Molecular and Functional Characterization of RecD, a Novel Member of the SF1 Family of Helicases, from Mycobacterium tuberculosis*

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Background: The heterotrimeric M. tuberculosis RecBCD complex, or each of its individual subunits, remains uncharacterized.

Results: MtRecD exists as a homodimer in solution, catalyzes ssDNA-dependent ATP hydrolysis, unwinding of DNA replication/recombination intermediates, and interacts with RecA.

Conclusion: MtRecD possesses strong 5′-3′- and weak 3′-5′-helicase activities.

Significance: These findings provide insights into the mechanism underlying DSB repair and homologous recombination in mycobacteria.

The annotated whole-genome sequence of Mycobacterium tuberculosis revealed the presence of a putative recD gene; however, the biochemical characteristics of its encoded protein product (MtRecD) remain largely unknown. Here, we show that MtRecD exists in solution as a stable homodimer. Protein–DNA binding assays revealed that MtRecD binds efficiently to single-stranded DNA and linear duplexes containing 5′ overhangs relative to the 3′ overhangs but not to blunt-ended duplex. Furthermore, MtRecD bound more robustly to a variety of Y-shaped DNA structures having ≥18-nucleotide overhangs but not to a similar substrate containing 5-nucleotide overhangs. MtRecD formed more salt-tolerant complexes with Y-shaped structures compared with linear duplex having 3′ overhangs. The intrinsic ATPase activity of MtRecD was stimulated by single-stranded DNA. Site-specific mutagenesis of Lys-179 in motif I abolished the ATPase activity of MtRecD. Interestingly, although MtRecD-catalyzed unwinding showed a markedly higher preference for duplex substrates with 5′ overhangs, it could also catalyze significant unwinding of substrates containing 3′ overhangs. These results support the notion that MtRecD is a bipolar helicase with strong 5′ → 3′ and weak 3′ → 5′ unwinding activities. The extent of unwinding of Y-shaped DNA structures was ~3-fold lower compared with duplexes with 5′ overhangs. Notably, direct interaction between MtRecD and its cognate RecA led to inhibition of DNA strand exchange promoted by RecA. Altogether, these studies provide the first detailed characterization of MtRecD and present important insights into the type of DNA structure the enzyme is likely to act upon during the processes of DNA repair or homologous recombination.

Double strand breaks (DSBs)3 are routinely inflicted by endogenous sources such as reactive by-products of cellular metabolism, blocked replication forks, and after exposure of cells to DSB-inducing agents (1, 2). The repair of DSBs and a multitude of other complex DNA lesions is critical for the maintenance of chromosome integrity and cell survival (1, 2). Most cells employ homologous recombination (HR) machinery to repair the damaged duplex, and hence counter the lethal effects of DSBs. Genetic and biochemical studies in prokaryotes have defined that the first step of HR is catalyzed by a helicase-nuclease that resects the DSB ends to generate a 3′-single-stranded tail, onto which RecA protein is loaded to form a nucleoprotein filament (3–6). In many Gram-negative bacteria, including Escherichia coli, the heterotrimeric multienzymatic RecBCD complex serves as the major resection nuclease in specific processes related to DSB repair and early steps in HR (6, 7).

In Gram-positive bacteria like Bacillus subtilis, which is devoid of the prototype RecBCD-like nuclease-helicase, the resection of DSBs is orchestrated by a heterodimeric helicase-nuclease complex, AddAB (also referred to as RexAB in Lactobacillus lactis) (7–12). Consequently, bacterial species that possess genes encoding RecBCD/AddAB enzymes can be grouped into two clades as follows: those that contain an E. coli-type RecBCD enzyme but not AddAB, and those that have enzymes analogous to B. subtilis AddAB helicase-nuclease but not RecBCD (7, 13). It is intriguing that both Mycobacterium tuberculosis and Mycobacterium smegmatis possess, in addition to homologues of E. coli recB, recC, and recD (14, 15), genes that encode a new family of heterodimeric DSB end-processing enzyme, called AdnAB, with distinctive enzymatic properties vis-à-vis RecBCD and AddAB (16). Additional studies have indicated a role for M. smegmatis AdnAB in the resection of DSBs.

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3 The abbreviations used are: DSB, double-strand break; AFM, atomic force microscopy; dsDNA, double-stranded DNA; HR, homologous recombination; MtRecD, M. tuberculosis RecD; MtRecA, M. tuberculosis RecA; nt, nucletotide; ODN, oligonucleotide; ssDNA, single-stranded DNA; Ni2+ -NTA, Ni2+ -nitrilotriacetic acid.
(17–20); its ability to participate in HR via the Chi-dependent pathway, however, remains to be investigated.

During the human phase of the *M. tuberculosis* life cycle, it is exposed to genotoxic stress originating from both antimicrobial host defense mechanisms and the environment. The survival within the infected macrophage and reactivation of the tubercle bacillus from the dormant state are key features of infection; therefore, the maintenance of genome integrity is of critical importance in the biology of *M. tuberculosis* (21). New insights are beginning to emerge concerning the role of DSB repair in the survival of *M. tuberculosis* in hosts (21). The most severe genotoxic lesions are DSBs, which are also important intermediates in different processes of DNA metabolism. Intriguingly, the RecBCD complex, a crucial resection nuclease for HR in *E. coli* (6, 7), has no role in HR in *M. smegmatis*, and instead it is dedicated to the single strand annealing pathway (19, 20, 22). Furthermore, although mutations in *E. coli* recB, recC, or recD cause low cell viability and are very sensitive to DNA-damaging agents, including UV irradiation (23–28), *M. smegmatis* ΔrecBCD mutants show cell viability and UV resistance similar to that of wild-type *M. smegmatis* (19). However, it remains unknown whether or not *M. tuberculosis* ΔrecBCD mutants behave much like *M. smegmatis* ΔrecBCD mutants with respect to HR and DNA repair. In the absence of biochemical and biophysical data on the solution properties of mycobacterial RecBCD and the interpretation of genetic data remain speculative. The co-existence of genes encoding both RecBCD and AdnAB enzymes intrinsic to HR and DNA repair is consistent with the notion that they might play diverse and important roles in the maintenance of genomic integrity of cells (16, 17, 22). Notwithstanding, these findings raise important questions regarding the functional characteristics of *recB*, *recC*, and *recD* genes in *M. tuberculosis*. Specifically, it is not clear whether these putative genes are capable of encoding enzymatically active subunits of the RecBCD enzyme complex. Furthermore, the relative contributions and the importance of RecBCD and AdnAB enzymes to the maintenance of genome integrity remain uncharacterized.

Much of our mechanistic insight into the biological functions of RecD helicases is gained from studies of *E. coli* RecD, which is expressed at low levels, has poor solubility and yield, and is difficult to study in isolation (24–34). Purified *E. coli* RecD is not well behaved in solution unless as a part of the RecBCD complex (7, 34). Furthermore, many biochemical and mechanistic studies have been on the intact RecBCD enzyme complex, which is complicated by the fact that it contains two helicase subunits, RecB and RecC (7). Although the crystal structure of the N-terminal truncated form of *Deinococcus radiodurans* RecD2 helicase has been solved to 2.2-Å resolution (33), the structure of a full-length RecD remains to be determined. In this study, we have isolated the *M. tuberculosis* recD gene, generated its mutant allele, and purified the wild-type and mutant forms of its encoded product (MtRecD) to homogeneity from the soluble fraction of cells. We observed that MtRecD exhibited greater affinity and specificity to Y-shaped and linear duplexes containing a 5′ overhang. The unwinding mechanism proposed for SF1 helicases, based on the crystal structures of PcrA and UvrD, has been the subject of much debate because of the absence of direct evidence that monomers can catalyze DNA-unwinding reactions. Indeed, it has been conjectured that either a homodimer or higher order oligomeric form of SF1 helicase(s) is essential for driving efficient DNA unwinding activity (34). Thus, our results showing that MtRecD exists in solution as a homodimer, binds more robustly to Y-shaped DNA structures, and catalyzes ATP-dependent unwinding of DNA substrates in both 5′ → 3′ and 3′ → 5′ directions, albeit with different efficiencies, as well as inhibits RecA activity, are striking. In summary, these studies provide the first detailed characterization of MtRecD and present important insights into the type of DNA structures the enzyme is likely to act upon during the processes of DNA repair or HR.

### EXPERIMENTAL PROCEDURES

#### Reagents and Bacterial Strains

The chemicals used are of analytical grade. Restriction enzymes, T4 DNA ligase, T4 polynucleotide kinase, and *Pfu* polymerase were purchased from New England Biolabs. [γ-32P]ATP was purchased from the Bhabha Atomic Research Center, Mumbai, India. DNA gel extraction kit and Ni2+–NTA resin was purchased from Qiagen. Protein G-Sepharose, streptavidin, and biotin were purchased from Sigma. Fast performance liquid chromatography columns were purchased from GE Healthcare. Cloning primers, including ODNs, were purchased from Sigma Genosys. *E. coli* strains DH5α, RIL, C41, C43, and Rosetta2 (DE3)pLysS strain as well pET21a(+) plasmid were purchased from Invitrogen or Novagen. *Saccharomyces cerevisiae* Hop1 (35), *M. tuberculosis* RecA, anti-RecA antibody (36), *M. tuberculosis* SSB, and anti-SSB antibody (37) were prepared and characterized as described previously.

#### DNA Substrates

ODNs used in this study are listed in Table 1. ODNs were labeled at the 5′ end with [γ-32P]ATP and T4 polynucleotide kinase (38) and annealed in pairs to form DNA duplexes with various end structures (Table 2). Briefly, the reaction mixtures were heated for 5 min at 95 °C, followed by slow cooling to 4 °C over a period of 2 h. The annealed substrates were electrophoresed on a 10% (w/v) polyacrylamide gel in 0.5× TBE (1× TBE = 90 mM Tris, 89 mM boric acid, 1.98 mM EDTA, pH 8.3). The bands were excised from the gel and eluted into TE buffer (10 mM Tris–HCl, pH 7.5, and 1 mM EDTA).

#### Bioinformatics Analysis

The nucleotide sequences of *E. coli* and various *Mycobacterium* species were retrieved from TIGR Comprehensive Microbial Resource (CMR) database site. These sequences were analyzed for domain architecture and aligned using multiple alignment algorithm with the Clustal series of programs and visualized using Jalview.

#### Isolation of *M. tuberculosis* recD

The MTCY 20H10 cosm id, which bears the *recD* gene, was obtained from Institut Pasteur, Paris, France. The nucleotide sequence of *recD* ORF (accession number, Rv0629c) was identified using the Tuber cuList database. The predicted ORF corresponding to *M. tuberculosis* recD was PCR-amplified from MTCY 20H10 cosm id using the following primers: forward primer 5′-AAGGCTG-CACATATGAAAGCTACC-3′ and reverse primer 5′-ATC-CCCCCTCGAAGTCAGCCACACC-3′ that contained Ndel and XhoI (underlined) restriction sites. A single PCR cycle consisted of initial denaturation at 95 °C for 5 min, followed by 30
Mycobacterium tuberculosis RecD

### Table 1

Sequences of oligonucleotides used in this study

| ODN  | Length* | Sequence (5’–3’) |
|------|---------|-----------------|
| ODN 1 | 41      | GGCGTGATCACCAGATGCTGAGTTAGCAAGAACCATTCCGCAAGTCT |
| ODN 2 | 41      | GACGTGCGCCAAAGTTTCTCAGTCTGACATGAGCTGACCATG |
| ODN 3 | 41      | GCCATGACCTGCTGACATGCTTCTGTTGACTGACCATG |
| ODN 4 | 41      | GGCGATGAGCCATGACATGCTTCTGTTGACTGACCATG |
| ODN 5 | 41      | ACCTGCGAGCTGCACTGAGTTAGCTGAGAAGATGTTAGT |
| ODN 6 | 21      | AGCTGACCTGCTGACATGCTTCTGTTGACTGACCATG |
| ODN 7 | 21      | AGCTGACCTGCTGACATGCTTCTGTTGACTGACCATG |
| ODN 8 | 83      | AAGGATAGGTCGAATTTCATTTTAAAATGAGAAAATTCGACCTATCCT |
| ODN 9 | 52      | ODN 15 27 |
| ODN 10 | 52     | ODN 16 51 |
| ODN 11 | 52     | ODN 17 52 |
| ODN 12 | 52     | ODN 18 25 |
| ODN 13 | 52     | ODN 19 26 |

* This denotes length in nucleotide residues.

cycles of amplification with each cycle consisting of denaturation at 95 °C for 60 s, then annealing at 45 °C for 45 s, and extension at 72 °C for 90 s. The PCR product was cloned directly into the pET21a expression vector, using terminal NdeI and XhoI restriction sites. The identity of the cloned M. tuberculosis recD gene was confirmed by restriction mapping and nucleotide sequencing. The PCR products were gel-purified. Both the purified fragments were pooled, and another round of PCRs in which gene-specific primers were used, to generate the full-length mutants. The PCR product was cloned directionally into the pET21a expression vector, using terminal NdeI and mutant primer 5’-ATCCGCTAGTCGACCATG. In the second set of PCRs, the following primers were used: reverse primer 5’-TGCTGAGCTGCACTGAGTTAGCTGAGAAGATGTTAGT and mutant primer 5’-TGCTGAGCTGCACTGAGTTAGCTGAGAAGATGTTAGT. The PCR products were gel-purified. Both the purified fragments were pooled, and another round of PCRs in which gene-specific primers were used, to generate the full-length mutants. The PCR product was cloned directionally into the pET21a (+) expression vector, using terminal NdeI and XhoI restriction sites and named pMtRD. The identity of M. tuberculosis recD mutant (pMtRD) was confirmed by nucleotide sequencing.

Expression and Purification of MtRecD and MtRecD<sup>K179A</sup>—MtRecD was overexpressed in E. coli host strain Rosetta 2 (DE3)pLysS bearing pMtRD. Fresh LB medium containing 100 µg/ml ampicillin and 34 µg/ml chloramphenicol was inoculated with overnight culture grown under the same conditions at a ratio of 1:100. Cells were grown at 37 °C until the A<sub>600</sub> was 0.6 was achieved. Isopropyl 1-thio-β-D-galactopyranoside was then added to a final concentration of 0.5 mM for overexpression of MtRecD at 18 °C for 12 h. Bacteria were harvested by centrifugation, and the pellet was washed with buffer A (10 mM Tris-HCl, pH 7.5, 100 mM NaCl, and 1 mM EDTA). The pellet was resuspended in the buffer B (200 mM Tris-HCl, pH 8, 1 mM NaCl, and 5% glycerol), frozen, and stored at −80 °C. Thawed cell suspensions were lysed with sonication, and the resulting lysate was subjected to centrifugation at 30,000 rpm for 60 min at 4 °C. The supernatant was loaded onto a 5-ml Ni<sup>2+</sup>-NTA column and washed extensively (20-bed volume) with buffer B containing 20 mM imidazole. The bound protein was eluted using a linear gradient of 20–200 mM imidazole in buffer B. Fractions containing highly pure MtRecD (as monitored by SDS-PAGE) were pooled and precipitated by the addition of ammonium sulfate to 70% saturation. The precipitated protein was collected by centrifugation at 14,000 rpm for 30 min at 4 °C and dialyzed against buffer C (20 mM Tris-HCl, pH 8, 1.5 M NaCl, 5% glycerol, and 5 mM β-mercaptoethanol). The dialyzed protein was applied onto a Superdex-200 column and eluted using buffer C. Purified protein was dialyzed against buffer D (20 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM DTT, 20% glycerol, and 20 mM NaCl) and stored at −80 °C. Mutant MtRecD<sup>K179A</sup> was also purified as described above. The concentration of wild-type and mutant MtRecD was determined by the dye-binding method using bovine serum albumin as standard (40).

Gel Filtration Chromatography—Gel filtration chromatography was performed using a Superdex 200 10/300GL column (GE Healthcare) on a Bio-Rad FPLC system, with 0.5 ml/min flow rate at 18 °C. The column was equilibrated with 20 mM Tris-HCl, 100 mM NaCl, pH 7.5, 1 mM EDTA, 5% glycerol, and 5 mM β-mercaptoethanol. A set of protein standards of known molecular mass such as aprotinin (6.5 kDa), RNase A (13.7 kDa), carbonic anhydrase (29 kDa), ovalbumin (43 kDa), aldolase (158 kDa), ferritin (440 kDa), and thyroglobulin (669 kDa) was used to construct the standard curve. The indicated amounts of purified MtRecD were dialyzed in the column equilibration buffer and applied onto the column. The fractions were monitored by UV absorbance at 280 nm. The elution volume (V<sub>e</sub>) corresponding to the protein peak was determined, and the approximate molecular weight was deduced by interpolation on the standard curve based on its R<sub>e</sub> value.

Chemical Cross-linking of MtRecD—Purified MtRecD (2 µM) was dialyzed against buffer (20 mM Tris-HCl, pH 7.5, 20% glycerol, 1 mM DTT), and the protein was cross-linked with the indicated amounts of freshly diluted glutaraldehyde. After
incubation at 37 °C for 20 min, we added 2 µl of 5× SDS sample loading buffer (10 mM Tris-HCl, pH 6.8, 40% glycerol, 12.5% SDS, and 25 mM DTT containing 0.1% bromphenol blue), and incubation was extended for 5 min at 95 °C. Samples were separated by SDS-6% PAGE, and products were visualized by staining with silver nitrate.

Atomic Force Microscopy—MtRecD was diluted to a concentration of 0.5 µM in a buffer solution containing 20 mM Tris-HCl, pH 7.5, and 2 mM MgCl₂. 5 µl of reaction mixture was deposited on the surface of freshly cleaved Muscovite mica and allowed to remain for 1.5 min, then rinsed with ultrapure deionized water, and dried. AFM scans were performed on an Agilent atomic force microscope in tapping mode operating with a PicoView 1.8 SPN controller. We used nanosensors PPP-NCL 20 silicon cantilevers with nominal spring constant in the range of 21 and 98 newtons/m. The scan frequency was typically 1.5 Hz per line, and the amplitude of oscillation was 8–10 nm. We used first or second order polynomial function to remove the background slope. Molecular weights were derived from the measured volumes as described previously (41, 42).

Electrophoretic Mobility Shift Assays—Reaction mixtures contained 20 mM PIPES (pH 6.5), 1 mM DTT, 5 mM MgCl₂, 200 µg/ml BSA, 1 nm ³²P-labeled DNA, and the indicated concentrations of MtRecD. Reaction mixtures were incubated at 37 °C for 25 min and then with 0.015% glutaraldehyde for 10 min. Reaction was terminated by the addition of 2 µl of loading dye (0.1% (w/v) bromphenol blue and xylene cyanol in 20% glycerol). Samples were separated by electrophoresis on a 5% polyacrylamide gel in 0.25× TBE buffer at 80 V for 3 h at 4 °C. Gels were dried, and the bands were visualized using Fuji FLA-9000 PhosphorImager. The band intensities were quantified in UVItech gel documentation system using UVI-Band Map software (version 97.04) and plotted using GraphPad Prism (version 5.0).

UV Catalyzed Cross-linking of ATP to MtRecD—The assay was performed as described previously (37). Reaction mixtures containing 20 mM Tris-HCl, pH 7.5, 5 mM MgCl₂, 0.5 mM DTT, 1.6 pmol of [γ-³²P]ATP, and increasing amounts of MtRecD were incubated at 37 °C for 30 min. In parallel, reactions were also performed with a fixed concentration of MtRecD (1.5 µM) and increasing concentrations of [γ-³²P]ATP. Following incubation, samples were placed on ice at ~2 cm from the light source and subjected to irradiation at 254 nm for 5 min using a Hoefer Scientific cross-linker. After the addition of 2 µl of 5× SDS sample loading buffer (10 mM Tris-HCl, pH 6.8, 40% glycerol, 12.5% SDS, and 25 mM DTT containing 0.1% bromphenol blue), samples were incubated at 95 °C for 5 min and then separated by SDS-7.5% PAGE at 20 mA for 1 h. The gels were washed extensively with methanol/acetic acid/water (10:10:80 v/v/v) solution to remove free ATP and dried over a Whatman 3 MM filter at 80 °C (38). The radioactive bands were visualized on a Fuji FLA-9000 PhosphorImager, quantified in UVItech gel documentation system using UVI-Band Map software (version 97.04), and plotted against increasing concentrations of MtRecD or ATP. The K_d value for binding of ATP to MtRecD was estimated by fitting the data to the standard equation for binding data, Y = B_\text{max} \times X/(K_d + X), using GraphPad Prism (GraphPad Software, San Diego), where Y is the amount of ATP binding; X is the concentration of [γ-³²P]ATP; B_\text{max} is the maximum binding; and K_d is the concentration of [γ-³²P]ATP required to reach the half-maximum binding. The apparent K_d value was calculated from assays done in triplicate, and the standard errors were determined.

ATP Hydrolysis Assay—ATP hydrolysis was measured by thin layer chromatography on polyethyleneimine cellulose plates as described previously (43, 44). Reaction mixtures (10 µl) contained 20 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, 10 µM DNA or no DNA, 1 mM DTT, 5% glycerol, and the indicated amount of MtRecD. Reaction mixtures were preincubated at 37 °C for 5 min, prior to the addition of 5 µM [γ-³²P]ATP to start the reaction. After incubation for 60 min, reaction was terminated by the addition of EDTA to a final concentration of 15 mM, and 0.5-µl aliquots were spotted on the TLC plates. The plates were developed in a solution containing 0.5 M LiCl, 1 M HCOOH, and 1 mM EDTA. The spots were visualized using Fuji FLA-9000 PhosphorImager and quantified in UVItech gel documentation system using UVI-Band Map software (version 97.04). The data were plotted using GraphPad Prism (version 5.0). The K_cat value was determined by fitting the data to the Michaelis-Menten equation.

DNA Unwinding Assay—Reaction mixtures containing 20 mM Tris-HCl, pH 7.5, 1 mM MgCl₂, 1 mM DTT, 5% glycerol, 1 mM substrate, and the indicated concentrations of MtRecD were prepared and preincubated at 37 °C for 5 min. The reaction was started by the addition of 3.5 mM ATP, and incubation was continued for an additional 25 min. Reactions were quenched with 1× quenching buffer (40% glycerol, 2.4% (w/v) SDS, 100 mM EDTA, 0.12% (w/v) bromphenol blue), and the samples were separated on nondenaturing 8% polyacrylamide gels (29:1 acrylamide/bisacrylamide) in 0.5× TBE buffer at 150 V. Gels were dried, and the bands were visualized using the Fuji FLA-9000 PhosphorImager. The band intensities were quantified in UVItech gel documentation system using UVI-Band Map software (version 97.04) and plotted using GraphPad Prism (version 5.0).

Biotin/Streptavidin Blocking Assay—Reaction mixture contained 20 mM Tris-HCl, pH 7.5, 1 mM MgCl₂, 1 mM DTT, 0.2 mg/ml BSA, 5% glycerol, 1 mM substrate, and 100 mM streptavidin. After preincubation for 10 min at 37 °C, we added MtRecD together with 1 µM biotin (as a trap for free streptavidin), and incubation was extended for 5 min at 37 °C. The reaction was started with the addition of 3.5 mM ATP. Following incubation for an additional 25 min, reaction was quenched with 1× quenching buffer (40% glycerol, 2.4% (w/v) SDS, 100 mM EDTA, 0.12% (w/v) bromphenol blue). Samples were separated on 8% nondenaturing polyacrylamide gels (29:1 acrylamide/bisacrylamide) in 0.5× TBE buffer at 150 V for 5 h at 4 °C. The gels were dried and exposed to the phosphorimaging screen, and images were analyzed using the Fuji FLA-9000 PhosphorImager. The band intensities were quantified in UVI-Tech gel documentation station using UVI-Band Map software (version 97.04) and plotted using GraphPad Prism (version 5.0).

Far Western Analysis—Far Western analysis was performed as described previously, with minor modifications (45). The
bait protein (MtRecA, MtRecD, MtRecDK179A, MtSSB, or BSA) was loaded onto nitrocellulose membrane in increasing concentrations. The membrane was blocked in assay buffer (20 mM Tris-HCl, pH 7.5, 100 mM NaCl, 0.5 mM EDTA) with 5% nonfat milk for 1 h at 24 °C. MtRecA, MtRecD, or MtRecDK179A lysates were prepared, and the supernatant was treated with 50 μg/ml...
DNase I for $1 \text{h at } 37 ^\circ C$. The potential interacting proteins were allowed to interact by incubating overnight (12 h) at 4 °C with gentle agitation. The membrane was washed three times (10 min each) with assay buffer containing 0.05% Tween 20 followed three times (10 min each) with assay buffer alone. The membrane was incubated with anti-RecA antibody for 5 h at 4 °C with gentle agitation, followed by washing three times (10 min each) with assay buffer with and without 0.05% Tween 20. The membrane was incubated with secondary antibody for 1 h at room temperature with gentle agitation. The interacting protein was visualized using ECL Western blotting detection kit, and the image was captured on Fujifilm LAS4000 luminescence imager.

**Co-immunoprecipitation**—Assays were performed as described earlier (46). Whole-cell lysates (in 20 mM Tris-HCl, pH 7.5, 100 mM NaCl, and 0.5 mM EDTA) from *E. coli* cells expressing MtRecD and MtRecA were treated with 50 μg/ml DNase I at 37 °C for 1 h. The interaction between proteins in the whole-cell lysates was facilitated by incubation with gentle stirring for 6 h at 4 °C. RecA-RecD protein complexes were pulled down with anti-RecA antibody conjugated to protein G-Sepharose beads. Washing the beads with Tris-buffered saline three times was followed by boiling in SDS sample buffer. Proteins were resolved by 7.5% SDS-PAGE and transferred onto nitrocellulose membrane. The blot was incubated for 1 h with buffer containing 5% nonfat milk (46). The blot was further incubated with primary and secondary antibodies followed by exposure to ECL. Western blotting detection kit to visualize the interacting proteins partner. The image was captured using Fujifilm LAS4000 luminescence imager.

**DNA Strand Exchange Assay**—Assay was performed as described (46). Reaction mixture (10 μl) containing 20 mM Tris-HCl, pH 7.5, 3 mM ATP, 8 mM MgCl$_2$, 5 μM ssDNA (ODN9, Table 1), and 2.5 μM MtRecA was incubated in the presence of the ATP regeneration system (5 mM phosphocreatine and 10 units/ml creatine phosphokinase) at 37 °C for 5 min and then with MtRecD for 5 min at 37 °C. Strand transfer reaction was initiated by the addition of 1 μM $^{32}$P-labeled duplex DNA (ODN 8 annealed to ODN 9, see Table 1), and incubation was continued for an additional 10 min.
Reaction was stopped by the addition of 2.5 μl of 5× stop buffer (5% SDS and 100 mM EDTA) followed by the addition of 1.4 μl of 10× electrophoresis dye (50% glycerol, 0.42% (w/v) bromphenol blue, and 0.42% (w/v) xylene cyanol). Samples were loaded onto 10% polyacrylamide gel and electrophoresed in 45 mM Tris borate buffer, pH 8.3, containing 1 mM EDTA at 150 V for 7 h. The 32P-labeled DNA substrate and product were visualized by Fuji FLA-9000 PhosphorImager. The band intensities were quantified in UVI-Tech gel documentation station using UVI-Band Map software version 97.04 and plotted using GraphPad Prism (version 5.0).

**RESULTS**

**Bioinformatics Analysis of Mycobacterial RecD Subunits**—The BLAST search revealed the presence of a putative *E. coli recD* gene in various *Mycobacterium* species, including *M. tuberculosis* (Fig. 1) (47). Multiple alignment of *E. coli* RecD amino acid sequence with RecD from different *Mycobacterium* species revealed the existence of strongly conserved signature motifs of superfamily-1 (SF1) enzymes, which are distributed across the length of mycobacterial RecD proteins (Fig. 1, *enclosed in rectangles*). MtRecD contains seven signature helicase motifs, including the strongly conserved motif III sequence, GDXXQ, a structural hallmark of SF1 helicases, the ubiquitous Walker A and B motifs (I and II), and motif VI that contains an “arginine finger.” In addition to the conserved signature motifs, MtRecD shares 34% identity and 48.8% similarity with the prototype *E. coli* RecD. Likewise, a similar comparison of *M. tuberculosis* with *M. smegmatis*, *Mycobacterium ulcerans*, and *Mycobacterium avium* RecD showed 67.8, 65.5, and 70.2% identity, and 77.3, 76, and 77.6% similarity, respectively.

**Isolation of M. tuberculosis recD Gene**—An open reading frame annotated as recD (GenBank™ accession number, Rv0629c) in the MTCY 20H10 cosmid library of *M. tuberculosis* H37Rv was PCR-amplified using forward and reverse primers as described under “Experimental Procedures.” PCR amplification yielded the anticipated DNA fragment corresponding to the size of *M. tuberculosis recD* gene (data not shown). Similarly, PCR amplification was performed to generate MtRecD from different *Mycobacterium* species. The PCR products were cloned into pET21a(+) *E. coli* expression vector. Positive clones were identified by restriction analyses and confirmed by nucleotide sequencing (data not shown).

**Overexpression and Purification of MtRecD and MtRecDK179A**—To investigate whether *M. tuberculosis recD* gene product functions as an active enzyme, the plasmid vector containing *M. tuberculosis recD* gene, placed under the control of the lac promoter, was induced by the addition of isopropyl-1-thio-β-D-galactopyranoside to the culture medium. These studies showed maximum accumulation of soluble MtRecD at early log phase in the cultures of *E. coli* Rosetta2 (DE3)pLysS strain when grown at 18 °C (data not shown). Cultures grown at higher temperatures produced little or no MtRecD in the soluble fraction.

We developed a rapid protocol for the purification of MtRecD by using Ni2+ -charged metal affinity and size exclusion chromatography columns. MtRecD eluted from the gel filtration column at the volume expected for a homodimer (data not shown). Purified protein migrated as a single band during SDS-PAGE and was judged to be >97% pure (Fig. 2A). The mutant variant, MtRecDK179A, was expressed and purified in a manner similar to that of wild-type MtRecD (Fig. 2B). The yield of each protein was in the range of 4–5 mg from 4 liters of induced culture. The purity and identity of MtRecD and MtRecDK179A were assessed using MALDI electrospray ionization-MS, MALDI MS-MS, and peptide mass fingerprinting (data not shown). Both the proteins were found to be free of exon- and endonuclease contamination (data not shown).

**MtRecD Exists in Solution as a Homodimer**—The native state of SF1 helicases in solution and during DNA unwinding has been the subject of extensive debate. Among the members of the SF1 and SF2 helicases, some of the enzymes exist as monomers, but others occur either as dimers or oligomers. However, in the absence of partner proteins (such as SSB), monomers of Rep, and PcrA, which belong to SF1 group of helicases, fail to catalyze DNA unwinding in vitro (34). Accordingly, it has been suggested that either a homodimer or a higher order oligomer
might be the active species, consistent with the enhanced DNA unwinding activity of multimeric Dda helicase (a member of SF1 category). To determine the state of MtRecD in solution, we used three different methods. First, we performed size exclusion chromatography at three different concentrations of MtRecD (0.5, 1, and 1.5 μM) (Fig. 3A). MtRecD consistently
FIGURE 5. MtRecD binds robustly to Y-shaped DNA structures. Reaction mixtures containing the indicated $^{32}$P-labeled DNA (1 nM) was incubated in the absence (lane 1) or presence of 10, 25, 50, 75, 100, 200, 300, 400, and 500 nM MtRecD (lanes 2–11), respectively. After incubation at 37 °C for 25 min, samples were subjected to electrophoresis on native polyacrylamide gels and visualized as described under “Experimental Procedures.” Filled triangle on top of the gel images represents increasing concentrations of MtRecD. The positions of the free DNA and MtRecD-DNA complexes are indicated on the right. A–F, reactions performed with the indicated DNA substrates. G, graphic representation of the extent of the formation of MtRecD-DNA complexes with different substrates. The amount of MtRecD-DNA complexes in A–F was quantified and plotted against increasing concentrations of MtRecD. An asterisk denotes the $^{32}$P-labeled end. The error bars in G represent the deviation from the mean for data from three independent experiments.
eluted at a position corresponding to a molecular mass of 120 kDa, as deduced from the standard curve constructed using proteins of known molecular mass (Fig. 3B). Because the theoretical molecular mass of a monomer is 63 kDa, these results suggest that MtRecD exists in solution as a homodimer. Second, we used glutaraldehyde, a bi-functional zero-length cross-linker (48). Upon incubation of MtRecD with increasing concentrations of glutaraldehyde, we observed a slow migrating species, which corresponds to the size of a homodimer (Fig. 3D, lanes 3–7). Third, we directly visualized the state of MtRecD by tapping mode AFM as described previously (49). We observed two different types of particles having heights of 0.327 and 0.572 nm, in accordance with the size of a monomer and homodimer, respectively (Fig. 3D). Image-based quantitative determination revealed that the ratio of dimer to monomer was 4:1. Under these conditions, significant quantities of oligomers larger than dimers were absent, consistent with the data from cross-linking and gel filtration chromatography. To corroborate these findings, we used S. cerevisiae Hop1 (66 kDa) as a positive control, whose native state is a dimer in solution (50). To this end, we found that the radius and particle height of Hop1 were in the range of 0.38 to 0.58 nm (Fig. 3, E and F). These values are in excellent agreement with those obtained for MtRecD particles (Fig. 3C). Taken together, these results strongly suggest that MtRecD exists in solution as a homodimer.

MtRecD Exhibits Higher Binding Affinity for Linear Duplex DNA and Y-shaped Structures Containing 18-mer or Single-stranded Overhangs— It is necessary to delineate the structural requirements and the specificity of MtRecD with a
The formation of complex II resulted from interaction between two monomers already formed in solution, whereas the binding of MtRecD was cooperative, most likely resulting from binding to ssDNA (Fig. 4, lanes 2–9). We observed a similar pattern of MtRecD binding to DNA containing 19–20-mer ssDNA overhangs either at the 5’ or 3’ end. Reactions were performed with each substrate (1 nM) in the absence or presence of varying concentrations (10–500 nM) of MtRecD. The resulting products were separated by EMSA and visualized by phosphorimaging as described under "Experimental Procedures." Analyses of MtRecD binding to these substrates showed the formation of two complexes as follows: a faster moving complex and a slower moving complex, designated as CI and CII, respectively (Fig. 4, A and B). When the same experiment was performed with partial duplex DNA containing a 26-mer ssDNA overhang either on the 5’ or 3’ end, and a biotinylated nucleotide embedded at 4 or 2 positions from the ssDNA end, the extent of the formation of protein-DNA complexes was nearly identical (Fig. 4, C and D). We observed a similar pattern of MtRecD binding to ssDNA (Fig. 4E). Furthermore, addition of increasing concentrations of MtRecD caused a concomitant shift of CI to the CII position. By this assay, we found no significant binding activity of MtRecD with dsDNA, indicating that ssDNA plays an essential role in its binding to DNA (Fig. 4F). Analysis of the extent of the formation of MtRecD-DNA complexes suggested that binding was concentration-dependent and saturable (Fig. 4G). Altogether, these results support the view that binding of MtRecD was cooperative, most likely resulting from binding of a monomer or dimer already formed in solution, whereas the formation of complex II resulted from interaction between two DNA-bound protein dimers or monomers. However, further experiments are needed to distinguish between these two possibilities.

The above data suggested that MtRecD binds linear duplex DNA containing ssDNA overhangs at either end. E. coli RecBCD, a bipolar helicase, unwinds linear duplex DNA with a 3’ overhang to generate Y-shaped DNA structures. Therefore, we asked to what extent MtRecD by itself can bind Y-shaped DNA structures having varying fork lengths (Table 2). We observed robust binding of MtRecD to different Y-shaped DNA structures having >18-nt overhangs on either end resulting in the formation of CI and CII complexes (Fig. 5, A–F). However, binding to Y-shaped DNA structure having 18-nt overhangs somewhat decreased, although further shortening of the overhang lengths to 5 nt resulted in measurable but insignificant DNA binding activity (Fig. 5, E and F). Analysis of the extent of complex formation between MtRecD and various Y-shaped DNA structures, as a function of protein concentration, suggested that binding was saturable and that the binding affinity for forked DNA structures is higher compared with linear duplexes containing overhangs on either ends (compare Figs. 5G with 4G). To quantitatively compare the binding affinities of MtRecD, we determined the apparent $K_d$ values as described (38). The apparent $K_d$ value for interaction of MtRecD with Y-shaped structure was 58 nM, whereas the $K_d$ values for duplex DNA containing 3’ or 5’ overhang was 120 nM.

**Binding of MtRecD to Y-shaped Structure Produces a Salt-stable Complex**—To gain further insight into the nature of thermodynamic forces involved in MtRecD-DNA interaction, we tested the sensitivity of MtRecD-DNA complexes to increasing concentrations of NaCl. Assay was performed, and samples
were analyzed as described under “Experimental Procedures.”

Fig. 6 shows the salt concentration dependence of dissociation of DNA-MtRecD, which is a relative measure of the affinity of a protein for various DNA substrates. The results suggest important differences with respect to the stability of complexes formed with Y-shaped duplex compared with other DNA substrates. The pattern of dissociation of complexes was nearly identical for the substrates tested; the dissociation of CI proceeds via CII, indicating that CII is relatively more sensitive to salt compared with CI. Although 50% dissociation of the complexes formed between MtRecD-Y-shaped DNA occurred at >175 mM NaCl, the same concentration of salt caused complete dissociation of the MtRecD duplex containing either a 3’ or 5’ overhang as well as from ssDNA. The results are summarized in a graphic form in Fig. 6E. Together, these studies suggest that the interactions of MtRecD with Y-shaped DNA and charged residues are important for the binding to DNA.

ATP Binds to MtRecD—Binding reactions were performed at varying concentrations of MtRecD with a fixed amount of 32P-labeled ATP. In parallel, binding reactions were performed as described above except that the reactions contained increasing concentrations of [γ-32P]ATP and a fixed amount of MtRecD. Subsequently, reaction mixtures were subjected to UV radiation as described previously (51). The reactants and products were separated by SDS-PAGE, and ATP binding was analyzed as described under “Experimental Procedures.” Analysis of the band intensities indicated that binding increased with increasing concentrations of MtRecD (Fig. 7, A and B) or [γ-32P]ATP (Fig. 7, C and D), respectively. However, it should be noted that binding was somewhat better in reactions performed with increasing concentrations of ATP compared with MtRecD. The band intensities were quantified in the UVItech gel documentation system. We calculated $K_d$ values for the interaction between ATP and MtRecD. We made an assumption that one ATP molecule is bound to one MtRecD subunit. Analysis of binding data yielded $K_d$ values of 7.76 ± 2.26 and 10.01 ± 1.92 for reactions performed with increasing concentrations of MtRecD and ATP, respectively. Although the observed range of variation falls within experimental error, neither method is completely satisfactory; therefore, the values presented must be viewed with caution. However, we note that the $K_d$ value derived here is about 3-fold higher than that of E. coli RecD (52).

MtRecD Catalyzes ATP Hydrolysis That Is Stimulated by ssDNA but Not dsDNA—To investigate whether MtRecD is able to catalyze ATP hydrolysis, we performed 32Pi release assay as described

![FIGURE 8. Characterization of MtRecD-catalyzed ATPase activity. A, time course of ATPase activity by MtRecD. Reaction mixtures containing 5 μM [γ-32P]ATP, 20 μM ssDNA, and MtRecD (300 nM) were incubated for 5, 10, 20, 30, 60, 90, 120, and 180 min (lanes 2–9), respectively. Lane 1, reaction mixture incubated for 180 min in the absence of MtRecD. B, graphic representation of time course of ATP hydrolysis by MtRecD. C, ATP hydrolysis as a function of MtRecD concentration. Assay was performed with 5 μM [γ-32P]ATP in the absence (lane 1) or presence of (lanes 2–8) 25, 50, 100, 200, 300, 400, and 500 nM MtRecD, respectively. D, graphic representation of the extent of ssDNA-dependent ATPase activity as a function of increasing MtRecD concentration. E, graphic representation of single- or double-stranded DNA-dependent ATPase activity. Assay was performed with a fixed concentration of MtRecD (300 nM) in the absence or presence of 2.5, 5, 7.5, 10, 15, 20, and 30 μM single- or double-stranded DNA, respectively. The error bars represent the deviation from the mean for data from three independent experiments. Asterisks denote radiolabeled ATP or inorganic phosphate (Pi). C, reaction performed in the absence of MtRecD.](image-url)
For an initial characterization, ssDNA-stimulated ATPase activity was analyzed as a function of time. We found that MtRecD exhibited maximum ATPase activity around 30 min and plateaued at 40% (Fig. 8, A and B). In an analogous experiment, we explored the dependence between ATP hydrolysis and MtRecD. We found that the activity increased with increasing concentrations of MtRecD (Fig. 8, C and D). We next determined the effect of increasing concentrations of DNA cofactors on the ATPase activity of MtRecD. We observed that MtRecD ATPase activity was 8-fold higher in the presence of ssDNA compared with dsDNA (Fig. 8E). Furthermore, the difference in the extent of ATPase activity is consistent with its higher binding affinity for ssDNA.

**Kinetics Parameters of ATP Hydrolysis Catalyzed by MtRecD**—Following initial characterization of ATPase activity, the kinetic parameters for ATP hydrolysis, $k_{cat}$ and $K_m$, were determined. ATP hydrolysis catalyzed by MtRecD follows Michaelis-Menten kinetics (Fig. 9). The rate of the reaction was calculated from the slopes of such plots. The $K_m$ and $k_{cat}$ values were determined in reactions performed in the presence of excess of ssDNA and ATP. For all concentrations of ATP, we determined the initial velocities from multiple time courses over time ranges giving linear hydrolysis of ATP. Velocities were plotted as functions of ATP concentration and fitted to the Michaelis-Menten equation.

**MtRecD<sup>K179A</sup> binds partial duplex with a 5' overhang but contains weak ATPase activity.** A, reactions were performed with 1 nM 32P-labeled DNA and increasing concentrations of MtRecD<sup>K179A</sup> in the absence (lane 1) or presence of 10, 25, 50, 75, 100, 150, 200, 300, 400, and 500 nM MtRecD<sup>K179A</sup> (lanes 2–11), respectively. B, graphic representation of the extent of the formation of MtRecD<sup>K179A</sup>-DNA complexes (CI and CII). The data points represent the mean of three independent experiments. C, reactions were performed with a fixed concentration of ATP and of increasing concentrations of MtRecD<sup>K179A</sup> in the absence (lane 1) or presence of 25, 50, 100, 200, 300, 400, and 500 nM MtRecD<sup>K179A</sup> (lanes 2–8), respectively. C, reaction performed in the absence of MtRecD mutant protein. D, graphic representation of the extent of ATPase activity as a function of increasing concentration of MtRecD<sup>K179A</sup>. The intensity of bands was quantified and plotted against the indicated protein concentrations.
results from mobility shift assays suggested thatMtRecD binds fers Partial Linear Duplex DNA and Y-shaped DNA Structures dent on the lysine residue in the Walker A box.

ities are intrinsic to the wild-type MtRecD polypeptide and depen-
these results are consistent with the notion that the observed activ-
the highest protein concentration tested (Fig. 10, 1, 1.5, 2, 2.5, 3, 3.5, 5, 7.5 and 10 mM ATP (lanes 4 –12), respectively. Lane 1, heat-denatured substrate; lane 2, substrate alone. B, graphic representation of DNA unwinding activity as a function of ATP concentration. The data points represent the mean of three independent experiments. C, kinetics of MtRecD-catalyzed DNA unwinding. Reaction mixtures containing 1 mM linear duplex with a 5’ overhang and 0.3 µM MtRecD was incubated for a period of 0, 5, 10, 15, 20, 25, 30, and 35 min (lanes 3–10), respectively. Lane 1, heat-denatured substrate; lane 2, substrate alone. D, graphic representation of the kinetics of unwinding. The intensity of bands in A and C was quantified after correcting the background using UVI-Band Map software version 97.4 and plotted using GraphPad Prism version 5.0. The data points represent the mean of three independent experiments. C, reaction performed in the absence of MtRecD.

determined by nonlinear regression fitting of the data to the Michaelis-Menten equation with a range of ATP concentrations in the presence of excess ssDNA cofactor (Fig. 9). ATPase activity catalyzed by MtRecD exhibited hyperbolic dependence on the ATP concentration (Fig. 9B, inset). The data from the linear phase of the curve was fitted to a Lineweaver-Burk plot (Fig. 9B), which yielded values of $K_m = 1.5 \times 10^{-6}$ M, $V_{max} = 45 \times 10^{-6}$ mol/min/liter, and a $k_{cat}$ of 2 min$^{-1}$. Although MtRecD showed high affinity for ATP, the relatively low $k_{cat}$ value suggests that it possesses weak ssDNA-dependent ATPase activity.

Characterization of MtRecD$^{K179A}$—Protein sequence alignments between MtRecD and E. coli RecD indicated a number of amino acid residues likely to be involved in catalysis. We have mutated one such residue, K179A, to assess whether the above activities were intrinsic to MtRecD and not due to a contaminant in the protein preparation. To this end, we performed DNA binding and ATPase assays with MtRecD$^{K179A}$. Like wild-type MtRecD, MtRecD$^{K179A}$ could bind linear duplex DNA having a 5’ overhang in a concentration-dependent manner (Fig. 10, A and B). In agreement with the essential role of Lys-179 in the Walker A motif, mutant MtRecD catalyzed measurable but insignificant levels of ssDNA-dependent ATPase activity even at the highest protein concentration tested (Fig. 10, C and D). Thus, these results are consistent with the notion that the observed activities are intrinsic to the wild-type MtRecD polypeptide and dependent on the lysine residue in the Walker A box.

MtRecD Unwinds a Broad Range of DNA Substrates but Prefers Partial Linear Duplex DNA and Y-shaped DNA Structures Containing \(\geq 18\)-mer 3’ or 5’ Single-stranded Overhangs—The results from mobility shift assays suggested that MtRecD binds efficiently to linear duplex DNA with 5’ or 3’ ssDNA overhangs and more robustly to Y-shaped DNA structures containing \(\geq 18\)-nt overhangs at both the 3’ or 5’ ends. We next examined the unwinding activity of MtRecD using a variety of DNA substrates by gel electrophoresis assays. We tested the ability of MtRecD to unwind linear duplex DNA having a 26-nt ssDNA overhang at the 5’ end as a function of ATP (1–10 mM) and time (0–35 min). Assays were performed with a fixed amount (1 nM) of$^{32}$P-labeled DNA substrate and 300 nM MtRecD as a function of ATP concentration. Under similar conditions, assay was performed as a function of time. The products were separated by PAGE and visualized as described under “Experimental Procedures.” Analysis of the data from these assays showed that unwinding occurred over a broad range of ATP concentrations, reached a maximum around 3 mM (Fig. 11, A and B), and plateaued at \(\sim 30\) min (Fig. 11, C and D).

Extensive studies have established that E. coli RecBCD unwinds duplex DNA to generate Y-shaped DNA structures (6, 7, 34). Therefore, we characterized MtRecD helicase activity using a variety of linear duplex and Y-shaped DNA structures having 5’ and 3’ ssDNA overhangs of equal and unequal lengths (Table 2). The assay was performed in reaction mixtures containing 1 nM of the indicated$^{32}$P-labeled DNA substrate, 3.5 mM ATP, and increasing concentrations of MtRecD (50–500 nM). Fig. 12 shows the DNA unwinding activity of MtRecD with a variety of DNA structures. In the absence of MtRecD, unwinding was observed with all the substrates due to heat-induced denaturation, but the extent was fairly low (Fig. 12, A–H, lane 2). In the presence of MtRecD, we observed unwinding of increasing percentages of$^{32}$P-labeled linear duplex having a 5’ overhang, but also a similar substrate having 3’ over-
hang, albeit at lower efficiency (Fig. 12, compare A with B). This observation is in accord with the unwinding activity of *E. coli* RecBCD, which initiates unwinding on duplex molecules that have ssDNA tails of ≤25 nucleotides (6, 7). Under similar conditions, MtRecD could unwind a variety of Y-shaped DNA structures but with lower efficiency relative to linear duplex with a 5′ overhang (Fig. 12, C–H). The extent of unwinding was ~60% with linear duplex having 5′ overhang, and it declined 2–3-fold in efficiency with linear duplex having 3′ overhang and different Y-shaped DNA structures (Fig. 12I). Paradoxically, although MtRecD exhibited robust binding to Y-shaped duplexes, compared with linear duplexes with ssDNA overhangs, its capacity to unwind these substrates was inefficient. While considering the possible reasons for inefficient unwinding of Y-shaped DNA duplexes, we speculate that MtRecD may require the assistance of accessory proteins to unwind forked DNA substrates. The experiments are underway to test this possibility. Nonetheless, these data support the idea that MtRecD by itself possesses strong 5′ → 3′ and weak 3′ → 5′ helicase activities.

To further investigate the unwinding mechanism catalyzed by MtRecD, we used linear duplex DNA substrates containing a 26-mer ssDNA overhang either at the 5′ or 3′ end with biotinylated nucleotide at 2 or 4 positions from the 3′ or 5′ end. The rationale behind the experiment is that the presence of biotin/streptavidin would physically block MtRecD binding, and therefore may inhibit unwinding. Reactions were performed with a fixed amount (1 nM) of the indicated 32P-labeled DNA substrate and varying concentrations (50–500 nM) of MtRecD. We previously observed that MtRecD could bind and form two discrete protein-DNA complexes with these substrates (Fig. 4, C and D), and we catalyzed unwinding of these substrates to an extent similar to those substrates, which are devoid of biotinylated nucleotides (Fig. 13, A and B). By contrast, when a biotin/streptavidin block was included on the 5′ tail, unwinding by MtRecD was completely abolished (Fig. 13, C and E). Similarly, an identical block on the 3′ tail inhibited unwinding (Fig. 13, D and E). Altogether, these results are consistent with the notion that MtRecD is a bipolar helicase with a strong 5′ → 3′ and a weak 3′ → 5′ helicase activity.

*M. tuberculosis* RecD Forms a Stable Complex with RecA—Previous studies have demonstrated that *E. coli* RecBCD facilitates the loading of RecA protein onto ssDNA and that the C-terminal domain of the RecB subunit interacts with RecA (53). It remained possible that other subunits of RecBCD complex may also influence the loading and activities of RecA. To this end, we employed far Western blot and co-immunoprecipitation assays to investigate possible interactions between MtRecD with its cognate RecA. Increasing amounts of MtRecD and MtRecDK179A were immobilized on a nitrocellulose membrane. Similarly, increasing amounts of MtSSB and BSA were immobilized to serve as positive and negative controls, respectively. Subsequently, the membrane was incubated with MtRecA, washed extensively prior to Western blot analysis. We observed that MtRecA bound to both MtRecD and MtRecDK179A to a comparable extent, indicating that MtRecD interacts with its cognate RecA (Fig. 14A). Consistent with previous studies (37), the results of positive control and negative control confirmed the validity and efficacy of this approach (Fig. 14A). To assess the specificity of interaction between MtRecD and its cognate RecA, we performed reciprocal experiments, in which increasing amounts of MtRecA and BSA were immobilized onto nitrocellulose membranes. After immunostaining, we observed no immunological cross-reactivity between BSA and MtRecD (Fig. 14B, lower panel). By contrast, we observed both MtRecD and MtRecDK179A interacted with MtRecA in a concentration-dependent fashion (Fig. 14B, upper and middle panels).

To further corroborate the interaction of RecD with RecA in the *in vivo* context, we performed reciprocal co-immunoprecipitation assays using *E. coli* whole-cell lysates expressing MtRecA and MtRecD. To exclude indirect DNA-mediated interactions, lysates were treated with DNase I, and the removal of nucleic acid contaminations was verified prior to the binding experiments. Whole-cell lysates were incubated with anti-RecA and then immunoblotted with anti-MtRecD. Conversely, cell lysates were incubated with anti-MtRecD and immunoblotted with anti-RecA antibodies. As shown in Fig. 14C, Western blot analysis confirmed specific interaction of MtRecD with its cognate RecA. The observation that RecA specifically interacts with RecD *in vivo* is consistent with the notion that interaction between them is physiologically relevant.

MtRecD Inhibits MtRecA-mediated DNA Strand Exchange—Previously, genetic and biochemical studies have demonstrated that HR in *E. coli* requires loading of RecA by the RecBCD enzyme and that RecD subunit inhibits this reaction (see Ref. 30 and references therein). However, the mechanistic basis of RecD-promoted inhibition is poorly understood. To verify and extend the above conclusions, we used DNA strand exchange assay to gain insights into the potential biological significance of physical interaction between MtRecD and its cognate RecA. Fig. 15A depicts the schematic of strand labeling and progress of the strand exchange reaction. We first incubated 2.5 μM MtRecA with 5 μM 83-mer ssDNA in the presence of ATP and then with increasing concentrations of MtRecD (0.05–1 μM). Strand exchange reaction was started by the addition of 1 μM 32P-labeled 83-bp duplex DNA. After 10 min of incubation, the reactants and products were separated by native PAGE, and the
The band intensities of the two radioactive species, namely linear duplex DNA and the displaced ssDNA, were quantified as described under “Experimental Procedures.” We observed that strand exchange promoted by MtRecA was inhibited by MtRecD in a concentration-dependent manner (Fig. 15, B and C). Furthermore, DNA binding and unwinding reactions catalyzed by MtRecD were found to be bipolar, with DNA unwinding activities observed on both the 5' and 3' ends. The graphical representation of the extent of unwinding of DNA substrates in A–D plotted versus increasing concentrations of MtRecD showed a concentration-dependent inhibition by MtRecD of the linear duplex DNA overhangs in C and D. The black circle on linear duplex DNA overhangs in C and D corresponds to the position of biotinylated nucleotide. The PacMan symbol corresponds to streptavidin.
lyzed by MtRecD revealed that it neither binds blunt-ended duplex DNA nor catalyzes its unwinding. In line with these findings, our data support the view that MtRecD interacts directly with RecA, functions as an anti-recombinase, and negatively regulates HR.

**DISCUSSION**

The detailed characterization of the substrate specificity and unwinding activities of the RecBCD enzyme complex as well its interacting partner(s) is essential for our understanding of the role of this enzyme in *M. tuberculosis*. Our knowledge about the molecular and functional characteristics of this enzyme complex or its individual subunits in mycobacteria remains largely unknown. Here, we report that MtRecD exists in solution as a stable homodimer. We found that MtRecD exhibits similar DNA binding specificities for 3′- and 5′-recessed linear duplexes but more robust binding activity with Y-shaped DNA structures. Although these results reveal that MtRecD is a bipolar helicase, the extent of directional unwinding is not equivalent; 5′–3′ unwinding activity is much stronger relative to 3′–5′ unwinding activity. We note in this context that the *E. coli* RecBCD complex is a bipolar helicase, wherein RecB and RecD subunits of the enzyme complex unwind DNA in a 3′ → 5′ and 5′ → 3′ direction, respectively (7, 34). Furthermore, our data reveal that MtRecD interacts directly with RecA and functions as an anti-recombinase, consistent with the idea that MtRecD may negatively regulate HR.

The helicases are classified into six different superfamilies, namely SF1 to SF6 (7, 34). Among these, superfAMILY I contains the largest number of helicases, and they are involved in all aspects of DNA and RNA metabolism (34). The SF1 family of helicases is further subdivided into three families based on the polarity of their translocation on ssDNA. For example, SF1A helicases translocate in a 3′ to 5′ direction and SF1B helicases in a 5′ to 3′ direction, respectively. The well characterized members of SF1A helicase subfamily include PcrA, Rep, and UvrD, whereas RecD and Dda belong to SF1B category (54, 55). The oligomeric nature of the SF1 family of helicases has been the subject of extensive debate. *E. coli* Rep and UvrD function as dimers, whereas *Bacillus stearothermophilus* PcrA, phage T4 Dda, and TraI helicases are active in the monomeric form (56–62). *E. coli* and *D. radiodurans* RecD exist in solution as monomers (63–65). Using multiple complementary methods, we show that MtRecD exists in solution as a homodimer in the absence of DNA. The recombinant MtRecD contains the C-terminal His tag. Like *E. coli* recombinant RecD, which contains a His tag (29), His-tagged MtRecD showed similar enzymatic characteristics.

In general, DNA helicases show specific directionality in unwinding of duplex DNA substrates, defined as 5′ → 3′ or 3′ → 5′, depending on whether the substrate contains a 5′ or 3′ ssDNA overhang. The first step in the action of the RecBCD complex involves its binding to DNA and the manifestation of its nuclease/helicase activity in an ATP-dependent manner (7, 54, 55). A combination of approaches, including genetic, structural, biochemical, and single molecular measurements, have been employed to elucidate the mechanistic aspects of *E. coli* RecBCD helicase-nuclease complex in HR (7, 34). However, our understanding of the functional roles of individual subunits of the RecBCD enzyme complex has been seriously hampered due to the lack of active subunits. The purified *E. coli* RecD, reconstituted from inclusion bodies, has a low DNA unwinding activity but stimulated rapid DNA unwinding by the reconstituted RecBCD complex (29). One caveat of this study is that the resulting activity could be from two helicase subunits, RecB and RecD. The purified His-tagged *D. radiodurans* RecD unwinds 20-bp DNA duplexes with a 10–12 nt 5′ overhang or a 20-bp forked substrate but not blunt-ended or 3′-tailed duplex DNA (64). In other studies, the His-tagged *E. coli* RecD has been
shown to catalyze unwinding of 40-bp duplex having a 5' ssDNA overhang in the 5'–3' direction, but not a similar substrate with a 3' overhang or a blunt-ended duplex (65). To our knowledge, it is unknown whether *E. coli* RecD subunit can bind and catalyze unwinding of Y-shaped DNA structures. Crystal structures of the members of SF1 helicases have revealed the mechanism of their translocation on ssDNA, as in the case of PcrA and UvrD, or in the 5'–3' direction by RecD2 (33, 66). The crystal structure of *D. radiodurans* RecD2 is similar to that of *E. coli* RecD (33, 66). The enzyme binds an 8-nucleotide stretch of ssDNA in a cleft of the protein, which contains a tyrosine residue that acts as a “pin” for strand separation during DNA unwinding reactions.

The substrate specificity and the mechanistic aspects of DNA unwinding by the members of the RecD family have been minimally defined. In this study, we examined the ability of MtRecD to bind and unwind a variety of model DNA substrates. Protein-DNA interaction assays showed that MtRecD bound stably to a partial duplex DNA with 3' or 5' overhangs or Y-shaped DNA structures having ≥18-nt overhangs at either end, suggesting that partial duplexes having either 3' or 5' ssDNA overhang ends play an important role in the DNA substrate specificity of MtRecD. However, MtRecD was not able to bind blunt-ended or Y-shaped DNA having 5-nt overhangs. Although MtRecD can bind linear duplex having 5'– or 3' overhangs with nearly equal efficiency, unwinding of the 5' overhang substrate was more efficient than unwinding of substrates containing 3' overhangs. These results differ from those reported for *D. radiodurans* RecD1, *Pseudomonas syringae* RecD, and UvrD helicase (64, 67, 68). MtRecD also exhibited ATP binding as well as ssDNA-dependent ATPase activity, consistent with the activities of *E. coli* RecD and *D. radiodurans* RecD2 (52, 64). MtRecD<sup>K179A</sup> showed reduced levels of ATPase activity, suggesting that the lysine residue in the Walker A motif is involved in ATP binding/hydrolysis (7, 34, 69). Protein-DNA binding assays suggested that the DNA binding activity of MtRecD<sup>K179A</sup> was comparable with that of wild-type MtRecD, indicating that the mutation did not perturb protein folding.

The observation that RecA and RecD physically interact with each other raises the possibility that, under some in vivo conditions, RecD might exist independently rather than remain bound to the RecBC enzyme complex. Several lines of evidence suggest that the RecBCD resects linear duplex to generate the recombinogenic ssDNA substrate(s). However, the actual composition of RecBCD enzyme complex following the χ encounter has been the subject of a long and contentious debate. Several recent observations indicate that a number of proteins regulate the recombination-like activities of RecA. The possible mechanism of inhibition may involve direct or indirect interaction between RecA and the proteins that exert their effect at different stages of HR pathway. The proteins that negatively regulate RecA activity include UvrD, RecX, PcrA, and UvrA proteins (46, 70–76). However, genetic and biochemical evidence is consistent with the inhibitory effect of *E. coli* RecD on HR promoted by RecA. To this end, *E. coli* RecD has been shown to negatively regulate the loading of *E. coli* RecA onto ssDNA in a χ-regulated manner (30).

It seems that mycobacteria have evolved complex networks and dedicated enzymes for the repair of DSBs, complex DNA lesions, and to promote HR. The work from Glickman and coworkers (20) in *M. smegmatis* has shown that RecBCD is not essential for HR and to confer resistance to ionizing radiation, but it is indispensable for RecA-independent single strand DNA annealing pathway. However, AdnAB is involved in the RecA-dependent HR pathway and confers resistance to IR and oxidative DNA damage. These findings suggest important and
possibly distinctive features of the mycobacterial DSB repair machinery. In particular, the very fact that recB, recC, and recD genes are absent in M. leprae but are retained by M. tuberculosis emphasizes the need to understand the role(s) of each of the subunits of RecBCD enzyme complex. To this end, our studies provide both direct evidence and important insights into the mechanism and regulation of RecD catalyzed activities, thereby furthering our understanding of recombinational DNA repair in M. tuberculosis.

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