ATP-dependent motor activity of the transcription termination factor Rho from *Mycobacterium tuberculosis*

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ABSTRACT

The bacterial transcription termination factor Rho—a ring-shaped molecular motor displaying directional, ATP-dependent RNA helicase/translocase activity—is an interesting therapeutic target. Recently, Rho from *Mycobacterium tuberculosis* (*Mt*Rho) has been proposed to operate by a mechanism uncoupled from molecular motor action, suggesting that the manner used by Rho to dissociate transcriptional complexes is not conserved throughout the bacterial kingdom. Here, however, we demonstrate that *Mt*Rho is a *bona fide* molecular motor and directional helicase which requires a catalytic site competent for ATP hydrolysis to disrupt RNA duplexes or transcription elongation complexes. Moreover, we show that idiosyncratic features of the *Mt*Rho enzyme are conferred by a large, hydrophilic insertion in its N-terminal ‘RNA binding’ domain and by a non-canonical R-loop residue in its C-terminal ‘motor’ domain. We also show that the ‘motor’ domain of *Mt*Rho has a low apparent affinity for the Rho inhibitor bicyclomycin, thereby contributing to explain why *M. tuberculosis* is resistant to this drug. Overall, our findings support that, in spite of adjustments of the Rho motor to specific traits of its hosting bacterium, the basic principles of Rho action are conserved across species and could thus constitute pertinent screening criteria in high-throughput searches of new Rho inhibitors.

INTRODUCTION

RNA helicases are ubiquitous motor proteins that remodel RNA and RNA-protein structures and are involved in all steps of RNA metabolism (1,2). Their activities are often implicated in infection and disease and thus constitute attractive prognostic/diagnostic markers or potential drug targets (3). RNA helicases generally rely on one of two distinct mechanisms of action (1,4). Some RNA helicases are directional, NTP-fueled translocase enzymes exhibiting some degree of processivity. In this case, the helicase loads onto a single-stranded RNA segment and then uses the energy derived from ATP hydrolysis to migrate toward one end of the RNA chain, removing bound obstacles (such as a complementary strand) from its path. This mechanism is reminiscent of that used by DNA helicases (5). Other RNA helicases are local ‘disruptases’ that load on RNA and remodel/disrupt its local structure (or local interaction with an RNA-binding protein) in an ATP-dependent fashion. In this case, the RNA helicase acts as a non-directional and non-processive molecular switch. Local ‘disruptases’ are best exemplified by DEAD-box proteins which compose the largest group of RNA helicases. Upon ATP binding, DEAD-box proteins adopt a tight RNA-bound, ‘close’ state able to denature local RNA structures; upon release of the products of ATP hydrolysis, they revert to an RNA-free ‘open’ state allowing enzyme recycling (1,6).

The bacterial transcription termination factor Rho is an important model of ring-shaped helicase (4,5). Rho unwinds deleterious RNA:DNA duplexes (R-loops) that are formed behind RNA polymerase (RNAP) during transcription and dissociates transcription elongation complexes (TECs) at specific loci of bacterial genomes (7,8). The molecular mechanisms used by Rho to unwind duplexes and dissociate TECs have been studied extensively, although work has focused primarily on the Rho factor from *Escherichia coli* (*Ec*Rho) (7,9). These studies revealed that Rho uses a sophisticated translocation mechanism, called ‘tethered tracking’, to slide in a 5–3′ direction along nascent RNA, dissociating transcriptional R-loops and/or TECs in the process (Figure 1A). This mechanism invokes net translocation steps of one RNA base per hydrolyzed
ATP molecule (10–12) and conforms to the general idea that toroidal helicases directionally translocate nucleic acids within their central channel (5).

Rho is an interesting antibacterial target because it is widespread in bacteria and absent from eukaryotes (13). Bicyclomycin (BCM), a natural Rho inhibitor used in veterinary medicine (14), is mostly effective against Gram-negative bacteria (15). Until now, only one Gram-positive species—Micrococcus luteus—has been found to be sensitive to BCM (16). Bacterial resistance to BCM may have several origins. Rho may be dispensable in (as for Staphylococcus aureus) (17) or absent from (as for ~8% of bacterial species) (13) a given bacterium. Alternatively, the bacterium may use a specific resistance mechanism, for instance one conferred by a drug efflux protein (18) or by mutations in Rho that alter BCM binding (13,19). It is also conceivable that the mechanism of action of Rho is different and insensitive to BCM in some bacteria. BCM binds in a subsite of that of EcRho and reminiscent of that used by RNA helicases (36). A schematic of the RNA-bound Rho hexamer (7) is shown in the inset. (A) A model of the MtbRho hexamer. An homology model of the MtbRho subunit was built with Swiss-Model (http://www.swissmodel.expasy.org/) without pre-defined template. Then, the MtbRho hexamer was reconstructed by aligning modeled MtbRho subunits with subunits of the EcRho hexamer (PDB: 3ICE) using Pymol software (http://www.pymol.org). Apart from the insertion domain, the MtbRho and EcRho subunits are structurally similar (rmsd ~ 1.3 Å). Color coding is the same as in panel B.

Rho is considered essential for the growth of Mycobacterium tuberculosis (21–23), a species that is nonetheless resistant to BCM (24). A recent report suggests that M. tuberculosis’ Rho (MtbRho) has no duplex unwinding (helicase) activity and can dissociate TECs without efficient molecular motor action (25). The authors argue that only ATP binding to MtbRho is required for its transcription termination function because non-hydrolysable adenosine 5′-(βγ-imido)triphosphate (AMP-PNP) and ATPγS analogs stimulate the dissociation of artificially stalled TECs by MtbRho. They also observed that BCM inhibits the ATPase activity of MtbRho (IC_{50} = 30 μM) but fails to significantly affect transcription termination in the tested concentration range (up to 200 μM of BCM) (25). These unexpected findings suggest that MtbRho uses a mechanism that is different from that of EcRho and reminiscent of that used by RNA helicases working as local ‘disruptases’.

To test the possibility that MtbRho uses a molecular switch (e.g. local ‘disruptase’) mechanism that would make it intrinsically resistant to BCM, we have studied the motor activity of MtbRho in detail using in vitro ATPase, duplex unwinding and transcription termination assays as well as a catalytic MtbRho mutant and MtbRho/EcRho do-
main fusion chimera. We show that MtbRho is a [5′→3′]-directional, ATP-dependent RNA helicase which requires a catalytic site that is fully competent for ATP hydrolysis to promote duplex unwinding or transcription termination. Non-hydrolysable ATP analogs do not promote duplex unwinding by MtbRho nor change dramatically MtbRho affinity for RNA. Furthermore, both the helicase and transcription termination activities of MtbRho can be inhibited by BCM, albeit at concentrations that are significantly higher than for EcRho. Experiments with a domain deletion mutant and the MtbRho/EcRho chimera indicate that this low apparent BCM affinity is conferred principally by the C-terminal domain (CTD) of MtbRho while the N-terminal domain (NTD) specifies which regions of nascent RNA transcripts are utilized by MtbRho. Overall, our data argue against a scenario whereby MtbRho would rely on a distinctive, motor-independent mechanism (25) and demonstrate that MtbRho is an ATP-dependent translocase that is well adapted to the peculiar transcriptome and slow metabolism of M. tuberculosis. Our findings also suggest that inhibition of the ATPase-dependent activities of MtbRho could be a valid strategy in search of new antibiotics targeting M. tuberculosis.

MATERIALS AND METHODS

Materials

Chemicals and enzymes were purchased from Sigma-Aldrich and New England Biolabs, respectively. BCM was purchased from Santa Cruz Biotechnology. Sigma-saturated RNAP from E. coli was obtained from Epicentre Technologies. Nucleoside triphosphates were purchased from GE-Healthcare while ADP, AMP-PNP and ATPγS analogs were from Sigma-Aldrich. Synthetic oligonucleotides were obtained from Eurogentec. Polynucleotide fragment (> 300 nt) stocks were prepared as described previously (26) from polynucleotide batches obtained from Santa Cruz Biotechnology and Midland Certified Reagent Company (Midland, TX, USA). Streptavidin-coated magnetic beads were from Ademtech (Pessac, France). DNA templates used in in vitro transcription reactions were prepared by standard PCR procedures, as described previously (27). RNA substrates were obtained by in vitro transcription of PCR amplicons with T7 RNAP and purified by denaturing polyacrylamide gel electrophoresis (PAGE), as described (26). Plasmids for over-expression of single-point MtbRho mutants were prepared by Quickchange (Stratagene) mutagenesis of the pET28b-MtbRho plasmid (kindly provided by Dr. Ranjan Sen, Hyderabad, India). Plasmids for over-expression of the MtbRho deletion mutant (wherein amino acids 76–219 of MtbRho are replaced by a Val-Pro dipeptide) and the [Mtb-Ec]Rho (wherein amino acids 1–308 of MtbRho are fused to amino acids 131–419 of EcRho) and [Ec-Mtb]Rho (wherein amino acids 1–130 of EcRho are fused to amino acids 309–602 of MtbRho) chimeras were obtained by inserting synthetic DNA fragments (obtained by overhang extension PCR using the pET28b-Rho (26) and/or pET28b-MtbRho plasmids as starting templates) between the NdeI and XhoI sites of the pET28b plasmid (Novagen) following standard cloning procedures (see Supplementary Table S1 for details). The sequences of DNA templates and plasmids were verified by capillary DNA sequencing (Genoscreen, France).

Preparation of proteins

The EcRho protein was prepared and purified as described previously (26). Wild-type (WT) and mutant MtbRho factors as well as MtbEc protein chimeras were over-expressed as N-terminal His tag fusions (25) in Rosetta 2(DE3) cells (Merck-Millipore) harboring the appropriate pET28b derivative and were purified following published procedures (28). Briefly, the MtbRho proteins were affinity selected on His-Pur cobalt resin (Thermo scientific) and were subsequently purified by cation exchange chromatography on a SP sepharose FF column (GE-Healthcare) with a 0.15-1M NaCl gradient and by gel filtration chromatography on a Sephacryl S-300 HR column (GE-Healthcare) equilibrated in GF buffer (150 mM NaCl, 20 mM HEPES, pH 7.5, 2 mM β-mercaptoethanol). Protein preparations were controlled by liquid chromatography-high-resolution mass spectrometry using an UltiMate 3000 NanoRS LC system (Dionex, Sunnyvale, CA, USA) connected to a 4-GHz MaXis ultra-high resolution quadrupole-TOF spectrometer (Bruker Daltonics) equipped with an electrospray ion source. All proteins were stored at −20°C as micromolar solutions in 100 mM KCl, 10 mM Tris-HCl, pH 7.9, 0.1 mM EDTA, 0.1 mM DTT and 50% (v/v) glycerol. Concentrations of EcRho and MtbRho proteins are expressed in hexamers throughout the manuscript. Purified Ded1 protein was kindly provided by Prof. Eckhard Jankowsky (Case Western Reserve University, Cleveland, USA).

Preparation of duplex substrates

Duplexes were assembled by mixing 10 pmoles of RNA transcript with 12 pmoles of complementary oligonucleotide (bearing a 6-carboxyfluorescein label at the 5′-end) in annealing buffer (150 mM potassium acetate, 20 mM HEPES, pH 7.5, 1 mM EDTA). Mixtures were heated at 95°C and slowly cooled to 20°C. Duplexes were then purified by native 7% PAGE and stored at −20°C in helicase buffer (20 mM HEPES, pH 7.5, 0.1 mM EDTA, 0.5 mM DTT and 150 mM potassium glutamate) (26).

NTP hydrolysis assay

The NTP hydrolysis activities of the Rho enzymes were determined with the EnzCheck Phosphate Assay kit (Molecular Probes) as described previously (27,28). Briefly, two quartz cuvettes containing equal volumes of a solution of 20 nM Rho, RNA cofactor (e.g. poly[υC]) at a standard concentration of 10μM in Rcs buffer (0.4 mM 2-amino-6-mercaptopurine riboside and 1 U/mL of purine nucleoside phosphorylase in NTPase buffer (1 mM MgCl2, 50 mM Tris-HCl, pH 7.5, 0.1 mM sodium azide) were placed in the control and sample beam holders of an Uvikon-XL UV-spectrophotometer equipped with a 37°C circulating water bath. After equilibration at 37°C for 5 min, equal volumes of water and NTP (1 mM, final concentration)
were added, respectively, to the control and reaction cuvettes and absorbance at 360 nm was recorded as a function of time. The NTPase rates were deduced from the initial first-derivative maxima that were obtained after smoothing of the time-dependent $A_{305}$ curves with Kaleidagraph software. The experiments were calibrated with standard samples containing known amounts of phosphate, as recommended by the kit manufacturer.

Equilibrium binding assays

Equilibrium Rho-RNA dissociation constants were determined using a filter-binding assay, as described previously (29,30). Briefly, ∼10 fmol of $^{32}$P-labeled RNA substrate were mixed with various amounts of Rho in 100 μl of binding buffer (20 mM HEPES, pH 7.5, 0.1 mM EDTA, 0.5 mM DTT, 150 mM potassium acetate and 20 μg/ml BSA) containing 0 or 1 mM ATP (or AMP-PNP). After incubation for 10 min at 30°C, the samples were filtered through stacked [top] nitrocellulose (Amersham Protran) and [bottom] cationic nylon (Pall Biodyne B) membranes using a Bio-dot SF apparatus (Biorad). No significant differences were observed in control experiments performed with 30 min incubation times (not shown). The fractions of free and Rho-bound RNA (retained on the nylon and nitrocellulose membranes, respectively) as a function of Rho concentration were then determined by phosphorimaging of the membranes using a Typhoon-Trio imager and dedicated ImageQuant TL v8.1 software (GE healthcare).

Duplex unwinding assay

Helicase reactions were performed as described previously (31) with 6-carboxyfluorescein labeled duplex substrates (control experiments with $^{32}$P-labeled substrates supported that the fluorescein moiety does not affect $\text{Mth}_\text{Rho}$ behavior) (28). Briefly, 5 nM substrate was mixed with 50 or 500 nM Rho and 0–300 μM BCM in helicase buffer and incubated for 3 min at 30°C. Then, 1 mM MgCl₂, 1 mM nucleotide cofactor (ATP, ADP, ATPγS, AMP-PNP, ADP-BeFx or ADP-αIF₄) and 400 nM oligo trap (unlabeled oligonucleotide having the same sequence than the released ‘reporter’ strand) were added to the helicase mixture before further incubation at 30°C. Reaction aliquots were taken at various times and mixed with two volumes of quench buffer (10 mM EDTA, 1.5% SDS, 300 mM sodium acetate, 6% Ficoll-400) before being loaded on 9% polyacrylamide gels that contained 1XTBE and 0.5% SDS. Detection and quantification of gel bands were performed by fluorescence imaging with a Typhoon-Trio imager as described (28). Helicase reaction parameters were obtained by fitting data points to an equation describing the kinetic regimen uncovered previously for $\text{E}_{c}\text{Rho}$:

$$F_p = A \times (1 - e^{-k_{\text{exp}}t}) + k_{\text{lin}} \times t$$

where $F_p$ is the fraction of product formed, $A$ is the amplitude of the exponential (burst) phase of the reaction, and $k_{\text{exp}}$ and $k_{\text{lin}}$ are the rate constants of the exponential and linear phases of the reaction, respectively (30,32).

Transcription termination experiments

Transcription termination experiments were performed under single-round ‘chase’ conditions as described previously (33) with minor modifications. Briefly, biotinylated DNA templates were prepared by PCR amplification of pT7A1-αcro DNA template (34) using a 5‘-biotinylated reverse primer. To allow preparation of TECs halted at +24, a forward mutagenic PCR primer was also used to introduce a C→T mutation at position +6 of the pT7A1-αcro DNA template. The resulting mAS97Bt template (30 nM) was mixed with E. coli RNAP holoenzyme (36 nM) in transcription initiation buffer (40 mM Tris-HCl, pH 7.9, 50 mM KC1, 5 mM MgCl₂, 1 mM DTT, 0.05 mg/ml BSA, 0.4U/μl Superase-In [Ambion]) and incubated at 37°C for 5 min. The mixture was then supplemented with 12 μM ApU, 6 μM ATP, 6μM GTP, 0.95μM UTP, and 1 μCi/μl $[\text{o}^{32}\text{P}]$-UTP and incubated for 10 min at 37°C. Then, the mixture was chilled on ice. KCl concentration was raised to 0.1 M and the halted transcription complexes were purified by filtration through a microspin G50 column pre-equilibrated with transcription elongation buffer (40 mM Tris-HCl, pH 7.9, 100 mM KC1, 5 mM MgCl₂, 1 mM DTT, 0.1 mg/ml BSA, 0.2U/μl Superase-In). The complexes were then immobilized on streptavidin beads (Supplementary Figure S4) or used directly in single-run chase reactions. In the latter case, an aliquot of the solution of halted transcription complexes (~15 nM) was supplemented with 70 nM Rho and 0–750 μM BCM in transcription elongation buffer and incubated for 10 min at 37°C. Then, rNTPs (75 μM each, final concentrations) and rifampicin (25 μg/ml) were added to initiate chase reactions. After incubation for 10 min at 37°C, the mixtures were mixed with 1 volume of denaturing buffer (95% formamide, 50 mM EDTA) and resolved by 8–10% denaturing PAGE. Gels were analyzed by Typhoon-Trio phosphorimaging. Apparent global termination efficiencies ($\text{TE}_{\text{app}}$) were defined as:

$$\text{TE}_{\text{app}} = \frac{\sum I_{\text{term}}}{\sum I_{\text{term}} + \sum I_{\text{runoff}}} \times 100$$

where $\sum I_{\text{term}}$ is the sum of the intensities of the bands corresponding to termination products while $\sum I_{\text{runoff}}$ is the sum of the intensities of the bands corresponding to runoff products. IC₅₀ values for BCM inhibition were obtained by non-linear least-square fitting of $\text{TE}_{\text{app}}$ values obtained as a function of BCM concentration to a standard sigmoid inhibition equation:

$$\text{TE}_{\text{app}} = (\text{TE}_{\text{app}})_0 - F_{\text{max}} \times \frac{[\text{BCM}]^p}{[\text{BCM}]^p + [\text{IC}_{50}]^p}$$

where (TE$_{\text{app}}$)$_0$ is the value of TE$_{\text{app}}$ at 0 μM BCM, $F_{\text{max}}$ is the maximal fraction of TE$_{\text{app}}$ that is sensitive to BCM inhibition and $n$ is an empirical parameter that defines the sigmoid shape of inhibition (35).

Data reporting

All data values are reported as mean ± standard deviation calculated from at least two independent experimental replicates (whenever number of replicates $N_R > 2$ is indicated in figure legends).
RESULTS

Selection of protein mutants and chimeras to probe MtbRho features

The typical EcRho ring is made of six identical protein subunits, each divided in two distinct domains (7–9,36,37). The NTD contains primary binding site (PBS) motifs involved in the recruitment of RNA †Rut (Rho utilization) sites by the Rho hexamer (Figure 1A and B). PBS motifs include residues forming a cleft only large enough to accommodate a 5′-YC dimer (Y and C being pyrimidine and cytosine residues, respectively), thereby explaining the preference of EcRho for single-stranded C-rich Rut sites (7–9,37). The CTD contains residues required for intersubunit cohesion and for ‘molecular motor’ function. Essential CTD motifs include Walker A and B motifs forming the ATP binding pockets at subunit interfaces, ‘catalytic Glu’, ‘Arg valve’ and ‘Arg finger’ residues required for the catalysis of ATP hydrolysis as well as Q-loop and R-loop residues forming the ‘Arg finger’ residues required for the catalysis of ATP hydrolysis (10,38).

Figure 1. Comparison of ATPase activity of MtbRho with EcRho. (A) Comparison of the ATPase rate of WT MtbRho with rates measured for various NTP substrates and RNA cofactors (number of replicates, Nk = 4 for 10 µM poly[rC]). (B) Comparison of the ATPase rate of WT MtbRho with rates measured for MtbRho variants (Nk = 3 for ΔMtbRho and Nk = 4 for WT MtbRho).

Importantly, changes in the chemical state of the ATPase domains critical for Rho mechanochemistry.

Secondary binding site (SBS) for RNA (Figure 1B) (10,38). The non-conservative mutation of MtbRho (E211A mutation) does not affect ATP binding or protein oligomerization but completely abolishes ATP hydrolysis (38).

Tight allosteric coupling within the MtbRho enzyme

To assess the substrate/cofactor preferences of MtbRho, we performed standard measurements of its NTP hydrolysis rate (27) in the presence of various NTP substrates and polynucleotide cofactors. In agreement with previous reports (25,39), we found that an RNA cofactor is required to activate NTP hydrolysis by MtbRho (Figure 2A and data not shown). Poly[rC] is the preferred polynucleotide cofactor with a Km of ~0.7 µM (Figure 2A) while ATP is the preferred substrate (Figure 2B). Hydrolysis of CTP is highly inefficient (Figure 2B) at a concentration (1 mM) that is saturating for ATP (Km, ATP ~ 70 µM) (25), a feature that has also been observed for the Rho factors of M. luteus (MlRho) (40) and Streptomyces lividans (41), two evolutionary relatives. By contrast, the phylogodivergent EcRho displays little preference for a specific NTP (40).

Under our standard conditions (see ‘Materials and Methods’ section), the rate of ATPase turnover is about 3-fold lower for MtbRho than for EcRho or for the MtbRho chimera (Figure 2C), suggesting that components in the CTD of MtbRho are suboptimal. Deletion of the NTD insertion subdomain of MtbRho (ΔMtbRho) reduces the ATPase rate by half under standard conditions (Figure 2C). However, a higher concentration of poly[rC] cofactor is required to reach maximal ATPase turnover with the ΔMtbRho variant (data not shown), which is consistent with an RNA binding defect induced by the deletion of the NTD subdomain (see Table 1 and below). At saturating poly[rC]
To probe the motor properties of MtbRho, we performed ATP-dependent fashion the MtbRho and EcRho enzymes. One should recall, however, that Thr301 has a less important role in MtbRho than has the corresponding Lys326 side chain in the R-loop of EcRho (the K326A mutation reduces the ATPase activity of EcRho by ~75%) (10,27). In sharp contrast, non-conservative mutation of the catalytic Glu386 residue (E386A mutant) completely inhibits ATP hydrolysis by MtbRho, even when concentrations of ATP and poly[rC] are increased to 5 and 30 μM, respectively (Figure 2C and data not shown). This dramatic effect induced by the E386A mutation is not matched by a drastic change of the oligomeric state or capacity to bind ATP or RNA (Supplementary Figure S2 and Table 1).

Thus, the E386A phenotype is comparable to that of the inactivating E211A mutation in EcRho (38) which supports that the conserved catalytic glutamate residue (Supplementary Figure S1) is also required in MtbRho for polarization of the water molecule attacking the γ-phosphoryl group of ATP.

Taken together, these data establish that coupling between RNA binding and ATP hydrolysis is tight within the MtbRho ring and most likely relies on similar principles in the MtbRho and EcRho enzymes. One should recall, however, that the rate of ATP hydrolysis is a measure of fuel consumption but by no means a measure of motor efficiency. The latter depends on multiple factors governing the mechanochemical transduction mechanism and yield (42), some of which may vary between the MtbRho and EcRho motors.

**MtbRho unwinds short RNA duplexes in a directional, ATP-dependent fashion**

To probe the motor properties of MtbRho, we performed duplex unwinding (helicase) experiments with model substrates (26). We prepared RNA:DNA substrates containing a 57 bp (substrate A) or 34 bp (substrate B) duplex region located downstream from a single-stranded RNA (ssRNA) tail encompassing a synthetic Rut site (Figure 3, inset) (30). We also prepared a 5′-ssRNA tailed substrate containing a shorter duplex (14 bp, substrate C) by pairing an oligo(2′-O-methylribonucleotide) to the RNA strand (Figure 3, inset). The 2′-O-methyl modifications, which increase RNA duplex stability significantly (43), prevent spontaneous denaturation of substrate C under our experimental conditions (Figure 3, ADP lanes in bottom gels).

We incubated each substrate with a 10-fold molar excess of Rho hexamers in the presence of ATP under 'standard' helicase reaction conditions (see ‘Material and Methods’ section) (30,32). As expected, EcRho unwinds the three duplex substrates with high efficiencies (Figure 3, left gels and graph). Substrate C is slightly less reactive than the longer duplex substrates, probably because its 2′-O-methyl moieties favor an A-form duplex conformation and non-productive interactions with EcRho (31). By contrast, MtbRho unwinds substrate C most efficiently, unwinds substrate B very inefficiently, and is completely unable to unwind the 57 bp-long duplex of substrate A (Figure 3, right gels and graph). Incubation of the substrates with larger excesses (100-fold) of MtbRho does not change unwinding kinetics significantly (data not shown), indicating that MtbRho was already present at a saturating concentration. These data demonstrate that MtbRho is a RNA helicase, albeit one that works on a narrower range of substrates than EcRho.

This narrow spectrum of activity may be due to suboptimal interactions between MtbRho and the substrates. Optimal Rut features for MtbRho are currently unknown and may not be adequately represented by our synthetic Rut sequence (Figure 3, inset), which had been optimized for EcRho (30,44). Furthermore, the interaction network promoting RNA translocation may be weakened in MtbRho by the K→T substitution at position 501 of the R-loop (corresponding to SBS residue Lys326 in EcRho; see above). To test these scenarios, we performed unwinding experiments with MtbRho protein variants. We observed that the [Mtb:Ec]Rho chimera unwinds the three substrates with effi-
These data combined with equilibrium binding measurements (Table 1) confirm that the NTD insertion subdomain (∆MtbRho variant) also significantly increase the capacity of MtbRho to unwind the 14 bp and 34 bp duplex substrates (Figure 4A, open circles). When present at a 10-fold higher concentration (500 nM), the ∆MtbRho variant can even unwind the 57 bp duplex substrate (Figure 4B). It has been shown for EcRho that the amplitude of the exponential phase depends on helicase processivity under similar unwinding reaction conditions (30, 32). These observations suggest that stabilizing, productive contacts are formed between the R-loop and RNA within the SBS of MtbRho but that these contacts are suboptimal unless Thr501 is replaced by a lysine. Significantly, a lysine or arginine is found at the corresponding R-loop position in the Rho factors from most species (e.g. Lys326 in EcRho) (13), with the notable exception of mycobacteria and coryneform relatives (data not shown). Taken together, these findings indicate that features weakening the MtbRho helicase (with respect to the EcRho prototype) are located both in its N-terminal ‘RNA anchoring’ and in its C-terminal ‘motor’ domains.

Selective unwinding of short duplexes by MtbRho is reminiscent of the activity of DEAD-box proteins which disrupt short RNA duplexes in a local, non-directional fashion. However, MtbRho is unable to unwind a substrate C derivative wherein the relative positions of the Rut-containing ss-RNA and 14bp duplex regions are permuted (Supplementary Figure S3). The EcRho enzyme also has no [3′→5′] unwinding activity (30) whereas DEAD-box protein Ded1 unwinds substrate C and its permuted derivative with similar high efficiencies (Supplementary Figure S3), as expected (45). These data thus support that MtbRho is a directional [5′→3′] helicase behaving more like the EcRho translocase than the local ‘disruptase’ Ded1.

**Table 1. RNA binding parameters**

<table>
<thead>
<tr>
<th>Rho factor</th>
<th>No nucleotides</th>
<th>+ AMP-PNP</th>
<th>+ ATP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( n )</td>
<td>( K_d ) (nM)</td>
<td>( n )</td>
</tr>
<tr>
<td>WT MtbRho</td>
<td>1.2 ± 0.2</td>
<td>3.5 ± 0.7</td>
<td>0.9 ± 0.2</td>
</tr>
<tr>
<td>E386A</td>
<td>1.5 ± 0.3</td>
<td>5.3 ± 0.8</td>
<td>0.9 ± 0.2</td>
</tr>
<tr>
<td>∆MtbRho</td>
<td>2.3 ± 0.6</td>
<td>20.4 ± 2.3</td>
<td>1.4 ± 0.2</td>
</tr>
</tbody>
</table>

Values were determined from the best non-linear least square fits of binding data to the Hill equation, \( F = F_{\infty} \times [\text{Rho}]^n/(K_d^n + [\text{Rho}]^n) \), where \( F \) is the fraction of labeled substrate retained on the nitrocellulose membrane, \( F_{\infty} \) is the maximal fraction of bound substrate, \( n \) is the Hill coefficient and \( K_d \) is the apparent dissociation constant. Experiments were performed with the Rut-containing RNA strand of helicase substrates (Figure 3) and the indicated MtbRho variants as described in methods.

**Figure 4. Helicase activities of the MtbRho protein variants.** Unwinding experiments were performed under standard conditions with substrate A, B or C (Figure 3, inset) and (A) the [Mtb:Ec]Rho chimera and ∆MtbRho deletion mutant or (B) single-point mutants T501A and T501K. To facilitate comparisons, reaction time-courses for WT MtbRho are depicted as dotted curves.

**ATP hydrolysis is required for duplex unwinding by MtbRho**

A hallmark of RNA translocases is their tight dependence on NTP hydrolysis. By contrast, local disruptases such as DEAD-box proteins often do not require ATP hydrolysis to unwind short RNA duplexes provided that their RNA clamp conformation is stabilized by ATP or a non-hydrolysable ATP analog (46, 47). A similar capacity has been proposed for MtbRho which is able to disrupt an artificially stalled TEC in the presence of AMP-PNP or ATPγS, albeit at rates that are only, respectively, ~7 and ~23% of the dissociation rate measured in the presence of ATP (25). We performed RNA affinity measurements to test this proposal but observed that MtbRho:RNA complexes are only marginally stabilized by nucleotides such as AMP-PNP or ATP (Table 1).

To explore the relationship between the ATPase activity and motor function of MtbRho, we also carried out unwinding experiments with the 14 bp duplex substrate C (Figure 3) in the presence of various analogs of ATP. We found that non-hydrolysable ATP analogs AMP-
PNP, ADP-BeF₃ (ground-state mimic), and ADP-AIF₄ (transition-state mimic) do not promote unwinding of substrate C by MₘₙRho (Figure 5A). Similarly, we observed that the ATPγS analog, which is hydrolyzed by MₘₙRho at a ~15-fold lower rate than ATP under our standard conditions (data not shown), does not stimulate the MₘₙRho helicase significantly (Figure 5A). UTP and CTP are also highly inefficient helicase cofactors whereas GTP is only slightly less efficient than ATP at promoting unwinding of substrate C by MₘₙRho (Figure 5A). Overall, the order of NTP efficiency is very similar for duplex unwinding (Figure 5A) and for NTP hydrolysis by MₘₙRho (Figure 2B).

The catalytic mutant E386A does not unwind substrate C significantly (Figure 5B) which confirms that ATP hydrolysis is required for the helicase function of MₘₙRho. Further support for this conclusion comes from the observation that BCM inhibits the MₘₙRho helicase in a dose-dependent fashion (Figure 5C), albeit with a half-maximal inhibitory concentration (IC₅₀ = [157 ± 20] μM) that is higher than for EcRho (IC₅₀ = [49 ± 5] μM). Altogether, these data support that MₘₙRho is a bona fide ATP-dependent molecular motor which requires ATP hydrolysis to trigger duplex unwinding.

**Transcription termination requires a catalytically-competent ATPase pocket in MₘₙRho**

To assess how the peculiar features of MₘₙRho affect its transcription termination activity, we performed in vitro transcription termination experiments with the RNAP of E. coli and a DNA template encoding the Rho-dependent terminator λr1 (Figure 6A) (27, 34). With this heterologous model system and under single-round ‘chase’ conditions (see ‘Material and Methods’), MₘₙRho triggers transcription termination with a global apparent efficiency (TE_app) that is comparable to that of EcRho (Figure 6B). However, the termination window (i.e. the region encompassing all RNA release sites) is large and starts much closer to the promoter than for EcRho (Figure 6B, compare gel lanes 2 and 3; see also Supplementary Figure S4A) which is consistent with previous observations (25).

Promoter-proximal (early) termination stop points are reminiscent of the behavior of the MinC Rho factor from M. luteus which also contains a large hydrophilic subdomain in its NTD (Supplementary Figure S1) (40). Deletion of this subdomain in MₘₙRho impairs early termination (48). We thus tested the termination activity of the ΔMₘₙRho variant, which lacks the NTD insertion subdomain of MₘₙRho, and observed that ΔMₘₙRho has a reduced capacity to trigger early termination (Figure 6B, lane 4; Supplementary Figures S4A and S5). By contrast, the [MₘₙEc]Rho chimera triggers strong early termination starting even earlier than WT MₘₙRho (Figure 6B, lane 5; Supplementary Figures S4 and S5).

Importantly, the catalytic mutant E386A is unable to trigger efficient transcript release (Figure 6B, lane 6; Supplementary Figure S4A) supporting that ATP hydrolysis is mandatory for transcription termination induced by MₘₙRho. By contrast, the T501A mutation only marginally affects transcription termination under our standard conditions by slightly favoring late over early termination (Figure 6B, lane 7; Supplementary Figures S4A and S5). Finally, the T501K mutant has an increased capacity to trigger early termination but its TE_app is not much different from that of WT MₘₙRho under our standard experimental conditions (Figure 6B, lane 8 and histogram; see also Supplementary Figures S4A and S5).

To confirm the importance of ATP hydrolysis for the termination activity of MₘₙRho, we performed transcription termination experiments in the presence of increasing concentrations of BCM. We observed that BCM inhibits transcription termination induced by MₘₙRho in a dose-dependent fashion (Figure 6C; see also Supplementary Figure S4B) but with a global IC₅₀ = [289 ± 51] μM that is one order of magnitude higher than for EcRho (IC₅₀ = [27 ± 3] μM). Quantification of termination efficiencies at individual termination positions yielded similar results with individual IC₅₀ values ranging between ~150 and ~300 μM for MₘₙRho and being lower than ~40 μM for EcRho (data not shown). Inhibition of the termination activity of the ΔMₘₙRho mutant also requires high BCM concentrations (global IC₅₀ = [281 ± 29] μM; Figure 6D), ruling out that BCM activity is perturbed significantly by the NTD insertion subdomain in MₘₙRho. By contrast, the [MₘₙEc]Rho chimera is inhibited at significantly lower BCM concentrations with a global IC₅₀ of [95 ± 13] μM (Figure 6D) and IC₅₀ values for individual termination positions as low as ~40 μM (Supplementary Figure S6). Taken together, these data indicate that MₘₙRho has a low apparent affinity for BCM that is due primarily to the composition of its CTD but confirm nonetheless that MₘₙRho requires the energy derived from ATP hydrolysis to trigger transcription termination.

Interestingly, the proportion of late (longer than ~220 nt) termination products formed with the [MₘₙEc]Rho chimera tend to increase at low BCM concentrations which contrasts with the evolution of early termination products (Figure 6D and Supplementary Figure S6). This suggests that sub-inhibitory concentrations of BCM trigger a downstream shift of the termination window, possibly because BCM slows down processive translocation of the [MₘₙEc]Rho chimera and alters its kinetic competition with RNAP (49). This phenomenon is not detected for WT MₘₙRho (Figure 6C and data not shown), which seems consistent with its low apparent processivity deduced from helicase experiments (compare Figures 3 and 4A). Hence, BCM-impaired MₘₙRho may not remain bound to the nascent transcript for sufficiently long times (and translocated distances) to catch up with RNAP at alternative pausing sites located further away from the initial Rhu loading site.

**DISCUSSION**

The ntbRho factor is a bona fide molecular motor

Transcription termination factor Rho is widespread in bacteria (13) where it supports important physiological functions linked to Rho-induced disruption of TECs or R-loops (7–9). Many Gram-negative species are sensitive to the antibiotic BCM (9), a naturally occurring, non-competitive inhibitor of Rho’s ATPase (20), whereas only one tested Gram-positive species—M. luteus—has been found to be sensitive to BCM (16). This spectrum of BCM activity suggested that Rho is dispensable in most Gram-positive bac-
Figure 5. ATP hydrolysis is required for duplex unwinding by MtbRho. Experiments were performed with substrate C under standard helicase conditions unless specified otherwise. (A) Comparison of the activation potential of various nucleotide cofactors (each present at a final concentration of 1 mM). (B) The ATPase-deficient mutant E386A does not unwind substrate C efficiently. (C) BCM-induced inhibition of the EcRho (open circles) and MtbRho (filled circles) helicases. The graph shows the normalized fractions of unwound duplex C formed after 60 min of incubation in the presence of Rho and ATP as a function of the concentration of BCM present in the reaction mixture. Note that BCM has complex effects on the kinetic regimes of the multirun helicase reactions (not shown) which prevent determination of reliable inhibitory parameters based on the evolution of unwinding reaction amplitudes or rates as a function of BCM concentration.

bacteria, a proposal that has been tested and verified for only a few specimens such as S. aureus (17). Recently, an alternative explanation has been proposed for mycobacterial resistance to BCM (25). Based on the observation that MtbRho disrupted stalled TECs in the presence of AMP-PNP or ATPγS and because the authors failed to detect a significant helicase activity for MtbRho, they proposed that the enzyme is able to trigger transcription termination by a mechanism uncoupled from molecular motor function (25). Such a mechanism would make MtbRho intrinsically resistant to BCM, which is what the authors observed for BCM concentrations up to 200 µM (25). Our data, however, do not corroborate this hypothesis and suggest alternative explanations as is discussed below.

Tight RNA binding by a protein is sometimes sufficient to destabilize proximal complexes on RNA. This principle is utilized by DEAD-box proteins which bind ATP and RNA cooperatively to disrupt short RNA duplexes (or RNA:protein complexes) in a non-directional fashion (1,6). However, MtbRho does not bind RNA much tighter in the presence of nucleotides (Table 1) and differs from DEAD-box proteins by two other important criteria. First, MtbRho is unable to unwind short duplex substrates in the presence of non-hydrolysable ATP analogs (Figure 5A). Second, MtbRho cannot unwind substrates wherein the duplex region is located upstream of the ssRNA loading (e.g. Rut site) (Supplementary Figure S3). Rather, the MtbRho enzyme behaves as a bona fide ATP-dependent [5′→3′] RNA translocase only able to unwind short RNA duplexes located downstream from a Rut site (Figure 3). This activity strictly requires ATP (or GTP) hydrolysis (Figure 5B), a condition confirmed by the inhibition of duplex unwinding induced by the non-conservative mutation of the catalytic Glu386 residue (Figure 5B, E386A mutation) or by high concentrations of BCM (Figure 5C). Importantly, in vitro transcription termination by MtbRho is similarly inhibited by the E386A mutation (Figure 6B) or by high BCM concentrations (Figure 6C), supporting that both the helicase and termination functions of MtbRho proceed by the same motor-dependent mechanism. This conclusion is in apparent contradiction with the previous observation that artificially stalled TECs can be dissociated by MtbRho in the presence of ATPγS or AMP-PNP (25). However, the reported TEC dissociation rates were low, especially in the presence of AMP-PNP (~7% of the ATP-dependent rate) (25), and may thus reflect residual motor-independent destabilization as much as experimental limitation(s) due, for instance, to slow hydrolysis of ATPγS by MtbRho (see ‘Results’ section).
transcription experiments performed under standard ‘chase’ conditions with *E. coli* RNAP and the λR1 terminator in the presence of various Rho factors (indicated above gel lanes). Intensity of the T501K lane had to be adjusted separately (as indicated by vertical dotted lines) because reactions with the T501K mutant consistently yielded lower product amounts. Apparent termination efficiencies (TEapp) are indicated below the gel (N_R = 3). (C) A higher concentration of BCM is required to inhibit MtbRho than EcRho under standard transcription termination ‘chase’ conditions (N_R = 3). (D) Comparison of the termination activities of the [Mtb:Ec]Rho and ΔMtbRho variants in the presence of BCM indicates that structural components conferring BCM resistance to MtbRho are located mostly in its CTD. In the figure, the intensity and gamma settings were adjusted to facilitate the comparison of termination signals in the various gel panels (separated by dotted lines). Gel images without gamma setting adjustments are presented in Supplementary Figures S6 and S7F.

or contamination of commercial AMP-PNP preparations by ATP (50).

Limited helicase processivity versus high termination efficiency of mtbRho

Although our data unambiguously demonstrate that MtbRho is a directional, ATP-fueled RNA helicase, they also show that the enzyme is much less efficient than its EcRho homolog at unwinding long duplexes (Figure 3). This low processivity of MtbRho derives from at least two specific structural features which are discussed below.

First, the presence of a threonine residue (instead of a basic lysine/arginine residue, as in most Rho homologs) (13) at the C-terminal position of the R-loop (Thr501) most likely weakens the SBS:RNA interaction network in MtbRho. Replacement of Thr501 by a lysine (T501K mutant) enhances the capacity of MtbRho to unwind longer duplexes whereas its replacement by alanine (T501A mutant) abrogates the unwinding burst observed with a short duplex (Figure 4B). These observations are consistent with Rho helicase processivity depending on optimal SBS:RNA interactions (11).

Second, the presence of a large, hydrophilic NTD insertion subdomain in MtbRho (Figure 1B) appears to perturb...
the coupling between RNA binding and motor functions. Deletion of this subdomain (ΔMtRR variant) weakens RNA binding (Table 1) but significantly increases the ATPase turnover and apparent duplex unwinding processivity of MtRR at saturating RNA concentrations (see ‘Results’ section and Figure 4A). Bulky NTD subdomain appendages (Figure 1C) may hamper the structural rearrangements driving enzyme activation and mecanochemoconversion (i.e. transformation of chemical energy into mechanical work) in the Rho ring (10,52). Such constraints are, however, much less detrimental in the [MtRR-EcRho] Rho chimera which displays processive unwinding (Figure 4A) and strong termination (Figure 6B) activities. The NTD subdomain thus appears to specifically exacerbate the suboptimal behavior of the MtRR motor.

We cannot totally exclude that the processivity of the MtRR helicase is also mitigated by suboptimal PBS interactions with the duplex substrates. Our synthetic Rut sequence has been optimized for EcRho (30,44) and may thus not adequately recapitulate the distinct features of M. tuberculosis transcripts (39). An optimal MtRR:Rut interaction crowning the Rho hexamer could enhance intersubunit cohesion (and ring stability) during tethered tracking, thereby favoring further RNA translocation over RNA exit from the Rho ring and dissociation (11,12). Comparison of the enzymatic activities of the MtRR and [MtRR-EcRho] factors (Figures 2C, 4A and 6) nonetheless supports that EcRho contains a stronger, more processive motor than MtRR. It thus remains to be seen whether MtRR will be able to disrupt stable R-loop structures under appropriate conditions. This capacity could have some value if MtRR has to deal with transcriptional R-loops in vivo, as is the case for EcRho (Figure 1A) (53), which is not known yet.

Although MtRR efficiently terminates transcription by E. coli’s RNAP under conditions that favor fast RNAP translocation (Figure 6A; Supplementary Figure S4), our data suggest that MtRR cannot operate far from its loading site on the nascent transcript. This proposal is notably supported by the lack of downstream shift of the MtRR termination window in the presence of sub-inhibitory concentrations of BCM (see ‘Results’ section). We thus propose that, while the processive EcRho has evolved to catch up with RNAP at sites located up to ~130 nt downstream from the Rut region (7–9), MtRR needs to promptly inactivate its RNAP target. This task could be facilitated by the significantly lower elongation rate of M. tuberculosis’ RNAP (54) or by direct interactions between Rho and RNAP (55) that could be stronger in the case of MtRR (e.g. due to the NTD insertion subdomain). Further work is necessary to rigorously test these intriguing possibilities.

Origin(s) of BCM resistance

The low apparent affinity of MtRR for BCM that was deduced from helicase (Figure 5C) and transcription termination (Figure 6B and C) experiments is intriguing given that MtRR does not contain mutations that are known to confer BCM resistance (13). These mutations usually disrupt molecular contacts between amino acids and BCM in the ATPase pocket of Rho, thereby destabilizing the Rho:BCM complex (20). We could not determine BCM affinities by microcalorimetry because of experimental constraints associated with weak ligand binding (56) and foaming of concentrated MtRR solutions in the microcalorimeter mixing chamber (our unpublished observations). Thus, weakening of BCM binding by unknown structural determinants in MtRR (e.g. ones that would alter the shape of, or access to, the ATPase pocket) cannot be formally ruled out. Other explanations based on distinctive mecanochemoconversion coupling in MtRR are also possible, although their rigorous testing is beyond the scope of the present study.

Conserved transcription termination mechanisms in M. tuberculosis

Pathogenic mycobacteria have long adapted their metabolisms and genetic programs to the colonization of harsh and changing environments such as host macrophages and granuloma (57). How this adaptation is enforced at the level of mycobacterial transcription is only fragmentarily understood. The two major pathways leading to transcription termination in bacteria—that is, intrinsic termination and Rho-dependent termination—have been proposed to be markedly distinct in M. tuberculosis (25,58). However, a recent study demonstrated that mycobacterial RNAP terminates transcription at canonical intrinsic terminators in a process which can be aided by NusG, as in other bacteria (54). Mycobacterial RNAP only displayed an increased capacity (as compared to E. coli’s RNAP) to use terminators with suboptimal U-tracts (54). In the present work, we demonstrate that MtRR operates by a canonical ‘molecular motor’ mechanism that resembles the one used by EcRho. It thus appears that both intrinsic and factor-dependent termination mechanisms are not fundamentally different in M. tuberculosis but adjusted to the characteristics of the bacterium that include a GC-rich genome and slow metabolism. Since MtRR is expressed in M. tuberculosis (23) and appears to be essential for growth (21,22), seeking MtRR inhibitors by using a screening strategy derived from ATP-dependent motor assays (such as the ones presented in this study) could thus prove useful in the fight against tuberculosis, a major and global health concern.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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