Structural Mechanism for Rifampicin Inhibition of Bacterial RNA Polymerase

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Summary

Rifampicin (Rif) is one of the most potent and broad spectrum antibiotics against bacterial pathogens and is a key component of anti-tuberculosis therapy, stemming from its inhibition of the bacterial RNA polymerase (RNAP). We determined the crystal structure of Thermus aquaticus core RNAP complexed with Rif. The inhibitor binds in a pocket of the RNAP β subunit deep within the DNA/RNA channel, but more than 12 Å away from the active site. The structure, combined with biochemical results, explains the effects of Rif on RNAP function and indicates that the inhibitor acts by directly blocking the path of the elongating RNA when the transcript becomes 2 to 3 nt in length.

Introduction

Each year, there are 8–10 million new cases of tuberculosis (TB), which is the leading cause of death in adults by an infectious agent (Raviglioni et al., 1995; Shinnick, 1996). With TB near epidemic proportions in some parts of the world and the rapid increase in multidrug-resistant strains of Mycobacterium tuberculosis, the World Health Organization declared TB to be a global public health emergency (Raviglioni et al., 1995).

Rifampicin (Rif; Sensi et al., 1960; Sensi, 1983) is one of the most potent and broad spectrum antibiotics against bacterial pathogens and is a key component of anti-TB therapy. The introduction of Rif in 1968 greatly shortened the duration of TB chemotherapy. Rif diffuses freely into tissues, living cells, and bacteria, making it extremely effective against intracellular pathogens like M. tuberculosis (Shinnick, 1996). However, bacteria develop resistance to Rif with high frequency, which has led the medical community in the United States to commit to a voluntary restriction of its use for treatment of TB or emergencies.

The bactericidal activity of Rif stems from its high-affinity binding to, and inhibition of, the bacterial DNA-dependent RNA polymerase (RNAP; Hartmann et al., 1967). The essential catalytic core RNAP of bacteria (subunit composition α2β′ω) has a molecular mass of around 400 kDa and is evolutionarily conserved among all cellular organisms (Archambault and Friesen, 1993).

Mutations conferring Rif resistance (Rif®) map almost exclusively to the rpoB gene (encoding the RNAP β subunit) in every organism tested, including E. coli (Ezekiel and Hutchins, 1988; Wehrli et al., 1988b; Heil and Zillig, 1970) and M. tuberculosis (Ramaswamy and Musser, 1998; Heep et al., 2000). Comprehensive genetic analyses have provided molecular details of amino acid alterations in β conferring Rif® (Figure 1; Ovchinnikov et al., 1983; Lisitsyn et al., 1984a, 1984b; Jin and Gross, 1988; Severinov et al., 1993; Severinov et al., 1994).

High-resolution structural studies of the Rif-RNAP complex should lead to insights into Rif binding, the mechanism of inhibition, and also the mechanism by which mutations lead to Rif®. This could shed light on the transcription mechanism itself, as well as provide the basis for the development of drugs that selectively inhibit bacterial RNAPs but are less prone to single amino acid substitutions giving rise to resistance. The recent determination of the crystal structure of core RNAP from Thermus aquaticus (Taq; Zhang et al., 1999) has opened the door to further studies of RNAP structure, function, and interactions with substrates, ligands, and inhibitors. Here we describe the 3.3 Å crystal structure of Taq core RNAP complexed with Rif. The structure explains the effects of Rif on RNAP function. In combination with a model of the ternary transcription complex (Korzheva et al., 2000) and biochemical experiments, the data indicate that the predominant effect of Rif is to directly block the path of the elongating RNA transcript at the 5′ end when the transcript becomes either 2 or 3 nt in length.

Results

Rifampicin Inhibition of Taq RNAP

From a biochemical perspective, the interaction of Rif with RNAP has been extensively characterized using E. coli RNAP, which served as a prototype for bacterial pathogens (Honore et al., 1993; Nolte, 1997; Ramaswamy and Musser, 1998; Drancourt and Raoult, 1999; Heep et al., 1999; Morse et al., 1999; Padayachee and Musser, 1998; Drancourt and Raoult, 1999; Severinov et al., 1993; Severinov et al., 1994).

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Figure 1. The Rif-Resistant Regions of the RNAP β Subunit

The bar on top schematically represents the E. coli β subunit primary sequence with amino acid numbering shown directly above. Gray boxes indicate evolutionarily conserved regions among all prokaryotic, chloroplast, archaeabacterial, and eukaryotic sequences (labeled A–I at the top; Allison et al., 1985; Sweetser et al., 1987). Red markings indicate the four clusters where RifR mutations have been identified in E. coli (Ovchinnikov et al., 1983; Lisitsyn et al., 1984a, 1984b; Jin and Gross, 1988; Severinov et al., 1993; Severinov et al., 1994), denoted as the N-terminal cluster (N), and clusters I, II, and III (I, II, III). Directly below is a sequence alignment spanning these regions of the E. coli (E.c.), T. aquaticus (T.a.), and M. tuberculosis (M.t.) RNAP β subunits. Amino acids that are identical to E. coli are shaded dark gray, and those that are homologous (ST, RK, DE, NQ, FYWIV) are shaded light gray. Mutations that confer RifR in E. coli and M. tuberculosis are indicated directly above (for E. coli) or below (for M. tuberculosis) as follows: Δ for deletions, + for insertions, and colored dots for amino acid substitutions (substitutions at each position are indicated in single amino acid code in columns above or below the positions). Color coding for the amino acid substitutions (for reference to subsequent figures) is as follows: yellow, residues that interact directly with the bound Rif (see Figure 4); green, residues that are too far away from the Rif for direct interaction (see Figure 5); purple, three positions that are substituted with high frequency (noted as a % immediately below the substitutions) in clinical isolates of RifR M. tuberculosis (Ramaswamy and Musser, 1998).

Below the three prokaryotic sequences is a sequence alignment of three eukaryotic sequences with shading as above. The dots indicate a gap in the alignment.

A plate assay (see Experimental Procedures) showed that Taq cells were unable to grow on media supplemented with 50 μg/ml Rif (data not shown). For in vitro studies, Taq RNAP holoenzyme was reconstituted using Taq core RNAP (purified from Taq cells; Zhang et al., 1999) and recombinant Taq σ8 (overexpressed and purified from E. coli; Minakhin et al., 2001b). The enzyme initiated, elongated, and terminated transcripts efficiently from a template containing the T7A1 promoter and the terminator (Figure 2a; Nudler et al., 1994) at 37°C using the dinucleotide CpA as the initiating primer. The major RNA products, a trimeric abortive transcript (CpApU), a 105 nt terminated transcript (Term), and a 127 nt runoff transcript (Run off), were the same as those produced by E. coli RNAP (Figure 2a, lanes 1 and 8). Since E. coli σ8 is totally inactive when combined with Taq core RNAP in this assay (Minakhin et al., 2001b), the possibility of trace contamination with E. coli RNAP, and the disposition of the Rif site with respect to the universally conserved active for Taq RNAP. Quantitatively, the two RNAPs responded very differently to Rif; the K_i (estimated from the Rif concentration where the production of long transcripts was inhibited by 50%) for E. coli RNAP was about 0.1 μM while for Taq RNAP it was about 10 μM, a 100-fold difference in sensitivity (the Rif sensitivity of the thermophilic RNAP decreased at higher assay temperatures; data not shown). Qualitatively, however, both RNAPs responded the same way, with an increase in the production of the trimeric product and a concurrent precipitous drop in the production of the long transcripts (Figure 2a).

Mustaev et al. (1994) used chimeric Rif-nucleotide compounds to measure the distance between the initiating nucleotide binding site (the i-site) and the Rif binding site. By varying the linker between the Rif and the nucleotide and testing for maximal transcription initiation activity, the optimal length was found that allowed binding of each moiety in its respective site. This experiment was used to compare the disposition of the Rif and i-sites in E. coli and Taq RNAP. In both cases, optimal initiation activity was observed when the linker comprised five (CH₂) groups (Figure 2b). Thus, in spite of the fact that Taq RNAP requires a 100-fold higher concentration of Rif for inhibition, we conclude that Taq RNAP binds Rif and is inhibited through the same biochemical mechanism as E. coli RNAP, and the disposition of the Rif site with respect to the universally conserved active site is identical. We conclude that Taq RNAP can serve as a model for Rif interactions with other RNAPs.

Rif-RNAP Structure Determination and Refinement

Tetragonal crystals of Taq core RNAP (Zhang et al., 1999) were incubated overnight in stabilization buffer with 0.1 mM Rif, followed by a 30 min. soak in cryo-
Figure 2. Rif Inhibition of Taq RNAP

(a) Autoradiographs showing the radioactive RNA produced by Taq (lanes 1–7) and E. coli (lanes 8–13) RNAP holoenzymes transcribing a template containing the T7 A1 promoter and the tR2 terminator, analyzed on a 15% polyacrylamide gel and quantitated by phosphorimagery. In the absence of Rif (lanes 1 and 8), the major RNA products from each RNAP correspond to a trimeric abortive product (CpApU), a 105 nt terminated transcript (Term), and a 127 nt runoff transcript (Run off). Lanes 2–7 and 9–13 show the effects of increasing concentrations of Rif. The quantitated results are shown on the right, where the amounts of each product (normalized to 100% for the Run off and Term transcripts in the absence of Rif, and for CpApU at the highest concentration of Rif) are plotted as a function of Rif concentration.

(b) The distance between the bound Rif and the initiating substrate (i-site) of E. coli and Taq RNAP holoenzymes was measured using chimeric Rif-nucleotide compounds as previously described (Mustaev et al., 1994). Rif-nucleotide compounds (Rif-(CH2)n−Ap) with different linker lengths, n (indicated above each lane), were bound to RNAP, then extended in a specific transcription reaction with −[32P]UTP by the RNAP catalytic activity. The products were analyzed on a 23% polyacrylamide gel, visualized by autoradiography, and quantitated by phosphorimagery. The quantitated results are shown directly below, where the product yield (as % activity normalized to 100% at the highest level) is plotted as a function of the Rif-nucleotide linker length (n).
Overall Structure
As expected from the fact that all mapped Rif mutants occur in rpoB (Figure 1), Rif makes contacts only with the RNAP β subunit in a close complementary fit to its binding pocket deep within the main DNA/RNA channel. Clearly, Rif does not bind directly at the RNAP active site (Figure 3b). The closest approach of Rif to the active site, defined as the distance between the active site Mg\(^{2+}\) and Rif C38 (Figure 3c), is 12.1 Å.

Detailed Interactions
A large number of Rif derivatives have been investigated for antimicrobial activity. In general, modification of the ansa bridge, or modifications that alter the conformation of the ansa bridge, reduce activity. Other structural features of the antibiotic that are particularly critical for activity include the napthol ring with oxygen atoms (O1 and O2) at C1 and C8, and unsubstituted hydroxyls (O10 and O9) at C21 and C23 (Figure 3c; Brufani et al., 1974; Lancini and Zanichelli, 1977; Arora, 1981, 1983, 1985; Sensi, 1983; Arora and Main, 1984). Most Rif modifications that retain activity involve substitutions at C3 of the napthol ring, which have only modulatory effects on in vitro activity.

These results are explained by the structural details of the Rif-RNAP complex (Figures 4 and 5). A cluster of hydrophobic residues (L391, L413, G414, I452) line one wall of the Rif binding pocket and make van der Waals contact with the napthol ring and the methyl group at C7. One end of the binding pocket (the bottom in Figure 4) is formed by Q390. The alkyk chain of Q390 makes van der Waals contact with Rif C28 and C29, while the polar head group may interact with O5. Protein groups are positioned to make hydrogen bonds with each of the four critical hydroxyls of Rif: R409 with O1, Q393 and S411 with O2, and D396 and H406 with O10. O9 and O10 are also in position to interact with the backbone amide of F394, respectively. Also positioned to make a potential hydrogen bond with the backbone amide of F394 is O8.

D396 contributes to the binding interface in several ways. In addition to forming a potential hydrogen bond with O10, it forms the top end of the binding pocket (in Figure 4) by making van der Waals contact with C18-C21, and C31. Moreover, the negative charge of D396 may be important for neutralizing the positive charges of two nearby side chains, R405 (not shown) and F409 (Figure 4), each about 6 Å away. The charge neutralization might be important for the binding of the relatively apolar Rif. Most Rif mutants at 396 substitute a large, bulky group that would likely interfere with Rif binding and would not have the correct geometry for hydrogen bonding O10 (Y), or else apolar groups (V, G, or A) with no hydrogen bonding ability. One of these mutants, D396V (position 516 in E. coli), was among the original, strong Rif mutants mapped by Ovchinnikov et al. (1983), pointing to the importance of this residue in forming the Rif binding interface. Another mutant identified in E. coli, however (D396N), is isosteric with Asp and would likely maintain the hydrogen bond with O10. Nevertheless, this substitution yields weak Rif (Lisitsyn et al., 1984a), which may be caused by the loss of negative charge at this position.

Rif has a partial + charge, localized at N4 (Figure 3c). A negatively charged residue, E445, is situated nearby and may contribute to the Rif binding site by neutralizing this charge. This is not likely to be a strong effect, as many Rif derivatives with equal or stronger activity than Rif do not have this partial charge. E445 is the only residue close enough to Rif to be involved in potentially direct interactions (Figure 4) for which a Rif mutant has not been reported. However, this residue is universally conserved as either Glu or Asp in a segment of β region D that is invariantly present in prokaryotes, chloroplast, archaebacteria, and eukaryotes (Allison et al., 1985; Sweetser et al., 1987), pointing to its importance for the basic function of RNAP.

Thus, of the 12 residues that are close enough to Rif to make direct interactions (including backbone interactions with F394; Figure 4), 11 mutate to a Rif phenotype. The 12th position, E445, is highly conserved so that its substitution would likely be lethal and consequently not be detectable as Rif mutations.

Twelve additional positions have been identified at which substitution gives rise to Rif (Figure 1). These residues surround the Rif binding pocket but do not make direct interactions with the antibiotic (Figure 5). In every case, the Rif mutations involve replacement by a different sized amino acid side chain (almost always substituting a small residue with a more bulky one), or else involve adding or removing a Pro residue. These substitutions would likely affect the folding or packing of the protein in the local vicinity of the substituted residue, causing distortions of the Rif binding pocket.

Mechanism of RNAP Inhibition by Rif
The effects of Rif on RNAP in each stage of the transcription cycle have been probed using detailed kinetic analy-
Figure 3. Rif-RNAP Cocrystal Structure
(a) Stereo view of the Rif binding pocket of Taq core RNAP, generated using O (Jones et al., 1991). Carbon atoms of the RNAP β subunit are cyan or yellow (residues within 4 Å of the Rif), while carbon atoms of the inhibitor are orange. Oxygen atoms are red, nitrogen atoms are blue, and sulfur atoms are green. Electron density, calculated using \(|F_o \text{Rif} - F_o \text{nat}|\) coefficients (Rif denotes the Rif-RNAP cocrystal, native denotes the native core RNAP crystal), is shown (orange) for the Rif only (contoured at 3.5 σ), and was computed using phases from the final refined RNAP model with the Rif omitted.

(b) Three-dimensional structure of Taq core RNAP in complex with Rif, generated using GRASP (Nicholls et al., 1991). The backbone of the RNAP structure is shown as tubes, along with the color coded transparent molecular surface (β, cyan; β', pink; α, white; the α subunits are behind the RNAP and are not visible). The Mg²⁺ ion chelated at the active site is shown as a magenta sphere. The Rif is shown as CPK atoms (carbon, orange; oxygen, red; nitrogen, blue).

(c) Structural formula of Rif. Features of the structure discussed in the text are color coded (ansa bridge, blue; napthol ring, green). The four oxygen atoms critical for Rif activity (Brufani et al., 1974; Lancini and Zanichelli, 1977; Arora, 1981, 1983, 1985; Sensi, 1983; Arora and Main, 1984) are shaded with red circles.

Rif has essentially no effect on specific promoter binding and open complex formation (Hinkle et al., 1972; McClure and Cech, 1978). A small increase (about 2-fold) in the apparent \(K_m\) for initiating substrate binding in the enzyme’s i-site (the 5’ nucleotide) was observed, but the binding of the incoming nucleotide substrate in the i+1 site (the 3’ nucleotide) and the formation of the first phosphodiester bond were largely unaffected (McClure and Cech, 1978). The dominant effect of Rif binding on RNAP activity was a total blockage of synthesis of the second (when transcription was initiated with a nucleoside triphosphate) or third (when transcription was initiated with a nucleoside di- or monophosphate) phosphodiester bond (McClure and Cech, 1978). Since synthesis of the first and second phosphodiester bonds can occur in the presence of Rif, the antibiotic does not interfere with substrate binding, catalytic activity, or the intrinsic translocation mechanism of the RNAP. After
RNAP has synthesized a long transcript and entered the elongation phase, it becomes totally resistant to Rif. These properties led to the proposal that Rif inhibits RNAP through a simple steric block of the path of the elongating RNA at the 5’ end (McClure and Cech, 1978). Whether Rif directly blocked the path of the RNA or if blockage was an indirect effect due to a conformational change in the RNAP induced by Rif binding could not
Table 1. Crystallographic Data and Structural Model

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Structural Model

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Refinement

| R<sub>cryst</sub> (%) | 28.1 |
| R<sub>free</sub> (%)  | 35.9 |

<sup>a</sup>R<sub>m-age</sub> = Σ||I<sub>_o</sub> - <I<sub>_j</sub>/2I<sub>_o</sub>.

<sup>b</sup>Also included in the model was one Mg<sup>2+</sup> and Zn<sup>2+</sup> ion. (Zhang et al., 1999), and one Rif molecule (Brufani et al., 1964).

be distinguished. Alternatively, others have proposed that Rif exerts its effect allosterically by decreasing the affinity of the RNAP for short RNA transcripts (Schulz and Zillig, 1981).

The Rif-RNAP crystal structure explains the results described above and strongly supports the simple steric block mechanism (McCure and Cech, 1978). Rif directly abuts the base of a loop that comprises the C-terminal part of β conserved region D (residues 443-451, shaded red in Figure 5; βD loop II in Korzheva et al. [2000]), and a cluster of Rif<sup>5</sup> mutants, Rif cluster I (Figure 1), flanks this region. Modeling suggests that this loop, which contains several nearly universally conserved residues, participates in forming the binding site for the base pair at +1 in the transcription complex (Korzheva et al., 2000), so effects of Rif on the K<sub>cat</sub> for the initiating substrate are not surprising. However, Rif does not directly contact the end of this loop. In addition, conformational changes of the protein in this region are not indicated from the structural data, consistent with the observation that the effect of Rif on this region is small.

The principal effect of Rif is seen in the context of a model of the transcriptionally active ternary complex (Korzheva et al., 2000) containing RNAP, DNA template, and RNA transcript (Figure 6). In this figure, only the RNAP active site Mg<sup>2+</sup> and the 9 bp RNA/DNA hybrid (from +1 to −7) from the ternary complex model are shown. The rest of the RNAP and nucleic acids are omitted for clarity. Also shown is the atomic model of Rif as it would be positioned in its binding site on the β subunit.

It can be seen that the two substrate nucleotides, at +1 (green) and −1, are not directly affected by the presence of Rif, so that RNAP can bind and catalyze the formation of a phosphodiester bond between the two substrates in the presence of the antibiotic. With a transcript length of 3 nt, however, the 5′ phosphates of the 5′ nucleotide (at −2) sterically clash with Rif, and the nucleotides further upstream (−3 to −5) severely clash with Rif. At the same time, Rif does not interfere with the DNA (gray). Thus, the structure, in combination with the ternary complex model, explains the biochemical data on the mechanism of Rif inhibition, provides strong support for the proposal that Rif sterically blocks the path of the elongating RNA transcript at the 5′ end, and indicates that the blockage is a direct consequence of Rif binding in its site. The model even suggests why transcripts initiated with nucleoside triphosphates are blocked after the first phosphodiester bond, while transcripts initiated with nucleoside di- or monophosphates are blocked after the second phosphodiester bond. In the model, the nucleoside monophosphate in the transcript at the −2 position clashes only slightly with Rif, while the presence of a 5′ triphosphate at the −2 position would extend into Rif. The confluence of the structural and biochemical data also lends support to the ternary complex model of Korzheva et al. (2000).

Core RNAP can bind a preformed “minimal nucleic acid scaffold” of RNA/DNA oligonucleotides (Figure 6b, top) to yield functional ternary elongation complexes (Korzheva et al., 2000). We performed order of addition experiments using this system in order to assess whether Rif and RNA binding were competitive (Figure 6b). The DNA component of the scaffold was annealed with varying lengths of RNA transcript, and the effect of Rif, added before or after the oligonucleotides, on the sequence-dependent extension of RNA by one nucleotide (radioactively labeled CTP) was assayed at room temperature. In the case of <i>E. coli</i> core RNAP in the absence of Rif, the RNA transcript was extended with nearly equal efficiency regardless of its length within a range of 3–7 nt (Figure 6b, lanes 11–15). When
Rif was added prior to the nucleotide scaffold, the RNAP was unable to extend any of the RNA oligos, regardless of length (lanes 1–5), indicating that Rif occupied its site and blocked the extension and/or binding of all of the transcripts. When the scaffold was added prior to Rif addition, Rif was able to occupy its site and block the extension of the 3 nt transcript (lane 6), but had no effect on the extension of the longer transcripts (lanes 7–10), presumably because Rif could not access its binding site blocked by the longer RNA transcripts (Figure 6a). These results are consistent with the early data that Rif inhibits the RNA extension from 2 to 3 nt if the 5’ nucleoside is tri-phosphorylated, but inhibits extension from 3 to 4 nt if the 5’ nucleoside is mono- or di-phosphorylated (McCleure and Cech, 1978) since the synthetic RNA oligos lack 5’ phosphates.

Similar experiments were performed with *Taq* core RNAP (Figure 6b, lanes 16–30). In the absence of Rif, the efficiency of transcript extension was strongly dependent on the transcript length (lanes 26–30). Extension of the shortest transcripts was barely detectable, suggesting that, unlike *E. coli* RNAP, *Taq* core RNAP does not bind and stabilize the short, intrinsically unstable RNA/DNA hybrids. In the presence of Rif, a generalized inhibition of transcript extension was observed regardless of the order of addition or of the transcript length (lanes 16–25). We explain these results by the low binding affinity of *Taq* core RNAP for both Rif and scaffold blocked by the longer RNA transcripts (Figure 6a).

**Discussion**

In summary, we have shown that, although *Taq* RNAP is relatively insensitive to Rif, at sufficiently high concentrations, the antibiotic binds and inhibits the enzyme. Inhibition of *Taq* RNAP occurs through the same biochemical mechanism as *E. coli* RNAP, and the disposi-
Structure of the Rifampicin-RNA Polymerase Complex

Figure 6. Mechanism of RNAP Inhibition by Rif
(a) The RNAP active site Mg\(^{2+}\) (magenta sphere) and the 9 bp RNA/DNA hybrid (from +1 to –8) from a model of the ternary elongation complex (Korzheva et al., 2000) are shown. The RNAP itself and the rest of the nucleic acids are omitted for clarity. The incoming nucleotide substrate at the +1 position is colored green, the –1 and –2 positions, which can be accommodated in the presence of Rif, are colored yellow. The RNA further upstream (–3 to –8), which cannot be accommodated in the presence of Rif, is colored pink. The template strand of the DNA is colored gray. Also shown is a CPK representation of Rif as it would be positioned in its binding site on the β subunit (carbon atoms, orange; oxygen, red; nitrogen, blue). The Rif is partially transparent, illustrating the RNA nucleotides at –3 to –5 that sterically clash. Generated using GRASP (Nicholls et al., 1991).

(b) The structure of the minimal scaffold systems with RNA lengths from 3–7 nt (labeled above the RNA chain; Korzheva et al., 2000). The results are presented below as autoradiographs of the radioactive RNAs produced by E. coli (lanes 1–15) or Taq (lanes 16–30) core RNAPs transcribing the minimal scaffolds with the indicated lengths of RNA ("X") and analyzed on a 23% polyacrylamide gel. Lanes 1–10 and 16–25 demonstrate the effect of Rif inhibition of transcription when it was bound by RNAP either before (lanes 1–5 and 16–20) or after (lanes 6–10 and lanes 21–25) addition of the scaffold. Lanes 11–15 and 26–30 show elongation of the same scaffolds in the absence of Rif. The RNA with the critical length of 3 nt, which cannot be elongated by E. coli RNAP in the presence of Rif regardless of the order of Rif and scaffold addition (lanes 1 and 6), is colored red. The RNAs of 4–7 nt (colored green) were extended by E. coli RNAP when added before Rif (lanes 6–10).
futile cycle begins again. If the 5′ nucleoside contains a di- or a monophosphate at its 5′ end (or if it’s unphosphorylated), then after the synthesis of the first phosphodiester bond, the RNAP can translocate normally and the steric clash of the transcript with Rif occurs during the translocation of the 3 nt transcript following the synthesis of the second phosphodiester bond.

The relative insensitivity of Taq RNAP to Rif is likely due to amino acid substitutions in Taq RNAP compared with other, more Rif-sensitive RNAPs. The 12 residues close enough to interact directly with Rif are identical between E. coli, Taq, and M. tuberculosis (marked yellow in Figure 1). Among the 11 positions that do not directly interact with Rif but likely affect Rif binding indirectly, 5 are substituted in Taq RNAP (387, 395, 398, 453, and 566; Figure 1). Although the effect of these residues on the structure of RNAP is difficult to assess with only one available structure, we can venture some suppositions. Three of these positions, 387, 398, and 453, contain amino acids that are not dramatically different in overall size from their E. coli and M. tuberculosis counterparts and we predict that these residues are not the origin of the Taq RNAP insensitivity to Rif. Position 566 is highly conserved among all RNAPs as either Lys or Arg (the homologous position is Arg in both E. coli and M. tuberculosis) but is Thr in Taq RNAP. This substitution is unlikely to be the main determinant of the Taq RNAP Rif insensitivity, however, since Minakhin et al. (2001b) mutated Taq Thr-566 to Arg, but this had little effect on the RifR of the enzyme assayed at 45°C. This leaves position 395, which is highly conserved as a hydrophobic residue among all bacterial RNAPs. In E. coli and M. tuberculosis, this position is a Met, but in Taq, it is a Lys. Taq Lys-395 appears to participate in buried salt bridges with Asp-124 and Asp-133 that may contribute to thermostability of the protein. This nonconservative substitution (Lys for Met) could affect the local path of the polypeptide backbone, and is immediately adjacent to Phe-394, the backbone amide and carboxyl of which appear to be involved in important interactions with the Rif (Figure 4).

All but one of the residues that are close enough to Rif to participate in direct interactions are known to mutate to strong RifR (Figure 4). However, additional residues could be important for the formation of the Rif binding pocket, but not revealed as RifR mutants if they are necessary for basic RNAP function. As mentioned above, the four regions of the β subunit that harbor RifR mutants are highly conserved among prokaryotes (Figure 1), but the much weaker homology with archaeobacterial and eukaryotic RNAPs, combined with the fact that so many RifR mutations have been discovered, indicate that these regions are not critical to RNAP function in vivo. Nevertheless, some RifR mutations do have profound functional effects (Landick et al., 1990; Jin and Gross, 1991), and E. coli strains with RifR RNAP have been shown to be at a competitive disadvantage to wt E. coli in the absence of Rif (Jin and Gross, 1989).

The clinical success of Rif proves that the bacterial RNAP is an excellent target for antimicrobials. The structure and available genetic and biochemical data suggest that the design of modified versions of Rif to overcome the effects of RifR mutations, while perhaps leading to incremental improvements, may be futile in the long run because of the apparently small functional penalties of mutating this region of the RNAP, and the variety of amino acid positions and mutations that result in RifR (Figure 1). Somewhat encouraging, however, are the findings from clinical isolates of RifR M. tuberculosis. Although the RifR mutations are spread over 15 positions of rpoB, 77% of all the mutations isolated involved substitutions at one of only two positions, corresponding to Taq 406 and 411, and an additional position (Taq 396) accounts for a combined 86% of all the mutants.

An important conclusion from these studies comes from the inhibition mechanism of Rif, a simple steric block of transcription elongation. Thus, the powerful effects of Rif do not stem from the details of its chemical structure, and do not involve interference with the catalytic activity of the RNAP, for instance by mimicking substrates or transition states of the polymerization reaction. Such an inhibitor would act on features that are highly conserved between prokaryotes and eukaryotes, rendering it useless as an antimicrobial agent. Rather, the effects of Rif depend only on its ability to bind tightly to a relatively nonconserved part of the structure, disrupting a critical RNAP function by virtue of its presence. Decades of functional studies (Chamberlin, 1993; Mustaev et al., 1997; Korzheva et al., 1998; Nudler, 1999), and more recent structural evidence (Mooney and Landick, 1999; Zhang et al., 1999; Cramer et al., 2000; Korzheva et al., 2000), indicate that cellular RNAPs operate as complex molecular machines, with extensive interactions with the template DNA, product RNA (Korzheva et al., 2000), and other regulatory molecules. It seems likely that many distinct sites exist where the tight binding of a small molecule would disrupt critical features of the functional mechanism.

Experimental Procedures

Purification and Crystallization

Native Taq core RNAP was purified and crystallized as described previously (Zhang et al., 1999). The crystals were then soaked in stabilization solution (2 M (NH₄)₂SO₄, 0.1 M Tris-HCl, pH 8.0, and 20 mM MgCl₂) with 0.1 mM Rif for at least 12 hr. The crystals were then prepared for cryo-crystallography by soaking in stabilization solution containing 50% (v/v) sucrose for 30 min before flash freezing in liquid nitrogen. Diffraction data was collected at the APS beamline SBC 191D using 0.3° oscillations, and processed using DENZO and SCALEPACK (Otwinowski, 1991).

Structure Determination

The native core RNAP structure (Zhang et al., 1999) was used as a starting model for rigid body refinement and positional refinement against the observed amplitudes from the Rif-RNAP complex crystal (F_{obs}) using CNS (Adams et al., 1997), yielding an initial R factor of 0.354 (Rfree = 0.41, where the identical reflections was set aside as for the R_{free} determination of the native structure) for data from 100-3.2 Å resolution. An initial Fourier difference map, calculated using [F_{obs} - F_{calc}] amplitude coefficients and using phases calculated from the native core RNAP structure (β′ subunit), clearly revealed density for the Rif molecule (Figure 3a). Multiple rounds of manual rebuilding against [2F_{obs} - |F_{calc}|] maps using O (Jones et al., 1991), and refinement using CNS (Adams et al., 1997), resulted in the current model (Table 1). At later stages of the refinement, The Rif X-ray crystal structure (Brufrani et al., 1974) was easily placed into the difference density. Included in the model is the recently determined sequence of the Taq β′ subunit (Minakhin et al., 2001a), modeled earlier as a polyalanine chain (Zhang et al., 1999). Still missing from the model is a 300 amino acid, nonconserved domain inserted between conserved regions A and B of the β′ subunit (Zhang et al., 1999).
Assays

Tag3 cells were tested for sensitivity to Rif on solid media. Plates containing 3% bactoagar and 1/5 dilution of Luria broth were poured with and without 50 μg/ml of Rif. Cells from frozen stock were then streaked onto plates and incubated at 65°C for 2 days and assessed for growth.

The transcription assay comparing Rif inhibition of E. coli and Tag3 RNAPs (Figure 2a) was performed as described (Nudler et al., 1994). Briefly, 0.1 pmol of purified Tag core RNAP (Zhang et al., 1999) was incubated with ω−(Minakhin et al., 2001b) in 20 μl of transcription buffer (40 mM Tris-HCl, pH 7.9, 40 mM KCl, 5 mM MgCl2) for 15 min at 37°C to form holoenzyme. Rif was added to the final concentrations indicated in Figure 2a and incubated another 5 min at 37°C, followed by addition of 0.15 pmol of T7A1 promoter fragment and incubation for 5 min at 37°C. RNA synthesis was initiated by the addition of Cpa primer (100 μM) and RNAP (either the annealed scaffold (10 pmol) with a molar equivalent of core London 45°C, followed by addition of 0.15 pmol of T7A1 promoter fragment and incubation for 5 min at 37°C. RNA synthesis was initiated by the addition of Cpa primer (100 μM), NTPs (25 μM each), and ω−[32P]UTP (0.3 μM), and the reaction was stopped after incubation for 10 min at 37°C. The assay for E. coli RNAP holoenzyme was the same except the Cpa primer was added to a concentration of 10 μM. Radioactive RNA products were analyzed on a 15% polyacrylamide sequencing gel.

Assays for extension of the Rif-nucleotide compounds (Figure 2b) were carried out as described (Mustaev et al., 1994) with minor modifications. After binary complex formation, transcription reactions were started by the addition 10 μM Rif-(CH2)n-A compound, with the “n” indicated in Figure 2b, and ω−[32P]UTP (0.3 μM). The reactions were incubated for 2 min at room temperature for E. coli RNAP and 3 min at 55°C for Tag. Under these conditions, the reaction was not complete, and the yield of the Rif-(CH2)n-ApU depended on the linker length. Radioactive RNA products were analyzed on a 23% polyacrylamide sequencing gel.

Transcription reactions on the minimal scaffold system shown (Figure 6b) were performed as described (Korzheva et al., 2000) with minor modifications. The RNA and DNA components of the scaffold (100 pmol of each) were mixed in 100 μl of transcription buffer at 45°C and the mixture was allowed to cool to room temperature over 30 min. RNAP/scaffold complexes were formed by incubation of the annealed scaffold (10 pmol) with a molar equivalent of core RNAP (either E. coli or Tag) preincubated with Rif (100 μM for E. coli, 200 μM for Taq) for 10 min to form the RNAP/scaffold complex. Extension of the RNA oligonucleotide was assayed by the addition of ω−[32P]CTP (0.3 μM) and 5 min incubation at room temperature. In Figure 6b, lanes 1–5 and 16–20, RNAP was preincubated with Rif (100 μM for E. coli, 200 μM for Taq), for 10 min, to form the RNAP/scaffold complex. In lanes 1–5 and 16–20, the RNAP/scaffold complexes formed in the absence of Rif were incubated with Rif (concentrations as above) for 10 min. Finally, in lanes 11–15 and 26–30, the RNAP or RNAP/scaffold complex was not exposed to Rif. Radioactive RNA products were analyzed on a 23% polyacrylamide sequencing gel.

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