Molecular insights into the mechanism of phenotypic tolerance to rifampicin conferred on mycobacterial RNA polymerase by MsRbpA

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The protein MsRbpA from Mycobacterium smegmatis rescues RNA polymerase (RNAP) from the inhibitory effect of rifampicin (Rif). We have reported previously that MsRbpA interacts with the β-subunit of RNAP and that the effect of MsRbpA on Rif-resistant (RifR) RNAP is minimal. Here we attempted to gain molecular insights into the mechanism of action of this protein with respect to its role in rescuing RNAP from Rif-mediated transcription inhibition. Our experimental approach comprised multiple-round transcription assays, fluorescence spectroscopy, MS and surface plasmon resonance in order to meet the above objective. Based on our molecular studies we propose here that Rif is released from its binding site in the RNAP–Rif complex in the presence of MsRbpA. Biophysical studies reveal that the location of MsRbpA on RNAP is at the junction of the β- and β′-subunits, close to the Rif-binding site and the (i+1) site on RNAP.

INTRODUCTION

Rifampicin (Rif) is a non-competitive inhibitor of bacterial RNA polymerase (RNAP). Knowledge of the mechanism of action of this drug has been derived from genetic and biochemical studies carried out on Escherichia coli RNAP (Rabussay & Zillig, 1969; McClure & Cech, 1978). A substantial amount of information about the mechanism of action of Rif on RNAP has been obtained from structural studies of the Thermus aquaticus core RNAP in complex with Rif (Campbell et al., 2001). Resistance to Rif has been attributed to mutations in its binding pocket lying within the β-subunit of RNAP (Jin & Gross, 1988). The steric mechanism of transcription inhibition by Rif is simple. This mechanism, however, falls short of accounting for certain observations reported in earlier studies. For instance, there are rifampicin-resistant (RifR) mutations that are incapable of forming direct contacts with Rif, and there are RNAP variants that show differential sensitivity to Rif with distinct tail-extensions of the invariant alpha ring and σ-dependent effects on Rif action (Jin & Gross, 1988; Michelhaus et al., 2001; Williams et al., 1998; Artsimovitch et al., 2005; Wegrzyn et al., 1998). These observations do not find sufficient explanation from the steric model of Rif action.

The phenomenon of molecular tolerance to this drug came to light with the discovery of differential inhibition by Rif of transcription from σ70 and σ32-dependent promoters (Wegrzyn et al., 1998). After this discovery, similar instances with RNAP-associated proteins were also reported. In all such cases, association with RNAP reduced susceptibility to Rif. Prominent among these proteins were RbpA from Streptomyces coelicolor (Newell et al., 2006), GroEL1 and other metabolic enzymes from Mycobacterium smegmatis (Msmeg) (Mukherjee & Chatterji, 2008), and DnaA from E. coli (Flätten et al., 2009).

On deciphering the role of the mycobacterial homologue of RbpA in Msmeg, MsRbpA, we found that it rescues RNAP from the inhibitory effect of Rif (Dey et al., 2010). In the present work we have endeavoured to investigate the molecular mechanism of phenotypic tolerance to Rif conferred by MsRbpA on mycobacterial RNAP. Our results show that the interaction of MsRbpA with RNAP not only rescues the transcription activity of mycobacterial RNAP in the presence of Rif but also prevents Rif from inhibiting further cycles of transcription. At this stage we felt that it was necessary to study the nature of the interaction of MsRbpA with RNAP, which provided some mechanistic details.

In order to determine the location of MsRbpA on RNAP we made use of the fact that MsRbpA has six lysines and a single cysteine. Thus, applying a combination of chemical cross-linking and fluorescence-based distance measurement we found that MsRbpA appears to be positioned...
close to the Rif-binding site on the β'-subunit and the (i+1) site on the β'-subunit. Binding affinity measurements revealed that MsRbpA has a 10-fold lower affinity for RNAP as compared with Rif. We obtained an intriguing set of results, whereby MsRbpA had a similar order of binding affinity to rifampicin-sensitive (Rif<sup>f</sup>) and Rif<sup>R</sup> RNAPs, in spite of MsRbpA not having a similar rescuing effect on Rif<sup>R</sup> RNAPs at their respective IC<sub>50</sub> concentrations for Rif (Dey et al., 2010), as reported previously. MsRbpA retained similar binding affinities for Rif<sup>f</sup> and Rif<sup>R</sup> RNAPs, thus indicating interesting possibilities with respect to the activity of MsRbpA in the transcription process.

**METHODS**

**Reagents.** Rif was purchased from Sigma Aldrich, 5-iodoacetamide fluorescein (5-IAF) was obtained from Molecular Probes, sulfo-N-hydroxysulfosuccinimidyl-4-azidobenzoate (sulfo-HSAB) was sourced from Pierce, [3H]Rif was procured from Moravek Biochemicals and γ-aminonaphthalene sulfonate (AmNS)-UTP was a gift from AstraZeneca, R&D, Bangalore.

**Bacterial strains, plasmids and growth conditions.** Mycobacterium tuberculosis (Mtbc) β and σ<sup>+</sup> were purified from the E. coli overexpressing strain BL21(DE3) housing pAZI0612A, pAZI0611 and pARC8171, respectively, while Msmsq α and σ subunits were purified from BL21(DE3) cells transformed with pETRNAPαs and pETOsm, respectively. MsRbpA was purified from BL21(DE3) cells transformed with pETMsrbpA (Dey et al., 2010). Msmeg strain SM07 was used for the purification of holo- and core RNAP (Mukherjee & Chatterji, 2008; Dey et al., 2010).

**Reconstitution of a heterologous core RNAP from Mtbc β, β' and Msmsq α, σ subunits, followed by reconstitution with Mtbc σ<sup>+</sup> for promoter-specific in vitro transcription.** The protocol followed that described previously (Dey et al., 2010). For the in vitro transcription assay, Mtbc σ<sup>+</sup> was reconstituted with the previously assembled heterologous mycobacterial core RNAP, and the holoenzyme thus obtained was used for multiple-round transcription assays. The Mtbc rel promoter was amplified from pVP16 (Jain et al., 2005) using primers PreF and PreR (Dey et al., 2010).

**Promoter-specific multiple-round transcription assays.** Mtbc σ<sup>+</sup> (200 nmol) and reconstituted mycobacterial core RNAP (100 nmol) were incubated at 37 °C for 10 min in transcription buffer [50 mM Tris/HCl (pH 7.9), 10 mM MgCl<sub>2</sub>, 0.1 mM EDTA, 1 mM DTT, 100 μg BSA ml<sup>-1</sup>] containing 400 mM potassium glutamate (Lee & Gralla, 2004). Ten nanomoles of PCR-amplified rel promoter fragment from pVP16 was added and incubated for 15 min at 37 °C. NTP mix [200 μM; 50 μM each NTP and 200 nCi (7.4 kBq) [3<sup>2</sup>P]UTP] was added and incubated for the appropriate time at 37 °C. Urease stop dye was added and the samples were run on 10% urea polyacrylamide gels with 1× Tris-buffer EDTA at 21 W for 90 min. Radioactive bands were visualized and quantified by phosphorimager analysis. Each experiment was conducted at least three times and bands were standardized for each set of experiments to the condition that displayed the highest mean signal. The order of addition of the transcription reaction components was set at constant time intervals of 10, 20, 40, 60 and 80 min. Thus, every set consisted of five reactions starting simultaneously. Experimental set A served as a positive control in which no Rif/MsRbpA was added. Experimental set B was a negative control in which the multiple rounds of transcription were stopped by adding 500 nmol Rif at the 20 min time point. In the case of set C, Rif (500 nmol) was added at the 20 min time point and MsRbpA (400 nmol) was added at the 40 min time point. In all the sets, the five reactions were stopped at 10, 20, 40, 60 and 80 min in a consecutive manner.

**Preparation of recovery marker.** The ampiclon obtained from primers PreF and PreR, after carrying out PCR on the plasmid pVP16, was subjected to digestion by Sau3AI. The digested amplicon was gel-purified. It was then radiolabelled by incubating with [3<sup>2</sup>P]UTP (2 μCi; 74 kBq), cold dNTPs (50 μM), 1 unit Klenow fragment in labelling buffer containing 10 mM Tris/HCl (pH 7.4), 100 mM NaCl and 0.2 mM EDTA for 45 min at 37 °C. The unincorporated [3<sup>2</sup>P]UTP and dNTPs were removed by a Qiagen PCR Clean-Up kit. For every sample, 10 ng recovery marker was added after stopping the transcription reaction. The RNA transcripts and recovery marker were later ethanol-precipitated and loaded on 10% urea polyacrylamide gels.

**Fluorescence-based assay for Rif release from the RNAP–Rif complex.**

**Binding of γ-AmNS-UTP to RNAP.** It had been reported earlier that γ-AmNS-UTP acts as a substrate for E. coli RNAP, the K<sub>d</sub> for which has been reported to be 3.6 μM (Yarbrough et al., 1979; Tyagi & Wu, 1987; Kumar et al., 1992). The dissociation constant was estimated by using a low concentration of enzyme (100 nM) and varying concentrations of the probe. All steady-state fluorescence experiments were performed in a buffer containing 50 mM Tris/HCl (pH 7.9), 10 mM MgCl<sub>2</sub>, 50 mM KCl and 0.2 mM DTT. The substrate (γ-AmNS-UTP) concentration was kept at 0.4 μM and the enzyme concentration in excess at 8 μM, so that at least 90% of the labelled substrate was in the complex. Analysis of data was carried out based on a similar kinetic approach reported elsewhere (Topal & Fresco, 1980), and is described below in Results and the supplementary data.

**Binding of Rif to the γ-AmNS-UTP–RNAP complex in the presence and absence of MsRbpA.** It is known that the Rif-binding site on RNAP is about 20 Å away from the (i+1) site (Kumar et al., 1992; Campbell et al., 2001; Artisimovitch et al., 2005). Our previous studies have shown that γ-AmNS-UTP bound at the (i+1) site and Rif bound at the Rif-binding site of RNAP make a Förster's donor–acceptor pair (Kumar et al., 1992). This is because Rif has an absorption peak at a longer wavelength (473 nm) that has a substantial spectral overlap with the γ-AmNS-UTP–RNAP emission spectrum. Thus, it was previously observed that the emission of the donor molecule is progressively quenched on the addition of Rif to γ-AmNS-UTP–RNAP (Kumar et al., 1992). Steady-state excitation and emission spectra acquisition of all the labelled proteins was carried out in a Fluorolog Tau-3 (Jobin Yvon) spectrophuorimeter, with a Xe lamp as an excitation source and a spectral correction facility. The titration curve approached saturation when the RNAP to Rif ratio approached unity. Thus, under these conditions, the quenching of emission of the donor molecule, γ-AmNS-UTP–RNAP, by Rif was followed by increasing the molar ratio of RNAP : MsRbpA in the assay mixture. Due to the strong absorption of Rif at about 330 nm, γ-AmNS-UTP–RNAP was routinely excited at 360 nm in all subsequent steady-state experiments, in both the presence and the absence of Rif. In this way, the inner filter effect due to the presence of Rif could be avoided, as Rif shows minimal absorption at 360 nm (Kumar et al., 1992). The absorption of the samples at the excitation wavelength was kept below 0.05 so as to avoid an inner filter effect. The assay mixtures were incubated at 37 °C and the measurements were then carried out at 24 °C with a 5 nm bandpass.

**Estimation of Förster's distance between single cytoxines on MsRbpA and the (i+1) site in Msmsq RNAP**

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Modification of the single cysteine on MsRbpA with 5-IAF. Purified MsRbpA was dialysed against labelling buffer containing 40 mM HEPES (pH 8.0), 500 mM NaCl and 5% (v/v) glycerol. After dialysis, the \( A_{280} \) of the protein sample was recorded and the concentration determined. Tris (2-carboxyethyl)phosphine (TCEP) was added in 10-fold molar excess to the protein to reduce all disulfides. Later, 5-IAF was added to the protein solution to a molar concentration ratio of 1:1. The samples were then incubated at 4 °C for 12 h in the dark. Excess DTT was added to stop the reaction and the samples were then subjected to gel filtration chromatography on Sephadex G75 to remove the unbound dye. The dye:protein ratio was calculated by measuring the absorbances at 280 and 492 nm and by taking the IAF molar absorption coefficient at 492 nm to be \( 80000 \) M\(^{-1}\) cm\(^{-1}\). The labelling was also confirmed by MS.

Measurement of Förster's distance. Förster's distance, \( R_0 \), at which the transfer efficiency is 50%, was calculated using the formula:

\[
R_0 = 9.79 	imes 10^{-10} \left( \frac{\langle |\langle J \rangle Q(n^{-4})(\kappa^2) \rangle \rangle^6}{4} \right)^{\frac{1}{6}}
\]

where \( n \) is the refractive index of the medium and was taken as 1.4 (Kumar & Chatterji, 1990), and \( Q \) is the quantum yield of the sample, which was calculated as described previously (Kumar & Chatterji, 1990). Quinine sulfate in 0.05 M H\(_2\)SO\(_4\) was employed as described earlier (Cantor & Schimmel, 1980). The spectral overlap integral, \( I \), was calculated as described previously (Kumar & Chatterji, 1990) using the fOrTRAN program described therein. The orientation factor \( \kappa^2 \) was taken to be \( \frac{3}{5} \), taking into account that there is free rotational diffusion of the donor as well as the acceptor prior to energy transfer (Lakowicz, 1983). In addition, a diffusion-controlled collision between the donor–acceptor pair is likely as the fluorophores are bound on two different molecules; hence, the possibility of having freely diffusing fluorophores is high. It should be noted here that \( R_0 \) shows a sixth-root dependence on \( \kappa^2 \) and the refractive index, \( n \). In our system, the donor was \( \gamma\)-AmNS-UTP bound to the (i + 1) site of RNAP and the acceptor was fluorescein attached to Cys-56 of MsRbpA.

Measurement of the distance between fluorophores. For the measurement of the distance in the presence of fluorescein–MsRbpA, \( \gamma\)-AmNS-UTP–RNAP was incubated with and without fluorescein–MsRbpA. The absorbance of the fluorophore \( \gamma\)-AmNS-UTP–RNAP was kept below 0.05 at its excitation wavelength (360 nm) to avoid any inner filter effect. The emission was monitored at 460 nm in each case. The distance between the two fluorophores was calculated using the formula (Förster, 1948; Stryer, 1978):

\[
r = \frac{R_0}{(1/T)^{\frac{1}{6}}}
\]

where \( T \) is the transfer efficiency, calculated by measuring the quantum yields of the fluorescence of \( \gamma\)-AmNS-UTP–RNAP in the presence \( (Q_{n,0}) \) and absence \( (Q_0) \) of fluorescein–MsRbpA at 460 nm using the expression \( T = 1 - (Q_{n,0}/Q_0) \). Due to the sixth-root dependence, the error due to inhomogeneity of \( \kappa^2 \) is not more than 10%.

Chemical cross-linking and tryptic digestion. Purified MsRbpA was derivatized by sulfo-HSAB (Dey et al., 2010) and the derivatization was verified by MS. The derivatized MsRbpA was then cross-linked with the Msmeg core RNAP. The labelled MsRbpA was incubated with a fivefold molar excess of RNAP at 37 °C for 30 min, followed by exposure to 254 nm UV light for 8 min. The reaction mix was immediately loaded on a 6% SDS polyacrylamide gel and the complex formed as a result of cross-linking was probed with antibody against the Mtb β-subunit. The bands corresponding to the MsRbpA, β and β-MsRbpA complex were excised and subjected to in-gel trypsin digestion for the identification of the cross-linked peptide.

MS analysis (identification of the cross-linking site on the β-subunit). Peptides were mixed with an equal volume of matrix solution (α-cyano-4-hydroxycinnamic acid), spotted for analysis by a MALDI-TOF-TOF (Ultraflex TOF-TOF, Bruker Daltonics) instrument equipped with a pulsed N\(_2\) laser, and analysed in the reflectron mode using a time delay of 90 ns and an accelerating voltage of 25 kV in the positive ion mode. Initially, spectra of 200 laser shots were acquired and the spectra were calibrated externally to a spectrum of peptides of known masses ranging from 1000 to 2500 Da. The most intense peaks in the spectrum were selected for fragmentation by laser-induced dissociation (LID) using the LIFT program of the Ultraflex TOF-TOF instrument. For tandem MS, 500 laser shots were accumulated and the spectrum was calibrated internally to the precursor ion mass. These MS–MS spectra were used for a sequence-specific search in the Mascot database (Matrix Science). In addition, peptide mass fingerprint (PMF)-based searches were done using only the set of peptide masses, in the same database without any constraints for pi and molecular mass, and with a cut-off of 30% for sequence coverage. The whole procedure, from the step of gel excision, was repeated several times to ensure correct protein identification.

Identification of cross-linked peptides. The peptides were also subjected to liquid chromatography (LC), wherein the sample was passed through a reverse-phase column (Zorbax 300 SB-C8, 2.1 mm × 100 mm, 3.5 μm) attached to an ESI mass spectrometer. Separation of peptides was effected in this column via a gradient elution using water and acetonitrile (ACN), each containing 0.1% formic acid, at a flow rate of 0.3 ml min\(^{-1}\), and the eluting peptides were characterized by conventional MS (LC-electrospray ionization MS; LC-ESI-MS) and MS/MS (LC-ESI-MS/MS). The mass spectrometer, an HCT Ultra PTM Discovery (Bruker Daltonics), had an ESI source and housed a classic ion trap (Paul type), using which, both MS (LC-ESI-MS) and tandem MS (LC-ESI-MS/MS) data were acquired. Doubly and triply charged ions were prefered over singly charged species for isolation and fragmentation. The cross-linked peptides after MS/MS fragmentation were identified by de novo sequencing.

Measurement of binding kinetics of Rif to RNAP by surface plasmon resonance (SPR). Preparation of 3-formyl rifampicin. Rif (100 mg) in 10 ml 1% HCl in a mixture of tetrahydrofuran (THF) and water (10:1) was kept stirring overnight in the dark at room temperature. The conversion of Rif to 3-formyl rifampicin was monitored by silica TLC (in 10% methanol in THF as a solvent) and by ESI-MS. The \( m/z \) of Rif is 821.2 Da, whereas the \( m/z \) of 3-formyl rifampicin is 724.1 Da in the negative ion mode [the molecular mass of 3-formyl rifampicin = Rif–(5Cl + 3N + 11H) + O]=[821.2–(5×12)+(3×14)+(11+1)+(16+724.2) Da]. The overnight reaction mixture was passed over a packed silica column and eluted with a gradient of 5–20% methanol in THF. The eluent fractions were collected and subjected to ESI-MS to ascertain the purity. The fractions corresponding to an \( m/z \) of 724.2 Da (which is the \( m/z \) of 3-formyl rifampicin) were pooled and dried in a Rotavac (Heidelberg). After the preparation of 3-formyl rifampicin, it was assayed for its transcription inhibitory activity.

SPR measurements. All SPR experiments were carried out in Biacore 3000 instruments at 35 °C using a research grade CM5 sensor chip.
(GE Healthcare Bio-Sciences). All buffer solutions were freshly prepared, passed through a 0.22 μm pore-size filter and degassed. 3-Formyl rifampicin was immobilized on a CM5 sensor chip following an aldehyde coupling procedure according to the manufacturer’s protocol. Briefly, EDC/NHS [EDC: 0.4 M 1-ethyl-3-(3-methylaminopropyl)carbodiimide in water; NHS: 0.1 M N-hydroxysuccinimide in water] at a 1:1 ratio was injected at a flow rate of 5 μl min⁻¹ (for 5 min) to yield reactive succinimide ester on the sensor surface; then 5 mM hydrazine in water was injected at a flow rate of 5 μl min⁻¹ (for 7 min) to introduce the hydrazide group, followed by 1 M ethanolamine-HCl (pH 8.5) injection at a flow rate of 5 μl min⁻¹ (for 5 min) to deactivate the excess reactive groups. Then, 3-formyl rifampicin in 10 mM sodium acetate buffer (pH 4.0) was injected at a flow rate of 5 μl min⁻¹ (for 5 min) for its immobilization. Finally, 0.1 M sodium cyanoborohydride in 0.1 M sodium acetate (pH 4.0) was injected at a flow rate of 2 μl min⁻¹ (for 20 min) to stabilize the complex. A control (blank) channel was prepared by activation with EDC/NHS and then treated with hydrazine and ethanolamine.

MsRbpA was immobilized on the CM5 sensor chip through amine coupling chemistry according to the manufacturer’s protocol. Briefly, the chip surface was activated by injecting EDC/NHS at a flow rate of 5 μl min⁻¹ for 10 min. MsRbpA in sodium acetate buffer (pH 4.0) was injected at a flow rate of 5 μl min⁻¹ (for 5 min), and finally 1 M ethanolamine-HCl (pH 8.5) was injected at a flow rate of 5 μl min⁻¹ (for 5 min) to deactivate excess reactive groups. A control (blank) channel was prepared by activation with EDC/NHS and then treated with ethanolamine.

All experiments with Rif6 and Rif8 RNAP with 3-formyl rifampicin were performed in running buffer A [10 mM HEPES (pH 7.5), 75 mM KCl, 10 mM MgCl₂, 0.05 mM EDTA], and those with MsRbpA were performed in running buffer B [10 mM HEPES (pH 7.5), 150 mM NaCl, 1 mM EDTA]. The flow rate for the analysis was kept at 10 μl min⁻¹ for 3-formyl rifampicin and 20 μl min⁻¹ for MsRbpA. The association and dissociation times were kept at 300 s for 3-formyl rifampicin and 120 s for MsRbpA.

After every injection of RNAP, the sensor chip surface was regenerated by rapid injection of 2–10 μl 0.01–0.04 % SDS at a flow rate of 100 μl min⁻¹, which was followed by thorough washing with the appropriate running buffer until the baseline returned to zero.

RNAP was passed simultaneously through the control channel and the test channel. All the response units (RU) thus obtained during analysis are RU corrected for the RU in the blank channel. All data were analysed by globally fitting both the association and the dissociation phases for various concentrations through BIAevaluation software version 4.1.

**RESULTS**

MsRbpA rescues RNAP for transcription reinitiation after transcription arrest by Rif

Our previous work (Dey et al., 2010) on MsRbpA showed the outcome of the interaction between MsRbpA and RNAP. In the context of Rif-mediated inhibition of transcription, it was found that in the presence of MsRbpA, Rif is not able to inhibit transcription. This effect was seen both in vitro and in vivo. In the multiple-round promoter-specific transcription assays, we attempted to mimic the in vivo conditions when an actively transcribing RNAP is exposed to Rif. Therefore, when we performed multiple-round transcription assays, our objective was to follow the transcription process in three aspects, consecutively, i.e.:

1. run-off transcription in the absence of Rif and MsRbpA;
2. transcription arrest caused by addition of Rif;
3. transcription rescue caused by subsequent addition of MsRbpA.

In our multiple-round transcription assays we carried out uninhibited initial rounds of run-off transcription, followed by addition of Rif, which arrests all further reinitiation, and finally addition of MsRbpA. The transcript levels were tracked throughout the process by stopping the reactions at various time points. In the multiple-round transcription assay, the main concern was to have a poised RNAP competent for reinitiation. For this purpose we made use of the fact that potassium glutamate frees RNAP for rapid reinitiation (Lee & Gralla, 2004). Thus, we carried out three sets of transcription reactions.

In set A, we did not add any Rif or MsRbpA, and the mRNA levels were followed at the time points 10, 20, 40, 60 and 80 min (Fig. 1a, set A). The level of transcripts showed an increase with time, and the results of densitometric analysis for a triplicate set of experiments were plotted with respect to the time points (shown in Fig. 1b, set A). The multiple-round transcription assays in set A served as a negative control for the role of Rif. This was because in set A, no Rif was added to cause the arrest of transcription reinitiation. Similarly, no MsRbpA was added later to observe its role following the Rif-mediated arrest of transcription reinitiation.

In set B, after the initiation of the transcription reaction, Rif was added in a fourfold molar excess with respect to RNAP at the 20 min time point. It can be seen in Fig. 1(a, b), set B, that the transcript levels stagnated after the addition of Rif. The multiple-round transcription assays in set B served as a positive control for the role of Rif. This was because they showed that after the addition of Rif at the 20 min time point, no further increase in transcript levels took place. This implies that throughout the remaining duration of the assay, Rif did not allow the reinitiation of transcription to take place. At this point we should mention that the rate of transcription elongation is known to be faster than that of initiation (Bandwar et al., 2006), such that the formation of the open complex has finished at the time of Rif addition, so that there would be no observable increase in the 130 nt transcript from pre-formed complexes at the time of Rif addition.

The transcription assays in set C served as the test for the role of MsRbpA in relieving RNAP from the transcription arrest caused by Rif. In Fig. 1(a, b), set C, the addition of Rif stopped further rounds of transcription, which is evident from the stagnant population of transcripts in lanes 12 and 13 (for the transcription reactions stopped at the 20 and 40 min time points, respectively). After the addition of...
Fig. 1. (a) Promoter-specific multiple-round gel-based transcription assay. The different sets are defined as follows. Set A: multiple-round transcription assay at the relA promoter with the transcription reaction stopped at the time points 10 min (lane 1), 20 min (lane 2), 40 min (lane 3), 60 min (lane 4) and 80 min (lane 5). The transcription reaction contained 200 nmol Mtb σ^A and 100 nmol reconstituted mycobacterial core RNAP and was incubated at 37 °C for 10 min in 1× transcription buffer (50 mM Tris-HCl, pH 7.9, 3 mM MgCl_2, 0.1 mM EDTA, 1 mM DTT, 100 μg BSA ml⁻¹) containing 400 mM potassium glutamate. Ten nanomoles of PCR-amplified relA promoter fragment from pVJP16 was added and incubated for 15 min at 37 °C. NTP mix (200 μM: 50 μM each NTP and 200 nCi (7.4 kBq) [γ^32P]dUTP) was added and incubated for the appropriate time at 37 °C. In this series of reactions, neither Rif nor MsRbpA was added at any point during the transcription reaction. Set B: multiple-round transcription assay (as in set A) with the transcription reaction stopped at the time points 10 min (lane 6), 20 min (lane 7), 40 min (lane 8), 60 min (lane 9) and 80 min (lane 10). In this series of reactions a fourfold molar excess of Rif was added after the 20 min time point during the transcription reaction. Set C: multiple-round transcription assay with the transcription reaction stopped at the
time points 10 min (lane 11), 20 min (lane 12), 40 min (lane 13), 60 min (lane 14) and 80 min (lane 15). In this series of reactions, a fourfold molar excess of Rif was added after the 20 min time point during the transcription reaction followed by the addition of a fourfold molar excess of MsRbpA after the 40 min time point. In all the lanes, a 310 nt recovery marker was added as a loading control. The lane on the extreme left shows an ethidium bromide-stained RNA ladder run in parallel. (b) Densitometric analysis of the multiple-round transcription assays plotted with respect to the various time points [shown in (a)]. Sets A, B, and C were analysed in three separate repeat assays.

MsRbpA at the 40 min time point, there was a concomitant increase in the transcript levels in lanes 14 and 15 (representing the 60 and 80 min time points, respectively (Fig. 1a, b, set C). A recovery marker of 310 nt was used as a loading control to judge the changes in the levels of transcripts. Thus, it appears that after the arrest of transcription reinitiation by Rif, addition of MsRbpA not only is able to rescue the transcription activity of RNAP from the inhibitory effect of Rif but also ensures further rounds of transcription even in the presence of Rif. The mode of action of MsRbpA in relieving RNAP from Rif-mediated inhibition is perhaps due to a conformational change in the binding site for Rif and/or shielding of the polymerase against Rif so that the interaction between RNAP and Rif cannot take place. We were, however, unable to detect any interaction between MsRbpA and Rif in vitro, thus ruling out any activity of MsRbpA towards Rif to render it ineffective (Supplementary Fig. S1).

MsRbpA displaces Rif from its RNA synthesis inhibition site

RNAP performs the fundamental enzymic activity of the transfer of the nucleotidyl moiety from the NTP substrate to the hydroxyl at the 3¢ terminus of the nascent transcript (Nudler, 2009). The RNAP catalytic centre therefore comprises the binding sites for the RNA 3¢ terminus (i-site) and the insertion site for the incoming nucleotide (i+1) site (also known as A-site). The phosphodiester bond formation is preceded by translocation of the newly formed 3¢ terminus from the (i+1) site to the i-site and the subsequent release of the pyrophosphate (PPi) product. Previous biophysical and structural studies have revealed that Rif is about 20 Å away from the (i+1) site of RNAP (Kumar et al., 1992; Campbell et al., 2001; Artsimovitch et al., 2005).

Some years ago we applied the technique of fluorescence resonance energy transfer to understand the spatial location of the (i+1) site with respect to the Rif-binding site (Kumar et al., 1992). This technique has been used as a spectroscopic ruler (Stryer, 1978) to reveal proximity relationships by employing two probes at two specific sites; the nature of these probes is such that the fluorescence emission spectrum of one (donor: γ-AmNS-UTP in this case) overlaps with the absorption spectrum of the other (acceptor: Rif in this case). Excitation of γ-AmNS-UTP then results in a dipolar energy transfer to the Rif molecule, provided that the two probes are situated about 10–60 Å from each other (Förster, 1948; Stryer, 1978). Thus, the presence of Rif in the binding pocket will quench the fluorescence of γ-AmNS-UTP, which is located at the (i+1) site of RNAP (Kumar et al., 1992; Chatterji & Gopal, 1996).

In the multiple-round transcription assays, we observed that the interaction of MsRbpA led to the reinitiation of transcription from Rif-arrested RNAP molecules. Based on the steric mechanism of Rif-mediated inhibition of transcription, the simplest explanation of the above observation is that Rif is occluded from its binding site on the RNA exit channel. The ability of Rif (bound to RNAP) to quench the fluorescence of γ-AmNS-UTP at the (i+1) site will be absent in the presence of MsRbpA.

The first objective was the confirmation of binding and the determination of the dissociation constant (KD) of γ-AmNS-UTP with RNAP. Msmeg RNAP from strain SM07 and MsRbpA were purified using the protocol described previously (Mukherjee & Chatterji, 2008; Dey et al., 2010). KD was determined using a low concentration of the RNAP (100 nM) and varying concentrations of γ-AmNS-UTP. It is worth mentioning that in the known crystal structures of RNAP from T. aquaticus, Thermus thermophilus and E. coli (Campbell et al., 2001; Artsimovitch et al., 2005; Opalka et al., 2010), the Trp residues lie within 10–60 Å of the active site (Supplementary Fig. S2a, Supplementary Table S1). RNAP was excited at 295 nm, at which the probe shows minimal absorption, and tryptophan emission at 340 nm was monitored as shown in Supplementary Fig. S2b, c. Upon incremental addition of γ-AmNS-UTP to the enzyme, a single isoemissive point was observed at 380 nm (Supplementary Fig. S2b), indicating the presence of only a single type of complex between γ-AmNS-UTP and RNAP. The stoichiometry (n) and KD of RNAP and substrate (γ-AmNS-UTP) were determined using the equation described previously (Topal & Fresco, 1980; Kumar & Chatterji, 1990) and were calculated as n=1 and KD=3 μM (Supplementary Fig. S3a, b). Subsequently, all the fluorescence experiments were performed in a concentration range in which more than 90% of the substrate was bound to the enzyme at equilibrium. The suitability of γ-AmNS-UTP as a substrate in carrying out transcription was checked in a multiple-round fluorescence-based transcription assay on the Mtbrn promoter, adapted from Bhat et al. (2006). The transcription activity of RNAP was detected as the release of 1-naphthylamine-5-sulfonic acid (accompanied by an increase in fluorescence intensity at 460 nm with time; Supplementary Fig. S3c). The inhibition of transcription activity by Rif was also checked in the same assay when an increase in the time-based acquisition of
fluorescence intensity attained a plateau after the addition of Rif (Supplementary Fig. S3c). Thus, after reassuring ourselves that the components used in the assay, i.e. RNAP, \(\gamma\)-AmNS-UTP and Rif, retained their roles in the transcription process, we probed the role of MsRbpA in influencing the complex between Rif and RNAP. In this set of assays, the Rif concentration was varied from 0 to 16 \(\mu\)M. The quenching of \(\gamma\)-AmNS-UTP fluorescence reached saturation at 8 \(\mu\)M, which was expected as the ratio of RNAP to Rif reached unity. Interestingly, we have noticed previously (Dey et al., 2010) that more MsRbpA is needed (MsRbpA : RNAP \(\geq 2\)) to resume the transcription by RNAP in the presence of Rif. As expected (Fig. 2), the quenching of \(\gamma\)-AmNS-UTP fluorescence recovered upon addition of excess MsRbpA, even in the presence of Rif. It is essential to mention here that the standard method to differentiate Rif binding amongst RifS and RifR RNAPs is the determination of \(^{3}\)H Rif binding to RNAP using the dextran-coated charcoal method (Wyss & Wehrli, 1976). We applied this method to differentiate the effect of MsRbpA on RNAP binding to \(^{3}\)H Rif. The results obtained are shown in Supplementary Fig. S3(d). We were able to observe that there was a marginal decrease in the binding of \(^{3}\)H Rif as a result of the Rif\(^{\beta}\) RNAP interaction with MsRbpA. This change was not as marked as that seen in the case of Rif\(^{\beta}\) RNAP. Therefore, to probe the effect of MsRbpA on the binding of Rif to its primary binding site on RNAP, we adopted a fluorimetric approach.

**Location of MsRbpA on Msmeq RNAP**

(i) Estimation of Förster’s distance between the single cysteine on MsRbpA and the (i+1) site on Msmeq RNAP. The knowledge of the amino acid sequence of MsRbpA has revealed the existence of a single cysteine residue (Dey *et al.*, 2010) in the protein at the 56th position from the N terminus. The (i+1) site (or A-site) of the active centre of RNAP is composed mostly of residues from the \(\beta’\)-subunit (Nudler, 2009).

It was shown some time ago in our laboratory that \(\gamma\)-AmNS-UTP bound to the (i+1) site lies 20 Å away from Rif, where AmNS and Rif act as a Förster’s donor–acceptor pair. We intended to carry out a similar measurement by labelling the single cysteine residue of MsRbpA with fluorescein (Supplementary Fig. S4a), and measured its distance from \(\gamma\)-AmNS-UTP as the two form another Förster’s donor–acceptor pair (\(\gamma\)-AmNS-UTP \(\lambda_{\text{excitation}}=360\) nm, \(\gamma\)-AmNS-UTP \(\lambda_{\text{emission}}=460\) nm; and 5-IAF \(\lambda_{\text{excitation}}=492\) nm, 5-IAF \(\lambda_{\text{emission}}=515\) nm), shown in Fig. 3(a). After labelling of the sole cysteine in MsRbpA with fluorescein, we carried out several experiments as controls which showed that Cys modification did not influence MsRbpA binding to RNAP (Supplementary Fig. S4b), the oligomeric status of MsRbpA (Supplementary Fig. S4c), the effects (positive or negative) of MsRbpA on Rif transcription (Supplementary Fig. S4d), or MsRbpA structure (Supplementary Fig. S4e).

Ideally one should measure the distance between fluorescein-labelled MsRbpA and Rif, but this was not possible, as Rif is ejected from its binding site on RNAP upon addition of MsRbpA. The stoichiometry of MsRbpA and RNAP is greater than unity, and it can be seen from Fig. 3(b) that the quenching of \(\gamma\)-AmNS-UTP fluorescence by fluorescein-labelled MsRbpA saturates in the molar ratio 4:1 (MsRbpA : RNAP). However, analysis of the binding curve using the equations described previously (Topal & Fresco, 1980; Kumar & Chatterji, 1990) revealed that the number of binding sites for MsRbpA on RNAP was one (Supplementary Fig. S5). The mean distance calculated

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**Fig. 2.** MsRbpA relieves the Rif-induced quenching of the fluorescence intensity of \(\gamma\)-AmNS-UTP bound to RNAP. The plots show the fluorescence titration of \(\gamma\)-AmNS-UTP bound to RNAP (8 \(\mu\)M) by varying concentrations of Rif: (●) in the absence of MsRbpA, (■) in the presence of a twofold molar excess of MsRbpA, (▲) in the presence of a fourfold molar excess of MsRbpA, and (◆) in the presence of an eightfold molar excess of MsRbpA.
(Table 1) was 18 Å, and this value made sense when we identified the cross-linking site of MsRbpA on RNAP described in the next section.

(ii) Identification of a cross-linking site for MsRbpA on the β-subunit of RNAP. The chemical cross-linker HSAB has been used to locate the binding site of the ω subunit to the β’-subunit on RNAP (Gentry & Burgess, 1993). We used the same strategy to locate the binding site of MsRbpA on Msmeg core RNAP purified from 30 h-old cultures of Msmeg strain SM07, and found that the binding partner of MsRbpA in RNAP was the β-subunit (Dey et al., 2010).

Table 1. Characteristics of donors and acceptors described in this study

<table>
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<tr>
<th>Sample</th>
<th>Excitation wavelength (nm)</th>
<th>Emission wavelength (nm)</th>
<th>Spectral overlap integral, J (M⁻¹ cm³)</th>
<th>Quantum yield, Q</th>
<th>Distance at 50 % energy transfer efficiency, R₀ (Å)</th>
<th>Energy transfer efficiency, T</th>
<th>Distance between donor and acceptor, R (Å)</th>
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<td>γ-AmNS-UTP</td>
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<td></td>
<td>0.002</td>
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<td>460</td>
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<td>0.001</td>
<td>18.2</td>
<td>0.55</td>
<td>18 ± 2</td>
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</table>

Fig. 3. (a) Spectral overlap between the fluorescence emission spectra of γ-AmNS-UTP (RNAP) and fluorescein–MsRbpA. (b) Quenching of RNAP-complexed γ-AmNS-UTP fluorescence by fluorescein-tagged MsRbpA.
With the objective of determining the cross-linking site of MsRbpA on the β-subunit we carried out cross-linking of MsRbpA to RNAP. This involved two steps: derivatizing MsRbpA with the hetero-bifunctional cross-linker sulfo-HSAB, followed by incubating the derivatized MsRbpA with core RNAP and exposing the mixture to UV light. The first step, derivatization of MsRbpA, added an azidobenzoate (AB) group to free amino groups on MsRbpA to make MsRbpA-AB. Six lysine residues in MsRbpA can be modified in this way. The product of derivatization was verified for change of mass by ESI-MS (Supplementary Fig. S6, Supplementary Table S2). The last step involved the actual cross-linking of MsRbpA to RNAP. When the phenyl azide groups (attached to MsRbpA-AB) were exposed to UV light (254 nm), there was formation of highly reactive phenyl-nitrene, which creates covalent linkages with a variety of molecules. The cross-linked adduct of β-MsRbpA was detected with anti-Mtb β antibody (Fig. 4). The bands on the gel corresponding to the MsRbpA–β adduct (B*), β-subunit (B) and MsRbpA were excised. The bands were subjected to in-gel trypsin digestion and the resulting peptides were spotted onto a MALDI-TOF plate. The resulting MsRbpA–β adduct spectrum was analysed for the absence of the peaks which are present in the MALDI-TOF spectra of the pure β-subunit and MsRbpA-AB (Fig. 5). All the peaks that were detected in the MALDI-TOF spectra of the β-subunit were compared with the peaks of MsRbpA–β. We observed a conspicuous absence of the peaks of m/z 1271 (corresponding to the sequence TVGELIQNQIR) and m/z 1540 (corresponding to the sequence LRTVGELIQNQIR) in the MsRbpA–β adduct spectra. Thus, it appears that TVGELIQNQIR is a cross-linking site for MsRbpA on the β-subunit. The peaks which we detected containing lysine on MsRbpA corresponded to peptides SVEELELLKER (m/z=1474) and NLEGTLIE- GDVPEPK (m/z=1669). On studying the spectrum of derivatized MsRbpA, we detected the peak of m/z 1669 decreasing in intensity with the concomitant appearance of a peak at m/z 1802, which pointed towards the derivatization of the lysine residue of NLEGTLIEGDVPEPK with an AB group (Supplementary Fig. S7). The laser shot appears to induce the release of N₂, leaving the peptide derivatized with the nitrene ion (m/z 1802).

In an effort to search for the cross-linking of the β-subunit peptide TVGELIQNQIR (m/z 1271) with the AB-derivatized lysine-containing peptide NLEGTLIEGDVPEPK, we made use of ESI-MS and ESI-MS/MS. The formation of an adduct between these peptides will lead to a larger peptide of mass 3075 Da (1829 + 1271–1728) caused by the addition reaction between the two peptides, leading to the formation of N₂ and the cross-linked peptide. The reaction of a nitrene (N₂) with another group is a non-specific reaction, and therefore we carried out de novo sequencing of MS/MS spectra of a peak corresponding to m/z 3075 to ascertain which amino acid in the β-subunit peptide TVGELIQNQIR was involved in the cross-link. The possible inter-peptide cross-links are shown in Supplementary Fig. S8. It being a large peptide, we made use of the capability of the ESI technique to detect the higher-charged state of 3075 Da mass (+3 charged state in this case), which makes the detection and MS/MS analysis of this large peptide feasible. The +3 state of 3075 showed a m/z of 1025 (Supplementary Fig. S9). The triply charged peak of m/z 1025 was later subjected to MS/MS analysis and the de novo sequencing of the cross-linked peptide was carried out, for both the +2 and +3 states. The sequencing was done from both ends for the b-ions and b'-ions [b represents ions counted from threonine (T) at the N terminal of the cross-linked peptide TVGELIQNQIR—KPEPVDGEILTGLEG to the asparagine (N) at the next N terminus of the same peptide; b'-ions are counted in the reverse direction (Supplementary Fig. S10)]. The de novo sequencing also showed that the arginine (R) of the β-subunit peptide TVGELIQNQIR formed the chemical cross-link with the lysine (K) of the MsRbpA peptide NLEGTLIEGDVPEPK. The results of sequencing of +3 state b- and b'-ions are shown in Fig. 6(a, b). On comparing the sequence of the regions around the β-subunit peptide TVGELIQNQIR with those of T. aquaticus, T. thermophilus, E. coli, Mtb and Msmeg, we observed that this sequence existed upstream of the cluster 1 amino acid sequence that has been implicated in the Rif-binding pocket (Jin & Gross, 1988; Campbell et al., 2001; Artsimovitch et al., 2005) (Fig. 7). The detailed MS analysis reported here showed that Arg381 of the Msmeg RNAP β-subunit is cross-linked to Lys73 of MsRbpA. Thus, the interaction between MsRbpA and the RNAP at this site probably triggers the release of Rif from the binding pocket.

**Comparison of the relative binding affinities of Rif with Rif<sup>S</sup> and Rif<sup>R</sup> Msmeg RNAP**

We measured the kinetics of binding of Rif to Rif<sup>S</sup> and Rif<sup>R</sup> RNAP using SPR. The 3-(4-methylpiperazinyl-iminomethyl) group of Rif was hydrolysed to the corresponding...
3-aldehyde, and this aldehyde moiety was used to immobilize Rif to the CM5 sensor chip through a Schiff’s base bond, as described in Methods (Calleja et al., 1998) (Supplementary Figs S11–S14). We found that Msmeg RifS RNAP had an equilibrium binding constant ($K_D$) of $3.37 \times 10^{-9}$ M for Rif (Fig. 8a, Table 2), which is similar to that of the RNAP from E. coli (Wehrli et al., 1976; Yarbrough et al., 1976), whereas the RifR RNAPs SM0747 and SM0734 had $K_D$ values of $1.19 \times 10^{-8}$ and $1.51 \times 10^{-8}$ M (Fig. 8b, c, Table 2), respectively, resulting in a 10-fold weaker binding to Rif. Interestingly, SM0748 RNAP had a higher $K_D$ (~$8.92 \times 10^{-6}$ M), which resulted in 1000-fold weaker binding as compared with Rif$^R$ RNAP (Fig. 8d, Table 2). We also observed that the difference in $K_D$ arose from the difference in association rate constant ($k_a$) for the complex formation, whereas the dissociation rate constant ($k_d$) remained largely unaltered (Table 2). Additionally, we assayed the modified Rif and showed that it inhibits transcription just like Rif. This reassures us that the modified Rif binds to the same site as normal Rif (Supplementary Fig. S14).

Comparison of the relative binding affinities of MsRbpA with Msmeg Rif$^S$ and Rif$^R$ RNAP

We reported previously (Dey et al., 2010) that the RNAPs isolated from the Msmeg Rif$^R$ strains do not show the expected interactions with MsRbpA. Thus we carried out SPR experiments by immobilizing MsRbpA on the CM5 sensor chip as described in Methods. Beforehand, we confirmed by Western blotting the absence of MsRbpA in the RNAP samples used for the binding assays (Supplementary Fig. S15). We found that the equilibrium binding constant ($K_D$) of the interaction of MsRbpA with Rif$^R$ RNAP was $5.06 \times 10^{-8}$ M, with an association rate constant ($k_a$) of $1.05 \times 10^5$ M$^{-1}$ s$^{-1}$ and a dissociation rate constant ($k_d$) of $5.34 \times 10^{-3}$ s$^{-1}$ (Fig. 9a, Table 3). In our experiments with Rif$^R$ RNAP we found that the RNAPs did not differ in the order of magnitude of their kinetic parameters while associating with MsRbpA (Fig. 9b–d, Table 3).

Comparison of the relative binding affinities of MsRbpA with wild-type RNAP in the presence and absence of bound Rif

We also tested via SPR whether Rif affects the binding parameters between MsRbpA and wild-type RNAP. As can be seen in Supplementary Fig. S16 and Table 3, we carried out the SPR experiments with MsRbpA bound to the CM5 chip and the RNAP–Rif complex flowing over it in order to determine the difference between the association and dissociation rates. The RNAP–Rif complex was prepared by incubating a fivefold molar excess of Rif with SMO7...
RNAP at 35 °C for 5 min, after which the complex was injected. When RNAP–Rif was passed over the immobilized MsRbpA (Supplementary Fig. S16), both the association rate constant (k_a) and the first-order dissociation rate constant (k_d) were found to be lower than those of the MsRbpA–RNAP complex (Table 3), although the apparent K_D was found to be comparable. We would argue that the difference in the rate is due to the steric effect of the Rif present in the RNAP–Rif complex. However, this experiment further indicates that the sites of interaction of Rif and MsRbpA on RNAP are distinct and not overlapping. Such a contention was further supported by data presented here for a [3H]Rif-binding assay (Supplementary Fig. S3) and by the non-inhibitory effect of MsRbpA on the transcription reaction (Dey et al., 2010).

**DISCUSSION**

Our experiments carried out to understand the binding of MsRbpA to RNAP in the presence of Rif have revealed the following aspects of the mechanism.

In the multiple-round promoter-specific transcription assays, we attempted to mimic the in vivo condition when an actively transcribing RNAP is exposed to Rif. It is known that an elongating RNAP is resistant to Rif (Hinkle et al., 1972; Carpousis & Gralla, 1985), but generally inside the cell the whole population of RNAP is not in an active transcription state. When a microbial cell is exposed to Rif, the molecule, being freely diffusible, has easy access to the cytoplasm. Once inside the cellular milieu, Rif binds to the non-transcribing population of RNAP. This renders them...
incapable of initiating further rounds of transcription. The remaining population of actively transcribing RNAP molecules are released after transcription from their DNA-complexed state, after which they undergo the same fate as that of their non-transcribing counterparts. Thus, all the RNAP molecules are prevented from reinitiating due to the presence of Rif blocking the path of elongating RNA into the RNA exit channel. In other words, the RNAP molecules are in a state of transcription arrest. At this point, the presence of MsRbpA leads to the rescue of the arrested RNAP and thus transcription resumes. As per the steric model of Rif inhibition of RNAP, such an event can occur when the interaction of MsRbpA with RNAP occludes Rif from its binding site so that RNA synthesis can resume. The binding site of Rif is close to the active site and blocks the path of elongating RNA into the RNA exit channel when the transcript is 2–3 nt long. The results obtained in the multiple-round transcription assays appear to confirm this. Thus, to verify the MsRbpA-mediated release of Rif from its binding site on RNAP, we attempted a fluorimetric approach, wherein we made use of the fact that if Rif is bound to its binding pocket in RNAP, then it will be able to quench the fluorescence of $\gamma$-AmNS-UTP bound at the active site $i+1$ of RNAP (Kumar et al., 1992). The addition of MsRbpA in this situation led to a decrease in the quenching of $\gamma$-AmNS-UTP fluorescence by Rif. This could only be possible if Rif was released from its binding site on Rif$^{\text{S}}$ RNAP. Thus, the molecular mechanism of Rif tolerance caused by MsRbpA on RNAP appears to be the removal of Rif from its binding site and...
the resumption of transcription from arrested polymerases. The transcription assays also showed that the presence of MsRbpA not only leads to the resumption of Rif-arrested transcription but also prevents Rif from inhibiting further rounds of transcription, as the transcript levels rise at subsequent time points of the assay.

It is important to discuss the probable reasons as to why the presence of MsRbpA did not cause a major decrease in the binding of [3H]Rif to RNAP in the standard Rif-binding assay (Supplementary Fig. S3d). Such instances are rare but do exist. The work carried out by Xu et al. (2005) applied a similar [3H]Rif-binding assay while studying the multidrug

Fig. 7. Sequence comparison of the cross-linking site of MsRbpA on the β-subunit and proximity to cluster 1 of the Rif-binding pocket among Msmeg, Mtb, E. coli (Eco) and T. aquaticus (Taq). The solid box represents the sequence alignment of the cross-linking site on the β-subunit. The dotted box represents cluster 1 of the Rif-binding pocket. The arginine residue which showed cross-linking with MsRbpA is shaded within the solid box. The highly mutated sites in RifR RNAP are shaded in the dotted box [i.e. Asp (D), His (H) and Ser (S)].

Fig. 8. Sensograms showing the kinetics of various concentrations of (a) SMO7 RNAP, (b) SMO747 RNAP, (c) SMO734 RNAP and (d) SMO748 RNAP with Rif. The association (0–300 s) and dissociation (300–600 s) phases are shown. The overlaid black lines show the 1:1 (Langmuir) fitting.
Table 2. Summary of the kinetic parameters of the interaction of Rif with RifS and RifR RNAPs

<table>
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<tr>
<th>Source strain for RNAP</th>
<th>$k_a$ (M$^{-1}$ s$^{-1}$)</th>
<th>$k_d$ (s$^{-1}$)</th>
<th>$K_D$ (M)</th>
<th>$\chi^2$ value</th>
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<td>SMO7</td>
<td>$1.36 \times 10^7$ (± 0.60)</td>
<td>$4.6 \times 10^{-4}$ (± 0.64)</td>
<td>$3.37 \times 10^{-9}$ (± 1.06)</td>
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<tr>
<td>SMO747</td>
<td>$1.04 \times 10^7$ (± 0.67)</td>
<td>$1.24 \times 10^{-3}$ (± 0.60)</td>
<td>$1.19 \times 10^{-8}$ (± 0.89)</td>
<td>0.161</td>
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<tr>
<td>SMO734</td>
<td>$9.3 \times 10^4$ (± 2.84)</td>
<td>$1.41 \times 10^{-3}$ (± 0.42)</td>
<td>$1.51 \times 10^{-8}$ (± 0.15)</td>
<td>0.218</td>
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<tr>
<td>SMO748</td>
<td>$8.2 \times 10^2$ (± 1.2)</td>
<td>$7.37 \times 10^{-3}$ (± 2.87)</td>
<td>$8.92 \times 10^{-6}$ (± 2.39)</td>
<td>0.548</td>
</tr>
</tbody>
</table>

resistance of E. coli RNAP. Those authors reported the existence of a RifR mutant RNAP, R687H, which was able to bind as efficiently as the wild-type RNAP. Another instance has been reported by Artsimovitch et al. (2005), whereby they showed that a RifR RNAP mutant, $\beta_{1235A}$, retained the ability to bind to [3H]Rif, while another mutant, $\beta_{2513P}$, did not, when compared with the wild-type. Artsimovitch et al. (2005) have subsequently presented experimental evidence in support of the hypothesis that the $\beta$Leu1235 residue is necessary to transmit the allosteric signal to cause Rif tolerance but is dispensable for Rif binding. The results obtained by us point in a similar direction. Although the interaction of MsRbpA with RNAP does not affect RNA synthesis, it may act as a similar allosteric signal to the Rif-binding site. The interaction at the cross-linking site probably causes tolerance to Rif without affecting Rif binding via a plausible allosteric mechanism. Upon further analysis of the RNAP–Rif co-crystal structures from $T$. aquaticus (PDB id: 1i6v) and $T$. thermophilus (PDB id: 2A68), we found that Arg345 (Arg381 in the Msmeg $\beta$-subunit) lies approximately 27 and 19 Å from the Rif-binding amino acids Asp396 and Ser411, respectively (Asp432 and Ser447 in the case of the Msmeg $\beta$-subunit). Thus, the interaction between MsRbpA and RNAP at this site probably triggers the release of Rif from the binding pocket. However, since the accessibility of Arg345 (Arg381 in Msmeg) is hampered by the N-terminal domain of the $\beta$-subunit, confirmation of this
interpretation must await crystallographic analysis of mycobacterial RNAP and MsRbpA. We have recently observed that MsRbpA does not interact with E. coli RNAP (unpublished data). When we consider this fact along with the exclusivity of RbpA homologues in actinobacteria, it makes us realize that protein–protein interactions are not conserved across species (Artsimovitch et al., 2000). Thus, in spite of these observed differences from available structures, the interpretation cannot be ruled out that this conformational change results in the occlusion of Rif from its binding site on mycobacterial RNAP. In the absence of crystallographic evidence, it is inappropriate to judge whether the mechanism of MsRbpA in mitigating the effect of Rif on RNAP is allosteric (Artsimovitch et al., 2005) or steric (Campbell et al., 2001). This is in part also because the methods adopted in this work have inherent limitations which prevent us from reaching a definitive conclusion on the pathway/mechanism.

Our SPR experiments, on the other hand, explained why a higher concentration of MsRbpA is needed to counter the effect of Rif on transcription. The affinity of MsRbpA for RNAP was found to be an order of magnitude lower than that of Rif. It was quite intriguing that the differential transcription rescue activity of MsRbpA with respect to Rif$^R$ and Rif$^R_R$ RNAPs (Dey et al., 2010) could not be explained on the basis of binding affinity. Also, the binding of MsRbpA to RNAP was marginally affected by the presence of Rif bound to RNAP. One more interesting result which we obtained though our SPR experiments was that the Rif$^R$ and Rif$^R_R$ RNAPs do not differ in their binding parameters while associating with MsRbpA. The ambiguities arising from the latter situation appear to require alternative models of the Rif mechanism. Considering the alternative model proposed by Artsimovitch et al. (2005), it seems that there are additional contacts of amino acids with Rif in the higher-resolution T. thermophilus RNAP structure. The binding of MsRbpA at the cross-linking site might not be able to propagate a similar allosteric effect in Rif$^R_R$ RNAPs because of the greater distance from the remaining clusters and the additional amino acids, and therefore the Rif$^R$ mutants are not rescued at high concentrations (~1 mg ml$^{-1}$) of Rif. At such high concentrations, Rif may be getting access to the binding pocket via the presence of these non-mutated but weakly interacting residues. This issue will be more clearly addressed when the changes in the complete primary sequence of the $\beta$-subunit are studied in greater detail. This argument stems from a recent criticism of the T. aquaticus RNAP–Rif subunit to the effect that the antibiotic sensitivity of an enzyme cannot be attributed solely to the regions in the protein that have a direct interaction with the antibiotic (Zenkin et al., 2005). Those authors speculate that the structure of the Rif pocket can be affected by nearby protein regions. They made this argument when they found a single Rif$^R$ mutation (position 146 in E. coli) in the N-terminal region of the $\beta$-subunit, which could affect Rif binding to RNAP (Hinkle et al., 1972; Lisitsyn et al., 1984). Mtb RNAP differs from E. coli RNAP at the adjacent position 145 (Ile is substituted for Val). Therefore, Rif binding to Mtb RNAP may be enhanced by this substitution (Zenkin et al., 2005). It is because of these observations that Zenkin et al. (2005) have speculated about the future discovery of new molecular mechanisms underlying Rif resistance in mycobacteria and other bacteria.

**REFERENCES**


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<th>Interaction</th>
<th>$k_d$ (M$^{-1}$ s$^{-1}$)</th>
<th>$k_d$ (s$^{-1}$)</th>
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<td>1.08 × 10$^3$ (± 2.51)</td>
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<tr>
<td>SMO734 RNAP–MsRbpA</td>
<td>1.49 × 10$^3$ (± 3.40)</td>
<td>2.55 × 10$^{-3}$ (± 0.36)</td>
<td>1.71 × 10$^{-8}$ (± 0.11)</td>
<td>0.619</td>
</tr>
<tr>
<td>SMO748 RNAP–MsRbpA</td>
<td>0.33 × 10$^3$ (± 1.69)</td>
<td>2.06 × 10$^{-3}$ (± 2.41)</td>
<td>6.12 × 10$^{-8}$ (± 1.42)</td>
<td>0.300</td>
</tr>
<tr>
<td>SMO7 RNAP–Rif–MsRbpA</td>
<td>0.43 × 10$^3$ (± 0.35)</td>
<td>1.74 × 10$^{-3}$ (± 0.45)</td>
<td>4.05 × 10$^{-8}$ (± 1.24)</td>
<td>0.172</td>
</tr>
</tbody>
</table>
Mechanism of MsRbpA-mediated rifampicin tolerance


Edited by: G. R. Stewart