition has also been proposed to activate RecA for DNA binding (13, 26).

Thus, as was the case for the strand exchange, there is only a partial similarity between Rad51p and RecA activities. Because Rad51p forms a helical filament with DNA very similar to the one formed with RecA, it is of interest to determine if ATP and Mg\(^{2+}\) are required to activate Rad51p for binding or to promote the formation of oligomers of Rad51p that then bind to DNA or whether ATP and Mg\(^{2+}\) influence the successive binding of monomers to already bound clusters of Rad51p. A relevant issue that needs to be investigated further is whether, like RecA, the association and dissociation of Rad51p with DNA occurs with a unique directionality.
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