Functional Characterization of the Precursor and Spliced Forms of RecA Protein of *Mycobacterium tuberculosis*†

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ABSTRACT: The *recA* locus of pathogenic mycobacteria differs from that of nonpathogenic species because it contains large intervening sequences nested in the RecA homology region that are excised by an unusual protein-splicing reaction. *In vivo* assays indicated that *Mycobacterium tuberculosis recA* partially complemented *Escherichia coli recA* mutants for recombination and mutagenesis. Further, splicing of the 85 kDa precursor to 38 kDa MtRecA protein was necessary for the display of its activity, *in vivo*. To gain insights into the molecular basis for partial and lack of complementation by MtRecA and 85 kDa proteins, respectively, we purified both of them to homogeneity. MtRecA protein, but not the 85 kDa form, bound stoichiometrically to single-stranded DNA in the presence of ATP. MtRecA protein was cross-linked to 8-azidoadenosine 5′-triphosphate with reduced efficiency, and kinetic analysis of ATPase activity suggested that it is due to decreased affinity for ATP. In contrast, the 85 kDa form was unable to bind ATP, in the presence or absence of ssDNA and, consequently, was entirely devoid of ATPase activity. Molecular modeling studies suggested that the decreased affinity of MtRecA protein for ATP and the reduced efficiency of its hydrolysis might be due to the widening of the cleft which alters the hydrogen bonds and the contact area between the enzyme and the substrate and changes in the disposition of the amino acid residues around the magnesium ion and the γ-phosphate. The formation of joint molecules promoted by MtRecA protein was stimulated by SSB when the former was added first. The probability of an association between the lack and partial levels of biological activity of RecA protein(s) to that of illegitimate recombination in pathogenic mycobacteria is considered.

Over the past decade, tuberculosis has been one of the most intensively researched infectious diseases consequent to its resurgence coinciding with that of the AIDS epidemic (Barnes *et al*., 1991). Because of the profound influence of major antigens on the host immune system, cloning of those antigens has been pursued as one of the approaches toward the development of a robust subunit vaccine. It is believed, therefore, that the success of this approach would benefit from the availability of recombination-deficient mutants of *Mycobacterium tuberculosis* in which the integrated DNA could be stably maintained (Young & Cole, 1988). Consequently, the ability to achieve a high frequency of homologous recombination would facilitate the understanding of pathogenesis and will permit the construction of attenuated mutants of *M. tuberculosis*.

The *rec*A gene of *M. tuberculosis* has been isolated and cloned, and when expressed, it partially complemented the defects of an *Escherichia coli recA* mutant for homologous recombination, DNA repair, and mutagenesis but not induction of phage λ (Davis *et al*., 1991, 1992). The intertwining of functional and phylogenetic preservation of size can best be illustrated by over 30 *rec*A genes cloned so far, from different organisms [reviewed by Roca and Cox (1990)]. Despite this conservation, the *M. tuberculosis* and *Mycobacterium leprae rec*A genes reveal a strikingly unusual structure; the *rec*A locus specifying a functional 38 kDa protein is comprised of a single open reading frame with the coding potential for 85 kDa and 79 kDa proteins, respectively (Davis *et al*., 1991, 1994). Further studies indicated that the 85 kDa protein undergoes an unusual protein-splicing reaction to produce mature 38 kDa MtRecA[1] protein in *E. coli* (Davis *et al*., 1992). More recently, it has been shown that, unlike the *M. tuberculosis Rec*A, the *M. leprae* 79 kDa RecA precursor was completely spliced in itself but not in *E. coli* (Davis *et al*., 1994).

The prototype *E. coli* RecA protein has been an exemplar for genetic, enzymological, and structural understanding of the process of homologous genetic recombination (Kowalczykowski *et al*., 1994; West, 1992; Radding, 1991; Griffith & Harris, 1988). The staging of all the reactions promoted by RecA protein *in vitro* is dependent on its binding to single-stranded DNA, in the presence of ATP or ATPγS, for

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[1] Abbreviations: form I DNA, supercoiled closed circular M13 DNA as isolated from *Escherichia coli*; ssDNA, circular single-stranded DNA; ATPγS, adenosine 5′-*O*-(thiotriphosphate); DTT, dithiothreitol; BSA, bovine serum albumin; bp, base pairs; MtRecA protein, spliced form of RecA protein of *Mycobacterium tuberculosis*; EcRecA protein, RecA protein of *E. coli*; SSB, single-stranded DNA-binding protein of *E. coli*.

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production of an active nucleoprotein filament [reviewed by Kowalczykowski *et al*. (1994), Egelman (1993), Radding (1991), and Roca and Cox (1990)]. The nucleoprotein filament promotes homologous pairing with linear duplex DNA in three kinetically distinguishable phases: (i) presynapsis, which involves the cooperative binding of RecA protein onto ssDNA; (ii) synapsis, during which the nucleoprotein filament searches for homology and subsequently establishes synapsis to produce either paranemic or plectonemic joint molecules; and (iii) strand exchange, the unidirectional displacement of an identical strand from DNA to generate extended lengths of heteroduplex DNA [reviewed by Radding (1991), West (1992), and Kowalczykowski *et al*. (1994)]. With respect to the generality of these observations, recent studies have shown phylogenetic conservation in the structure as well as in the overall mechanism of homologous recombination (West, 1992; Kowalczykowski & Eggelston, 1994). By contrast, the pathogenic mycobacteria use a biologically unique mechanism by producing an 85 kDa precursor and 38 kDa (MtRecA) mature forms of RecA protein whose biochemical activities are unknown.

We have purified both 85 and 38 kDa (MtRecA) RecA proteins of *M. tuberculosis* to ascertain the biochemical defect caused by the protein "intein" (Colston & Davis, 1994) on the activity of the 85 kDa protein and the molecular basis for the partial levels of complementation by MtRecA protein. Here we show that the 85 kDa protein is fully defective with respect to DNA-binding, ATP-binding, and ATPase activities, while MtRecA protein is partially defective in the latter two activities but proficient in homologous pairing.

EXPERIMENTAL PROCEDURES

Biochemicals, Enzymes, and DNA. *E. coli* RecA protein (Griffith & Shores, 1985) and SSB (Lohman *et al*., 1986) were purified, and their concentration (Tsang *et al*., 1985) was determined as described. *E. coli* RecA protein antisera were prepared in rabbits by using standard procedures. Form I DNA (negatively supercoiled) and circular single-stranded DNA from bacteriophages M13 and ΦX174 were prepared as described (Cunningham *et al*., 1980). Linear duplex [^{3}H]-DNA was prepared by cleaving form I M13 DNA by *Xho*I or *Bam*HI in reaction conditions as suggested by the manufacturer. The reactions were terminated with an excess of ethylenediaminetetraacetic acid (EDTA), phenol-extracted, and dialyzed. The concentration of DNA is expressed as moles of nucleotides. ATPγS was obtained from Boehringer-Mannheim. Nucleoside 5′-triphosphates were purchased from Pharmacia LKB Biotechnology Ltd. Single-stranded 47-mer DNA was synthesized using a Pharmacia gene assembler, and its sequence is as follows: 5′GATCT-CAATGGTTGTGGAATTCAGAATAGCTACT-TCTCCATTAATCA3′.

Purification of RecA Proteins of M. tuberculosis. *E. coli* strain KM4104 containing the plasmid pEJ135 (Davis *et al*., 1991) was grown at 37 °C in 6 L of Luria-Bertani (LB) medium in the presence of 100 μg/mL ampicillin. When the density of cells reached 0.4 at A_{595} (after about 2 h growth), we added IPTG to a final concentration of 5 mM, and the cells grew for an additional 6 h. The cells were harvested by centrifugation at 4 °C, and the pellet (36 g) was resuspended in 50 mM Tris-HCl (pH 8.0) containing 10% (w/v) sucrose, quickly frozen, and stored at −20 °C. All the steps during purification were carried out at 4 °C. To the frozen cell suspension, thawed overnight at 4 °C, were added NaCl and lysozyme to a final concentration of 0.1 M and 0.2 mg/mL, respectively, and the suspension was incubated for 30 min. The cell suspension was then sonicated in a pulse mode power setting at 8 for 10 min with an ultrasonic processor (Heat Systems, Switzerland) with an attached megaprobe. Cell debris was removed by centrifugation at 26 000 rpm for 75 min in a Beckman Ti45 rotor. To the supernatant, Polymin-P (pH 7.9) was added to a final concentration of 0.6% with continuous stirring over a period of 30 min. The pellet, collected by centrifugation at 10 000 rpm for 10 min in a SS34 rotor, was resuspended in 120 mL of buffer A [20 mM Tris-HCl (pH 7.5), 10% sucrose, 0.1 mM EDTA, and 5 mM 2-mercaptoethanol] containing 0.25 M NaCl. The solution was stirred for 30 min and centrifuged at 10 000 rpm for 10 min. The pellet was extracted with buffer A containing 0.7 M NaCl, and the supernatant was obtained by centrifugation as described above. Solid ammonium sulfate (0.42 g/mL) was added to the supernatant over a period of 30 min. After the solution was stirred for 30 min, the precipitate was collected by centrifugation at 10 000 rpm for 10 min. The pellet was resuspended in buffer B [20 mM potassium phosphate (pH 6.5), 10% glycerol, 0.1 mM EDTA, and 5 mM 2-mercaptoethanol] containing 0.2 M KCl and dialyzed against the same buffer (two changes of 1 L each) over a period of 18 h. The dialysate was loaded onto a phosphocellulose column (1.5 × 14 cm) that had been equilibrated with buffer B containing 0.2 M KCl. Under these conditions, 85 kDa and MtRecA proteins were eluted in the unadsorbed fractions and these were combined and dialyzed against 4 L of buffer C [20 mM Tris-acetate (pH 7.5), 10% glycerol, 0.1 mM EDTA, and 5 mM 2-mercaptoethanol] over a period of 18 h. MtRecA protein in the dialysate was microcrystallized by the addition of spermidine acetate (pH 7.5) to a final concentration of 7 mM. After incubation on ice for 60 min, the pellet was collected by centrifugation at 10 000 rpm for 10 min. The pellet contained MtRecA protein, and the 85 kDa protein was in the supernatant. The pellet was resuspended in 3.5 mL of buffer C containing 1 M NaCl and was loaded onto a column of Sephacryl S-200 (1.8 × 135 cm) that had been equilibrated with buffer C containing 1 M NaCl. The column was eluted with the same buffer, and fractions of 2 mL were collected at a flow rate of 12 mL/h. The peak fractions containing MtRecA protein were combined, dialyzed overnight against 2 L of a buffer containing 20 mM Tris-acetate (pH 7.5), 20% glycerol, 0.1 mM EDTA, and 1 mM DTT, and stored frozen at −70 °C.

The supernatant which contained 85 kDa protein was dialyzed against 2 L of buffer B containing 0.2 M KCl. The dialysate was applied onto a Biogel-HTP column (1.5 × 10 cm) which had been equilibrated with buffer B containing 0.2 M KCl. The column was washed with equilibration buffer until the A_{280} of the eluate dropped to 0.02. The 85 kDa protein was eluted with a linear gradient of 20 to 150 mM potassium phosphate (pH 6.5) in buffer B. The fractions containing 85 kDa protein were combined and concentrated by using a Centricon 30 microconcentrator. The concentrated sample was layered on a gradient of 15 to 35% glycerol prepared in a buffer containing 20 mM potassium phosphate (pH 6.5), 0.1 mM EDTA, 0.4 M KCl, and 0.1% Triton X-100. The gradients were centrifuged in a Beckman SW41

rotor at 40 000 rpm for 10 h. Fractions of 0.5 mL were collected and analyzed on SDS−polyacrylamide gels. Fractions containing 85 kDa protein were combined and dialyzed against buffer B. The 85 kDa protein was purified further by chromatography on a Bio-Rex 70 cation exchange column (1 × 6.5 cm) which had been equilibrated with buffer B. The column was washed with buffer B and then eluted with a linear (20 to 500 mM) phosphate gradient in buffer B. The 85 kDa protein was eluted at 180 mM phosphate in a homogeneous form. Fractions containing 85 kDa protein were pooled and concentrated by using a Centricon 30 microconcentrator, dialyzed against buffer B, and stored at 4 °C. From this procedure, we obtained 14 mg of MtRecA protein and 33 μg of the 85 kDa protein which were devoid of detectable exo- and endonuclease activity.

Protein Analysis. Protein concentrations were determined by the method of Bradford (1976) using BSA as the standard. An extinction coefficient for MtRecA protein was determined as described for EcRecA protein (Tsang *et al*., 1985). The value for the extinction coefficient of ϵ_{280} (after correction for light scattering) is 0.482. SDS−polyacrylamide gel electrophoresis (SDS−PAGE) was done as described (Laemmli, 1970). The gel was stained by Coomassie blue R-250. Alternatively, the proteins were transferred to a nitrocellulose filter and were detected using antiserum to *E. coli* RecA protein by chemiluminescence (Schneppenheim & Rautenberg, 1987).

Mobility Shift Assay for DNA Binding. Oligonucleotides were labeled at the 5′ end using polynucleotide kinase and [γ-^{32}P]ATP. The mobility shift assays were performed as described (Ye & Samuels, 1987). Reaction buffer (20 μL) contained 30 mM Tris-HCl (pH 7.5), 2.2 or 12 mM $MgCl_2$, 1.4 mM DTT, 0.1 mM ATPγS or 0.1 mM NTPs (where mentioned), and the indicated amounts of 85 kDa or MtRecA protein(s). An identical reaction was performed with EcRecA protein as a positive control. The stock solutions of these proteins were diluted before use in the reaction buffer. After incubation at 37 °C for 2.5 min, reaction was terminated by the addition of 3 μL of loading buffer [binding buffer with 20% glycerol and 0.12% (w/v) bromophenol blue and xylene cyanol]. Aliquots were immediately loaded onto a polyacrylamide gel (9%) and subjected to electrophoresis at 10 °C in 89 mM Tris-acetate buffer (pH 7.5) for 4 h. The voltage across the gel was 12 V/cm both during prerun (1 h) and after the samples were loaded. After electrophoresis, the gels were dried and visualized by autoradiography.

Nitrocellulose Filter Binding Assay. A filter binding assay with KOH-treated nitrocellulose filters was done as described (McEntee *et al*., 1981). Standard reaction mixtures (20 μL) contained the binding buffer as described above with the indicated amounts of RecA protein. Reaction mixtures were incubated at 37 °C for 10 min. Aliquots (8 μL) taken at the indicated time intervals were diluted with 3 mL of ice-cold buffer containing 10 mM Tris-HCl (pH 7.5), 50 mM KCl, and 20 mM $MgCl_2$. The sample was filtered immediately on a nitrocellulose filter under vacuum. The filter was washed with 6 mL of ice-cold dilution buffer and dried under a heat lamp. The amount of radioactivity bound to the filters was determined by scintillation counting. The data shown are the means of at least three independent determinations.

Photolabeling of RecA Proteins. UV cross-linking of azido-ATP to RecA proteins was performed as described (Knight & McEntee, 1985; Kowalczykowski, 1986). Briefly, reaction mixtures (50 μL) contained 20 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 20 mM KCl, 1 μM M13 ssDNA, 2.5 μM EcRecA, MtRecA, or 85 kDa proteins, and the indicated concentrations of γ-^{32}P-labeled azido-ATP (ICN Biomedicals, U.S.A.). Reaction mixtures were incubated at 4 °C for 60 min, and cross-linking was accomplished by using a middle-wave UV lamp (Model UVG-11, UVP Inc., San Gabriel, CA). Irradiation of samples was done from a height of 12 cm for 5 min at 4 °C. Following irradiation, the samples were held on ice for 30 min and then subjected to SDS−polyacrylamide gel (10%) electrophoresis (Laemmli, 1970). The gel was washed extensively with distilled water, to remove free azido-ATP, and dried, and the cross-linked complexes were visualized by autoradiography. The autoradiogram was quantitated by scanning with an LKB Ultroscan XL laser densitometer.

NTPase Assay. The assay was done as described (Weinstock *et al*., 1981). Reaction mixtures (20 μL) contained 35 mM Tris-HCl (pH 7.5), 90 μg/mL BSA, 12 mM $MgCl_2$, 1.5 mM DTT, 0.8 μM 85 kDa or MtRecA proteins, and the indicated concentrations of [α-^{32}P]dATP or [γ-^{32}P]ATP, in the presence (3 μM) or absence of M13 ssDNA. An identical reaction mixture containing EcRecA protein was included as a positive control. Reaction mixtures were incubated at 37 °C for 30 min. The reaction was stopped by the addition of a mixture (8 μL) containing 3 mM ADP, 3 mM unlabeled ATP, and 25 mM EDTA. Aliquots (5 μL) were spotted on polyethylene−iminecellulose strips containing markers and developed in 0.5 M NaH_2PO_4. The bands were identified with an ultraviolet lamp, cut out, and counted in scintillation solvent. In this system, ATP bands near the origin, ADP migrates about halfway to the solvent front, and P_i runs ahead of ADP.

Nitrocellulose Filter Binding Assay for Joint Molecules. The assay was done as described (Ramdas *et al*., 1989). Reaction mixtures contained 33 mM Tris-HCl (pH 7.5), 1 mM DTT, 1.5 mM ATP, 6 mM phosphocreatine, 12 mM $MgCl_2$, 10 units of phosphocreatine kinase/mL, and 0.1 mg/mL BSA. M13 ssDNA (12 μM) was incubated with 4 μM MtRecA or EcRecA protein for 5 min at 37 °C and then with 0.75 μM SSB to form nucleoprotein filaments of RecA protein−ssDNA. After 10 min, the formation of joint molecules was initiated by the addition of 7.5 μM M13 Goril DNA (prepared by digestion of form I DNA with *Eco*RI). At the indicated time intervals, aliquots were taken and added to 5 mL of ice-cold 0.15 M sodium citrate/1.5 M NaCl (pH 7.0) solution. The samples were filtered immediately on nitrocellulose filters (Sartorius, 0.45 μm), washed with 6 mL of the above solution, and dried under a heat lamp. The bound radioactivity was determined by liquid scintillation.

Molecular Modeling. The three-dimensional structure of MtRecA protein was built using the homology modeling technique (HOMOLOGY, Biosym Technologies, v 2.3.0) based on the crystal structure of EcRecA protein (Story *et al*., 1992) and the sequence alignment of Davis and coworkers (1991). The framework for model building included amino acid residues 3−156, 165−194, and 211−328. The amino acid residues in other regions were not included since they were found to be disordered in the crystal structure (Story *et al*., 1992).

Model building and energy minimization were carried out using HOMOLOGY and DISCOVER packages interfaced with INSIGHT-II (Biosym technologies, v 2.3.0 and 2.9.5).

A rotamer search was carried out for the side chains that differed from the *E. coli* sequence using the Ponder and Richards library (1987). A 5 Å shell of water molecules was added to surround the protein molecule, and the whole structure was subjected to energy minimization. ADP was docked into the minimized apo-structure of EcRecA and MtRecA proteins on the basis of the 3 Å crystal structure of the EcRecA−ADP complex (Story & Steitz, 1992), while in studies involving ATP, the γ-phosphate and the magnesium ion were placed as in the model proposed by Story and Steitz (1992). A distance constraint with a force constant of 10 kcal mole^{-1} Å^{-2} was applied to maintain the coordination distance between magnesium and an oxygen each of β- and γ-phosphates as well as with the side chain of Thr-73. These complexes were then subjected to energy minimization, again with a 5 Å shell of water molecules around them.

All energy minimizations were carried out with a 13 Å nonbonded cutoff and a dielectric constant of 1.0, initially using the steepest descent algorithm followed by conjugate gradients till the root mean square (rms) derivative was less than 0.4 kcal mol^{-1} Å^{-1}. An identical minimization with EcRecA protein was carried out as a control in every case.

RESULTS

Overexpression and Purification of M. tuberculosis RecA Proteins. Induction of KM4104, a Δ*recA* strain harboring the plasmid pEJ135, containing *M. tuberculosis recA* under *lacZ* promoter resulted in the appearance of an intense band of a polypeptide corresponding to the size of the prototype *E. coli* RecA protein. However, consistent with earlier observations (Davis *et al.*, 1991), there was no band corresponding to the size of the 85 kDa protein. Therefore, we tested a series of different induction protocols and found substantial accumulation of both 85 kDa and MtRecA proteins, only when cells were induced and harvested during the exponential phase of their growth (data not shown). The scheme developed for the purification of the 85 kDa and MtRecA proteins is described in detail in Experimental Procedures. Since the activity of the 85 kDa protein is unknown, Coomassie-stained gels and Western blotting, using antibodies to EcRecA protein, were used to guide the progress of purification. The final preparations of 85 kDa and MtRecA proteins were homogeneous as judged by electrophoresis on SDS−polyacrylamide gels. The identity of purified proteins was established by their cross-reaction with *E. coli* anti-RecA protein antibodies (Figure 1).

Interaction of 85 kDa and MtRecA Proteins with DNA. The purified 85 kDa and MtRecA proteins were tested to ascertain several functions that have been firmly established for the prototype EcRecA protein [reviewed in Kowalczykowski and Eggelston (1994), West (1992), Radding (1991), and Roca and Cox (1990)]. Among the activities tested are their ability to bind to 8-azido-ATP, propensity to interact with single- and double-stranded DNA, ssDNA-dependent hydrolysis of ATP, and homology-dependent formation of joint molecules.

The DNA-binding activity of 85 kDa and MtRecA proteins was tested, in comparison with that of EcRecA protein, by using two independent assays. First, we quantitated the extent of binding of these proteins, in the presence of ATPγS, to ^{32}P-labeled 47-mer DNA on nitrocellulose filters in a protein-based assay (Weinstock *et al.*, 1981). The rationale

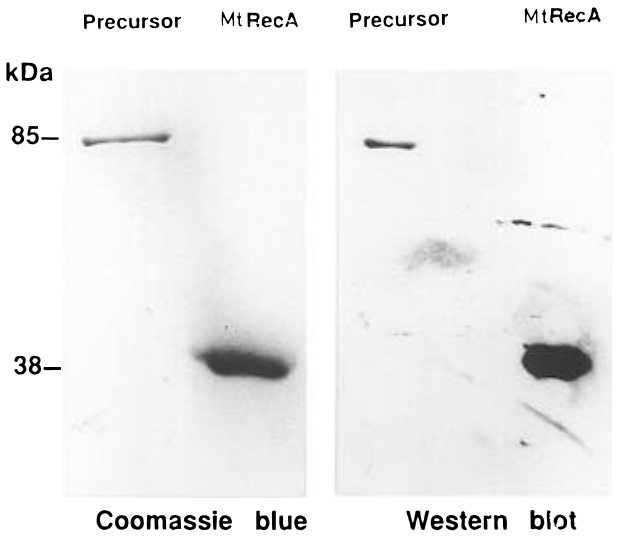

FIGURE 1: Electrophoretic analysis of RecA proteins of *M. tuberculosis*. See Experimental Procedures for details. Twenty micrograms each of the 85 kDa protein and MtRecA protein were analyzed on SDS−polyacrylamide gels. Proteins were visualized by staining with Coomassie blue (left panel) and by Western blot (right panel) using antibodies raised against *E. coli* RecA protein as described in Experimental Procedures.

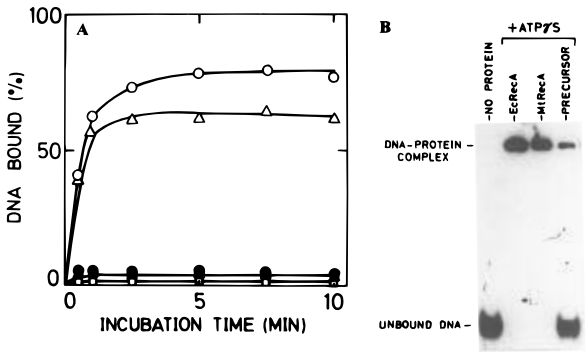

FIGURE 2: Single-stranded DNA-binding activity of *E. coli* and *M. tuberculosis* RecA proteins. (A) Nitrocellulose filter binding assay. Each of the RecA proteins (5 μM) was separately incubated with ^{32}P-labeled 47-mer DNA (15 μM), in the presence of 0.1 mM ATPγS as described in Experimental Procedures. Aliquots (20 μL) were removed, at the indicated time intervals, diluted with 3 mL of a buffer containing 10 mM Tris-HCl (pH 7.5), 50 mM NaCl, and 12 mM $MgCl_2$, and immediately filtered on nitrocellulose filters. The bound radioactivity was measured by liquid scintillation. Reactions done in the presence of ATPγS were (○) EcRecA protein, (△) MtRecA protein, and (□) 85 kDa protein. Reactions done in the absence of ATPγS were (●) MtRecA protein and (▲) 85 kDa protein. The data shown are the average of three independent determinations. (B) Mobility shift assay. The reaction mixtures (20 μL) contained the indicated RecA protein (5 μM), 15 μM ^{32}P-labeled 47-mer DNA, and 0.1 mM ATPγS. The samples were incubated and processed as described in Experimental Procedures. The protein−DNA complexes were electrophoretically separated from unbound ^{32}P-labeled DNA on polyacrylamide gels and visualized by autoradiography.

for these experiments is that EcRecA protein binds ATPγS with a very high affinity, and the use of an oligonucleotide as the substrate, with a minimum potential to form secondary structure, would make interpretations of results less complicated. Figure 2A shows the time-course of binding of EcRecA, 85 kDa, and MtRecA proteins, in the presence and absence of 0.1 mM ATP or ATPγS, to ^{32}P-labeled 47-mer DNA. The binding of both EcRecA and MtRecA proteins, under identical conditions, increased with time until a plateau was achieved by approximately 2.5 min. These data on

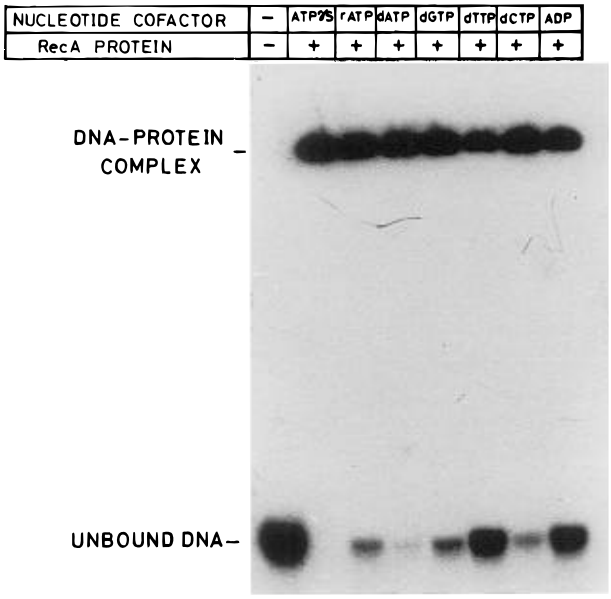

FIGURE 3: Nucleotide cofactor specificity of binding of MtRecA protein to single-stranded DNA. The DNA-binding activity was measured in the presence of the indicated NTPs (0.1 mM) as described in the legend to Figure 2B. After electrophoresis, the gel was dried and the DNA−protein complexes were visualized by autoradiography.

binding of EcRecA protein with 47-mer DNA as the substrate are similar to those obtained using other techniques [reviewed by West (1992) and Kowalczykowski *et al*. (1994)]. By contrast, under these conditions, in the presence or absence of ATPγS, 85 kDa protein showed no affinity for 47-mer DNA. Increasing the concentration of ATP to 1 mM showed no effect on the kinetics of binding of the respective proteins to DNA (data not shown). In titration experiments, we found that MtRecA protein binds to ssDNA at a stoichiometric ratio of one monomer per three nucleotide residues, similar to that of EcRecA protein (data not shown).

It is important to be certain that the 85 kDa protein−DNA complexes have not dissociated during binding and/or subsequent washing of nitrocellulose filters. Accordingly, parallel experiments were carried out to quantitate the binding using nondenaturing polyacrylamide gel electrophoresis. The basic premise of this method is that protein-bound DNA is retarded in mobility compared to unbound DNA. The results of a representative autoradiogram of a gel showing nucleoprotein complexes formed by all three proteins are illustrated in Figure 2B. While in the case of EcRecA and MtRecA proteins all of the ^{32}P-labeled 47-mer DNA was present in the form of nucleoprotein complexes, less than 10% was obtained with 85 kDa protein. Under identical conditions, 85 kDa protein did not bind to 230 bp ^{32}P-labeled duplex DNA (data not shown). Taken together, these results suggest that 85 kDa protein is quantitatively defective in binding to DNA.

Specificity of Nucleotide Cofactor for Binding of 85 kDa and MtRecA Proteins to Single-Stranded DNA. We next examined the relative affinity of MtRecA protein for ssDNA in the presence of a variety of nucleotide cofactors. We incubated stoichiometric amounts of MtRecA protein and ^{32}P-labeled 47-mer DNA with the indicated nucleotide cofactor(s). As shown in Figure 3, several nucleotide cofactors supported the binding of MtRecA protein to 47-mer DNA, albeit to a lesser extent, compared to ATPγS;

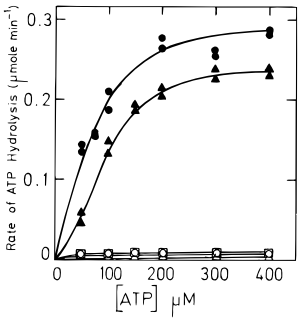

FIGURE 4: Comparison of EcRecA and MtRecA protein-catalyzed ssDNA-dependent ATP hydrolysis. ATPase activity was measured as described in Experimental Procedures. The data shown are the means of five independent experiments. Reactions done in the presence of ssDNA were (●) EcRecA proteins, (▲) MtRecA protein, and (□) 85 kDa protein. Reactions performed in the absence of ssDNA were (○) EcRecA protein and (△) MtRecA protein.

dATP supported more than 90% of binding activity while rATP, dGTP, and dCTP to an extent of approximately 80%. The other nucleotides examined, dTTP and ADP, supported to about half the efficiency of that of ATPγS. Since the simplest explanation for the lack of binding of 85 kDa protein to 47-mer DNA (Figure 2) is perhaps the presence of ATPγS, we performed assays under identical conditions in the presence of several natural nucleotide cofactors. The presence or absence of ribo- or deoxyriboside triphosphates, in the binding reaction, had no discernible effect on the binding of 85 kDa protein to single- or double-stranded DNA in the pH range of 7.5−10 (data not shown).

DNA-Dependent NTPase Activity of 85 kDa and MtRecA Proteins. Previous studies have shown that *E. coli* RecA protein hydrolyzes several nucleoside triphosphates, in the presence of DNA (Roberts *et al*., 1978; Ogawa *et al*., 1978); therefore, we wished to determine the NTPase activities of 85 kDa and MtRecA proteins. Figure 4 shows the rate of ATP hydrolysis as a function of substrate concentration, in the presence and absence of M13 ssDNA, by EcRecA, 85 kDa, and MtRecA proteins. Interestingly, the K_m for ATP was 179 μM in the case of MtRecA protein, approximately 3-fold higher than the K_m of 58 μM for EcRecA protein. Consistent with that of EcRecA protein, ATPase activity of MtRecA protein was not evident in the absence of ssDNA. In parallel experiments, we determined, under steady state conditions, the k_{cat} for ATP hydrolysis by EcRecA and MtRecA proteins. In agreement with earlier studies, we found that EcRecA protein hydrolyzed ATP with a monomer k_{cat} of 30 min^{-1} (Schutte & Cox, 1987; Menetski *et al*., 1988), while MtRecA protein hydrolyzed ATP with a monomer k_{cat} of 11 min^{-1}. To test whether 85 kDa protein catalyzed hydrolysis of ATP, we measured its ATPase activity under identical conditions. In contrast to the case of MtRecA protein, there was no discernible ATP hydrolysis by 85 kDa protein in the presence or absence of ssDNA. The extent of ATP hydrolysis in a wide range of pH (6−9) and Mg^{2+} concentrations (2−10 mM) was similar with either dATP or rATP as substrates, eliminating the possibility that the reaction conditions used are conducive only to EcRecA but not to MtRecA protein (data not shown). As described above, in addition to dATP, several NTPs supported the binding of MtRecA protein to ssDNA. To gain further insight into the relationship between NTP-dependent DNA binding and NTPase activity, we performed steady state

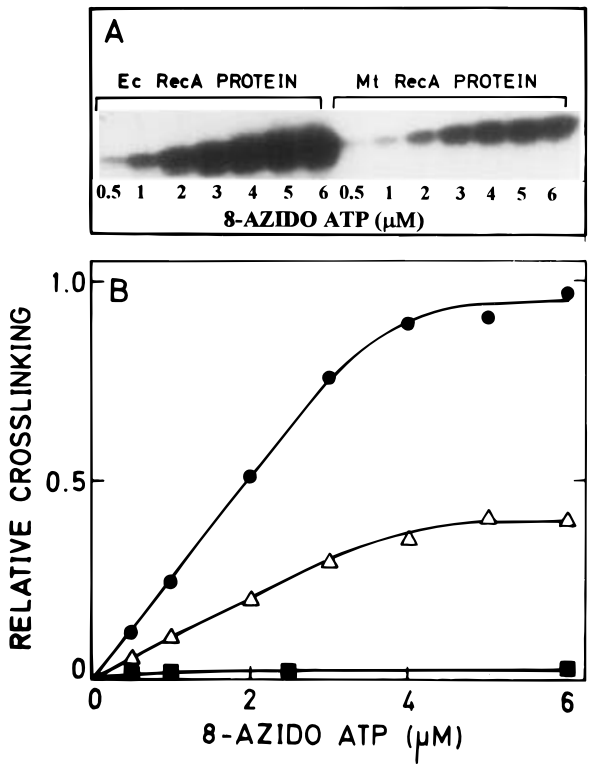

FIGURE 5: Photolabeling of EcRecA and MtRecA proteins as a function of azido-ATP concentration. Reaction mixtures contained the indicated amounts of azido-ATP and 2.5 μM each of the RecA proteins. Cross-linking was done as described in Experimental Procedures. (A) SDS−PAGE analysis of photolabeled RecA proteins. Under these conditions, photolabeling was not detectable with the 85 kDa protein and therefore is not shown. (B) Densitometric scanning of the autoradiogram: (●) EcRecA protein, (△) MtRecA protein, and (■) 85 kDa protein.

kinetic analysis of dCTP hydrolysis catalyzed by MtRecA protein. We have observed that the rate of hydrolysis promoted by MtRecA protein for dCTP was 4-fold less than that of EcRecA protein (data not shown).

Specific Binding of Azido-ATP to 85 kDa and MtRecA Proteins. Although the 85 kDa protein was devoid of ATPase activity, it is conceivable that it could bind ATP but is defective in catalysis. To investigate this possibility, we carried out standard reactions with increasing concentrations of azido-ATP, in the presence of ssDNA, and just prior to the termination of the reaction, azido-ATP was cross-linked to RecA protein(s) as described (Knight & McEntee, 1985; Banks & Sedgewick, 1986; Kowalczykowski, 1986). The resulting adduct was visualized by autoradiography after electrophoresis on SDS−polyacrylamide gels. As depicted in Figure 5A, with both EcRecA and MtRecA proteins, the extent of cross-linking increased with increasing azido-ATP concentration; however, the latter bound azido-ATP less efficiently than the former. Under identical conditions, 85 kDa protein did not bind azido-ATP, even after prolonged exposure (data not shown). To obtain an estimate of relative binding, the autoradiogram was scanned by densitometry, and the results are shown in Figure 5B. A comparison of the extent of photolabeling revealed that the binding of azido-ATP to EcRecA protein is close to the values reported previously (Kowalczykowski, 1986). However, binding of azido-ATP to MtRecA protein was reduced by approximately 60%. By contrast, 85 kDa protein showed absolutely no affinity for azido-ATP. Under these conditions, photolabeling of both MtRecA and EcRecA proteins was abolished in the presence of 0.1 mM unlabeled ATP or when BSA was substituted for EcRecA or MtRecA proteins (data not shown). We surveyed a variety of conditions such as pH, ionic conditions, and Mg^{2+} concentration and found that the conditions described in Experimental Procedures resulted in the optimal binding of azido-ATP (data not shown).

RecA Protein−Nucleotide Interaction at the Molecular Level. The EcRecA protein monomer in the crystal and the energy-minimized model of the molecule and that of MtRecA protein have the same overall structure. As expected, the deviations between the two models are observed in surface loops and chain termini. The interesting differences are, however, in the nucleotide-binding site, although the amino acid residues in this site are substantially conserved. These differences between EcRecA and MtRecA proteins have nearly the same nature in the models of the apo-enzyme and the ADP and ATP complexes. A comparison of the ADP complexes, illustrated in Figure 6, exemplifies them.

The nucleotide-binding site can be roughly divided into three regions (Figure 6): the phosphate-binding loop (P loop), comprised of amino acid residues 67−74, which interact primarily with the phosphate group (P); residues 100 and 103, involved in interactions with the base (B); and amino acid residues 240 and 262−264, which interact with the sugar hydroxyls (S) (the residue numbering corresponding to EcRecA sequence is used throughout). To estimate differences between regions, it is convenient to consider the centroid of all atoms which interact with ADP in one or more of the models, in each region. The inter-region distances estimated using centroids, and listed in Table 1, clearly show that the nucleotide-binding cleft is wider in MtRecA than in the EcRecA protein. In the EcRecA−ADP complex, 262-O, 263-O, and 264-N form hydrogen bonds with the O2′ hydroxyl of the sugar, while the side chain hydroxyl of Tyr-264 forms a hydrogen bond with the O3′ hydroxyl of the sugar. These hydrogen bonds do not exist in the MtRecA−ADP complex. The same is true about the MtRecA−ATP complexes, thus indicating weaker nucleotide binding.

Estimation of contact areas of ADP and ATP with the protein also leads to the same conclusion. In each case, the contact area was taken as the loss in solvent accessible area, calculated using the Connolly algorithm (Connolly, 1983), of the protein on nucleotide binding. In the case of ADP, the contact areas in the complexes with EcRecA and MtRecA proteins are 193 and 90 Å^2, while in the case of ATP, the contact areas are 111 and 67 Å^2, respectively. The interaction energies of ADP with EcRecA and MtRecA are −90 and −74 kcal/mol, respectively, while those of ATP complexes are −247 and −205 kcal/mole. These values also point to weaker nucleotide binding by MtRecA protein. Another set of interesting differences pertain to the neighborhood of the magnesium ion, and the terminal phosphates in ATP in the minimized structures of its complexes with EcRecA and MtRecA are shown in Figure 7. In the model proposed by Story and Steitz (1992), which formed the basis for energy minimization, an oxygen from each of the β- and γ-phosphates and the side chain oxygen of Thr-73 coordinate to the magnesium ion. During minimization, the ion moved to a different site where it is in contact with two oxygens of γ-phosphate and OE1 of Gln-194, in the EcRecA−ATP complex, while it remained nearly at the original position in

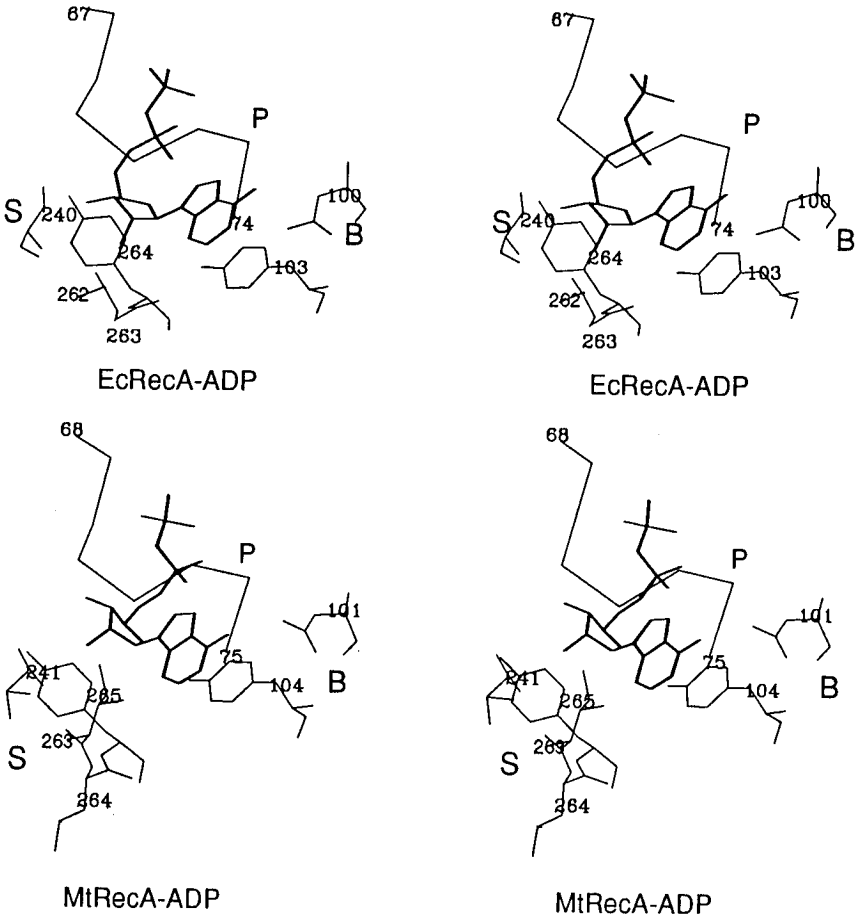

FIGURE 6: Stereoviews of the ADP-binding region in EcRecA protein−ADP and MtRecA protein−ADP complexes. The P loop is represented by α-carbons. The S and B regions are represented by main chain atoms and side chain atoms which interact with ADP. It may be noted that the residue number *n* in EcRecA protein corresponds to $n + 1$ in the MtRecA protein. ADP is highlighted in boldface.

Table 1: Distances (Å) among Different Regions of the Nucleotide-Binding Site in Different Structures[a]

distance	P−B	P−S	B−S
EcRecA protein	9.9	9.7	7.0
EcRecA protein−ADP	11.3	9.9	7.5
EcRecA protein−ATP	10.7	9.3	7.7
MtRecA protein	10.3	11.3	9.3
MtRecA protein−ADP	10.6	11.5	9.1
MtRecA protein−ATP	11.0	11.6	9.1

[a] See text for details.

the MtRecA−ATP complex. The calculations were repeated with magnesium at a random initial position where it is in contact only with an oxygen of γ-phosphate, but the same final results ensued. Thus, it appears that the magnesium ion has different preferred positions in the EcRecA and MtRecA proteins.

As is evident from Figure 6, other differences also exist in the disposition of residues in the neighborhood of the terminal phosphates of ATP. The catalytic and allosteric model proposed by Story and Steitz (1992) involves the activation of a water molecule for an in-line attack of the γ-phosphate. ATP hydrolysis destroys the hydrogen bond between the nucleotide and the side chain of Gln-194, which in turn would lead to a conformational change in the adjacent DNA-binding region. Despite the change in the position of the magnesium ion, the disposition of amino acid residues 96 and 194 in the minimized model of the EcRecA−ADP complex is such that the mechanism suggested by Story and Steitz (1992) is possible. In the MtRecA−ATP complex, however, the side chains of amino acid residues 96 and 194 move away from the nucleotide, making the proposed mechanism much less efficient.

ATP-Dependent Homologous Pairing between Single-Stranded and Linear Duplex DNA Promoted by MtRecA Protein. To examine the ability of MtRecA protein to promote homologous pairing, we preincubated stoichiometric amounts of MtRecA protein with M13 or ΦX174 circular single-stranded DNA and then added M13 Goril linear duplex [^{3}H]DNA. Homologous combination of substrates produced joint molecules, whereas heterologous combination did not (Figure 8). It has been established that the *E. coli* SSB contributes to the activities of RecA protein, and UvsX protein, through its effects at the level of ssDNA and not because of specific protein−protein interactions (Muniyappa *et al*., 1984; Kowalczykowski *et al*., 1987; Kodadek, 1990). However, in the absence of SSB, the extent of formation of joint molecules by EcRecA and MtRecA proteins was 48 and 28%, respectively. Consistent with earlier observations, in the presence of SSB, EcRecA protein produced more than 90% joint molecules. Correspondingly, we determined whether a helix-destabilizing protein would potentiate the formation of joint molecules by MtRecA protein. To this end, we preincubated M13 ssDNA with MtRecA protein, and then with SSB, prior to the addition of linear duplex DNA to start the reaction. As shown in Figure 8, SSB stimulated the formation of joint molecules with kinetics reminiscent of that of EcRecA protein.

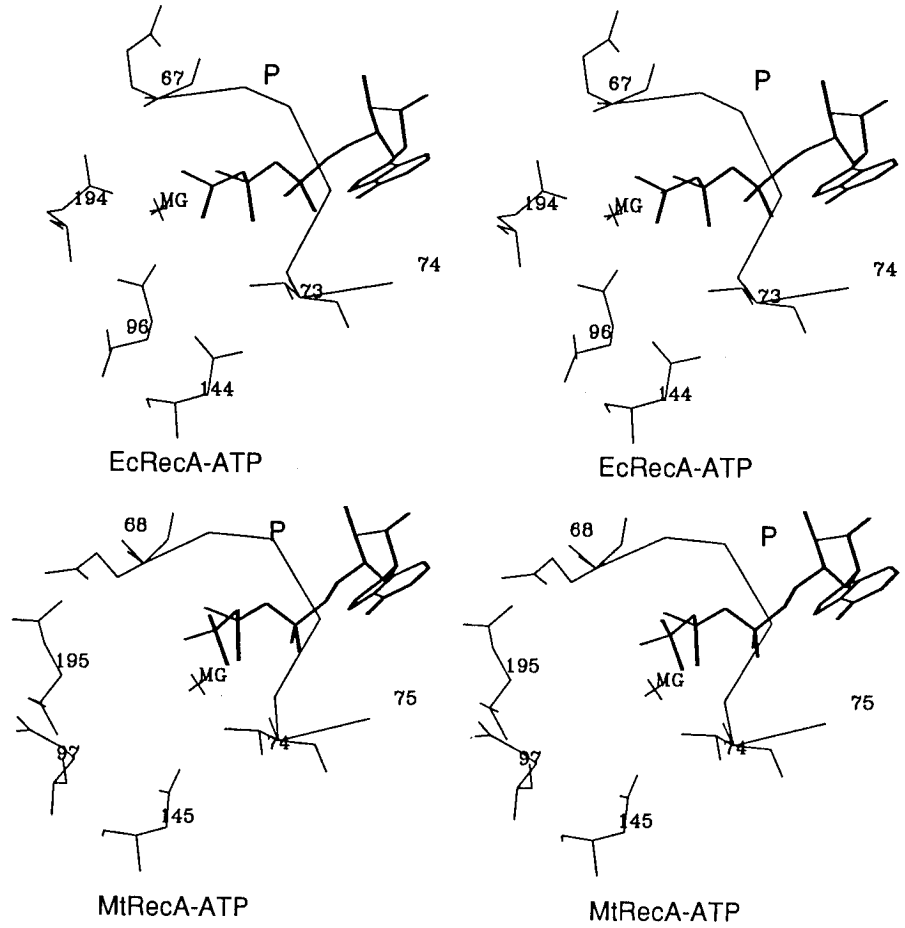

FIGURE 7: Neighborhood of the γ-phosphate and the magnesium ion in the complexes of ATP with EcRecA and MtRecA proteins. Residue number n in EcRecA corresponds to $n + 1$ in MtRecA protein. ATP is highlighted in boldface. MG is magnesium.

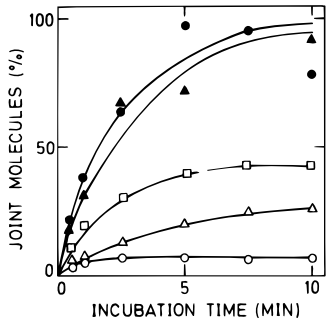

FIGURE 8: Comparison of the formation of joint molecules promoted by EcRecA and MtRecA proteins. The preparation of nucleoprotein filaments comprised of RecA protein-M13 ssDNA (or ΦX174 ssDNA) in an ATP regeneration system is described in Experimental Procedures. The formation of joint molecules was initiated by the addition of M13 linear duplex [^{3}H]DNA. At the indicated time intervals, aliquots were removed, added to 5 mL of ice-cold 1.5 M NaCl/0.15 M sodium citrate solution, and filtered immediately: (□) EcRecA protein alone, (●) EcRecA protein in the presence of *E. coli* SSB, (△) MtRecA protein alone, (▲) MtRecA protein in the presence of *E. coli* SSB, and (○) MtRecA protein in the presence of *E. coli* SSB and ϕX174 ssDNA (heterologous control).

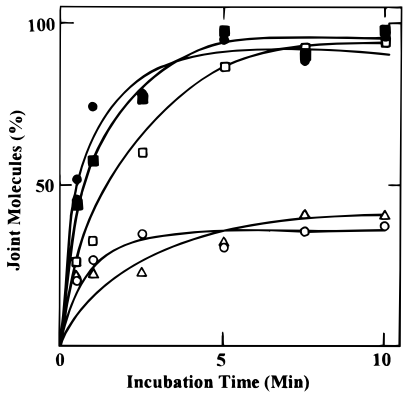

FIGURE 9: Preincubation of ssDNA with SSB, prior to MtRecA protein, does not stimulate the formation of joint molecules. The reaction and assay was done as described in the legend to Figure 8. Reactions done with EcRecA protein were (■) RecA protein prior to SSB and (□) SSB prior to the addition of RecA protein. Reactions performed with MtRecA protein were (●) RecA protein prior to SSB, (○) SSB prior to RecA protein, and (△) RecA protein in the absence of SSB.

It has been argued that the maximal stimulation of the activities of RecA protein by SSB requires the subsequent displacement of the latter from the nucleoprotein filament [reviewed by Kowalczykowski *et al.* (1994)]. To gain further insight into the molecular defect responsible for partial complementation of *E. coli recA* mutants by *M. tuberculosis recA*, we used the approach described below. We performed a series of experiments by using two different protocols. We preincubated ssDNA with SSB prior to MtRecA protein, or with MtRecA protein prior to SSB, to form nucleoprotein filaments. The data in Figure 9 show that preincubation of ssDNA with SSB prior to MtRecA protein resulted in the lack of stimulation in the formation of joint molecules (Figure 9, closed circles versus open circles). Since the displacement of SSB appears to be essential for attainment of maximal activity of RecA protein, together, these results suggest that the partial complementation of *E. coli recA* mutants by *M.*

tuberculosis recA may be due to its inability to displace SSB, reminiscent of that of *E. coli* recA56 protein (Lauder & Kowalczykowski, 1993).

DISCUSSION

In the present study, we have devised a strategy to purify both the 85 kDa and the MtRecA proteins to homogeneity. The identity of the purified RecA proteins was confirmed by cross-reaction with *E. coli* anti-RecA protein antibodies. Using a combination of assays, we have shown that the spliced form of MtRecA protein, but not 85 kDa protein, interacts with ssDNA to generate protein−DNA complexes. Interestingly, EcRecA protein binds to ssDNA in the presence or absence of ATP [reviewed by Roca and Cox (1990)]. MtRecA protein required the presence of ATP for efficient binding; however, binding was independent of ATP hydrolysis.

We found that the 85 kDa protein neither bound ATP nor catalyzed its hydrolysis in the presence or absence of ssDNA. By contrast, MtRecA protein, in the presence of ssDNA hydrolyzed ATP to ADP and P_i. The higher K_m for ATP by MtRecA protein in comparison to that of EcRecA protein would at first appear surprising in view of the fact that the amino acid residues in the nucleotide-binding site, including the P loop with consensus sequence (G/AXXXXGKT), found in nearly all ATP-binding proteins are substantially conserved in the two proteins (Walker *et al*., 1982). Indeed, the sequence homology between the two proteins is extensive, and it would have been surprising if the two had substantially different tertiary structures. The reduced affinity for ATP of MtRecA protein is not due to its origin from a Gram-positive organism since *Bacillus subtilis* RecA protein hydrolyzes ATP at a rate comparable to that of EcRecA protein (Lovett & Roberts, 1985). Thus, the cause of the reduced affinity should be due to some subtle structural variations which are indeed what our modeling studies indicate. These variations appear to be of two types. One is the widening of the nucleotide-binding cleft which results in the abolition of the possibility of hydrogen bonding between residues 262−264 and sugar hydroxyls and also in the reduction of contact area between the nucleotide and the protein. The fact that *recA* mutants in which Tyr-264 is replaced by either serine, alanine, or phenylalanine, although proficient in LexA repressor cleavage, exhibited less ATP binding and reduced rates of ATP hydrolysis (Knight & McEntee, 1985; Erikson *et al*., 1993) emphasizes the importance of the hydrogen bond involving the Tyr-264 hydroxyl for efficient binding and hydrolysis. The second variation is in relation to the disposition of amino acid residues around the magnesium ion and the γ-phosphate of ATP. The enzymatic mechanism proposed by Story and Steitz (1992) involves the activation of a water molecule by Glu-96 for an in-line attack of the γ-phosphate, the abolition of the interaction between nucleotide and Gln-194 on ATP hydrolysis, and the consequent conformational change in the DNA-binding region. The disposition of the concerned residues in the minimized model of the EcRecA protein−ATP complex is such as to facilitate this mechanism. In the model of the MtRecA protein−ATP complex, however, the side chains of Glu-96 and Gln-194 are too far removed from the γ-phosphate to make the proposed mechanism efficient.

Table 2: Comparison of the Activities of EcRecA and MtRecA Proteins[a]

RecA Protein	Size (kDa)	FUNCTIONAL ACTIVITIES					
		ATP		ssDNA-binding		Joint Molecules	
		Binding	Hydrolysis	-NaCl	+NaCl	-SSB	+SSB
Mycobacterium tuberculosis							
N IVS C	85	---	---	---	---	ND	ND
N C	38	+	+	+++	++	+	+++
Escherichia coli							
N C	38	+++	+++	+++	—	++	+++

[a] ND is not determined; IVS is intervening sequence. For details, see text.

The central question concerning MtRecA protein is whether the observed DNA-binding activities account for its role in homologous recombination. Conceivably, the nucleoprotein filament formed by MtRecA protein may serve as a scaffold in strand exchange reactions. With M13 linear duplex and ssDNA, MtRecA protein promoted the formation of joint molecules, albeit the extent was less than 30%. Nevertheless, in accordance with the demonstrated role of helix-destabilizing proteins in the synthesis of heteroduplex DNA, *E. coli* SSB abetted the formation of joint molecules. Interestingly, the partial levels of complementation of *E. coli recA* mutants by *M. tuberculosis recA* stems from the fact that MtRecA protein is defective in displacing SSB from ssDNA. Therefore, these results in conjunction with reduced affinity for ATP, and its hydrolysis, reveal the molecular basis of partial complementation of *E. coli recA* mutants.

Although a large number of microbial RecA proteins have been identified, in addition to the prototype EcRecA protein, the functions of three other microbial RecA proteins from *Proteus mirabilis* (West *et al*., 1983), *B. subtilis* (Lovett & Roberts, 1985), and *Thermus aquaticus* (Angov & Camerini-Otero, 1994) have been described. However, their *in vitro* biochemical functions such as binding to DNA, ATPase activity, and ability to promote the formation of joint molecules are largely similar to that of EcRecA protein. In contrast, the MtRecA protein shows significant differences in its requirements for binding to DNA, in its affinity for ATP, and in the formation of joint molecules. It has been proposed that the significance of excessive ATP hydrolysis of EcRecA protein in homologous recombination will become apparent by the identification and characterization of RecA protein analogues from different evolutionary origins (Cox, 1993). In this context, it is pertinent that MtRecA protein shows reduced affinity for ATP, and its hydrolysis, compared to that of EcRecA protein but is proficient in homologous pairing.

Genetic studies have established that the *M. tuberculosis* 85 kDa protein failed to complement *E. coli recA* mutants (Davis *et al*., 1991, 1992). The summary of the results presented in Table 2 reveals that the molecular lesion stems from the inability of the 85 kDa protein to interact with ssDNA, bind ATP, and promote ssDNA-dependent ATP hydrolysis, the crucial activities required for the staging of homologous recombination *in vitro*. Our results have also uncovered several features of MtRecA protein which might account for the partial complementation of *E. coli* RecA

mutants (Table 2). The occurrence of an unusual protein-splicing reaction in the maturation of MtRecA protein and the reduced efficiency of binding and hydrolysis of ATP suggest that the display of *recA* phenotype in *M. tuberculosis* is regulated at multiple steps for multiple reasons.

How are these *in vitro* findings related to the frequency of homologous recombination *in vivo*? In an effort to create attenuated mutants of *M. tuberculosis*, gene replacement strategies have been attempted. The preponderance of integration of recombinant DNA molecules into the chromosome at nonhomologous loci in the pathogenic *M. tuberculosis* (Kalpana *et al*., 1991), whereas at homologous loci in nonpathogenic *Mycobacterium smegmatis* (Husson *et al*., 1990), suggests that homologous recombination is a relatively inefficient process in slowly growing pathogenic mycobacteria. Intriguingly, only the clinical isolates of *M. tuberculosis* complex and *M. leprae* contain protein inteins in their RecA, while nonpathogenic mycobacteria do not (Davis *et al*., 1994). Clearly, the isolation and characterization of the 85 kDa and MtRecA proteins of *M. tuberculosis* will facilitate the understanding of the molecular basis of homologous recombination of the pathogen, the novel phenomenon of protein splicing, and the genetic engineering of attenuated strains for the production of improved vaccines.

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