

Structural Evidence for Rifampicin Monooxygenase Inactivating Rifampicin by Cleaving Its Ansa-Bridge

Li-Kai Liu,[†] Yumin Dai,[§] Heba Abdelwahab,[§] Pablo Sobrado,^{*,§} and John J. Tanner^{*,†,‡}

[†]Department of Biochemistry and [‡]Department of Chemistry, University of Missouri—Columbia, Columbia, Missouri 65211, United States

[§]Department of Biochemistry, Virginia Tech, Blacksburg, Virginia 24061, United States

Supporting Information

ABSTRACT: Rifampicin monooxygenase (RIFMO) decreases the potency of rifampicin (RIF) by converting it to oxidative products. Further decomposition of RIF has been observed in bacteria producing RIFMO and contributes to RIFMO-mediated drug resistance. Here we report the first crystal structure of RIFMO in complex with the hydroxylated RIF product. The 2.10 Å resolution structure reveals a breach of the ansa aliphatic chain of RIF between naphthoquinone C2 and amide N1. Our data suggest that RIFMO catalyzes the hydroxylation of RIF at the C2 atom followed by cleavage of the ansa linkage, which leads to inactivation of the antibiotic by preventing key contacts with the RNA polymerase target.

Microorganisms have evolved sophisticated strategies to evade antibiotics, such as efflux pumps, mutagenesis of proteins targeted by antibiotics, and enzyme-catalyzed chemical modification of antibiotics. Among the latter category, the enzyme rifampicin monooxygenase (RIFMO, also known as Rox) has been identified as a flavin-dependent monooxygenase that modifies rifampicin (RIF) (Figure 1A), an antibiotic used in combination therapy to treat mycobacterial and non-mycobacterial infections.^{1,2} The gene encoding RIFMO is present in a variety of environmental bacteria, notably *Nocardia*, *Streptomyces*, and *Rhodococcus* species.^{3–6} RIFMO is one of two known flavin monooxygenases that inactivate antibiotics, the other being TetX, which acts on tetracycline and has a protein fold different from that of RIFMO.^{7–9}

The mechanism of RIFMO is an area of active investigation. RIFMO (from *Nocardia farcinica*) was initially characterized as an NADPH-dependent *N*-monooxygenase proposed to hydroxylate RIF at the N2' atom to produce 2'-*N*-hydroxy-4-oxo-RIF. This hydroxylation was proposed to lead to subsequent RIF decomposition.^{3,4} However, direct structural evidence for this modification and degradation of RIF has remained elusive. Further, it was unclear how the addition of a hydroxyl group to N2' compromises the binding of RIF to its target, the β -subunit in RNA polymerase. Recently, Koteva et al. showed that RIFMO from *Streptomyces venezuelae*, in fact, catalyzes a different reaction involving monooxygenation of position 2 of the naphthyl group, followed by linearization of the antibiotic (Figure 1A).⁵ Direct structural evidence for the linearized product, however, is lacking.

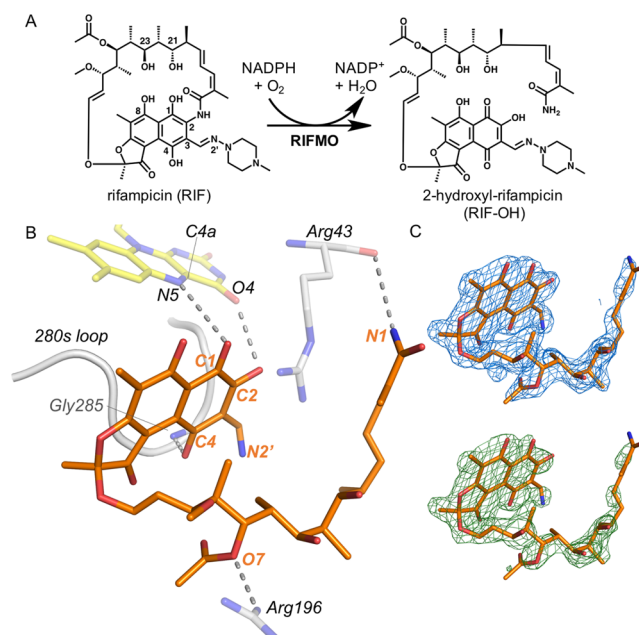


Figure 1. Structural evidence for cleavage of the RIF ansa-bridge by RIFMO. (A) Reaction catalyzed by RIFMO. (B) Pose of RIF-OH in the active site. (C) Electron density for RIF-OH. The top panel shows the final $2F_o - F_c$ map contoured at 1.0σ . The bottom panel shows a simulated annealing $F_o - F_c$ omit map contoured at 2.5σ .

Herein, we report the first crystal structure of RIFMO complexed with its product. The structure provides direct evidence for the Koteva et al. linearization mechanism. We also provide nuclear magnetic resonance (NMR) analysis further supporting this mechanism.

A 2.10 Å resolution crystal structure of RIFMO from *N. farcinica* (74% identical to the *S. venezuelae* enzyme) was determined from a crystal prepared in the presence of RIF and NADPH [Table S1, Protein Data Bank (PDB) entry 6C7S]. The electron density maps clearly show a form of RIF bound in the active site (Figure 1C). As in our previous structure of RIFMO complexed with RIF,¹⁰ strong electron density places the naphthoquinone ring system next to the FAD N5 atom edge of the flavin isoalloxazine (Figure 1B). However, the

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density suggested that the bond between C2 of the naphthoquinone and N1 of the ansa-bridge was broken, resulting in a gap of 7.0 Å between these two atoms (Figure 1C). The maps further indicated an atom bound to C2, which was modeled as O and presumed to be OH of the product 2-hydroxyl-rifampicin (RIF-OH) based on NMR analysis presented below. Assuming the bound species represents the product of RIFMO catalysis, the structure suggests that RIFMO converts RIF from a macrocyclic compound into a linear molecule, consistent with the mechanism of Koteva et al.⁵

Transformation of the substrate into the product results in profound conformational changes (Figure 2A). No longer

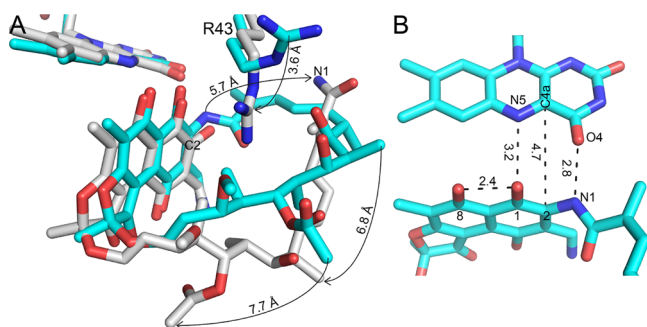


Figure 2. Comparison of the substrate and product complexes of RIFMO. (A) Superposition of RIFMO complexed with the substrate RIF (cyan, PDB entry 5K0X) and the product RIF-OH (white, PDB entry 6C7S). The arrows indicate the conformational changes that occur as a result of catalysis. (B) Structure of RIFMO complexed with RIF, highlighting interactions between the FAD and RIF (PDB entry 5K0X).

constrained by the covalent bond with C2, the N1 atom shifts by 5.7 Å, creating a gap of 7.0 Å between the formerly bonded atoms. The rupture of the C2–N1 bond allows the ansa-bridge to relax, with atoms of the bridge moving by 7–8 Å. The protein structure also changes during catalysis. In particular, upon product formation, Arg43 moves into the space vacated by the ansa-bridge (Figure 2A).

Previously, we have shown by HPLC and spectral analyses that the reaction of RIFMO with RIF occurs via the formation of an intermediate (P*), which converts over time to the final RIF-OH product.⁴ We reanalyzed the ¹H NMR assignments of RIF-OH and compared it to RIF. We also acquired electrospray ionization mass spectrometry, ¹H–¹³C HMBC, and ¹³C NMR data for P* and RIF-OH for further characterization. Analysis of the mass showed that the intermediate and final product most likely are tautomers, as an ion with an *m/z* value of 839.5 consistent with the addition of a hydroxyl to RIF was observed

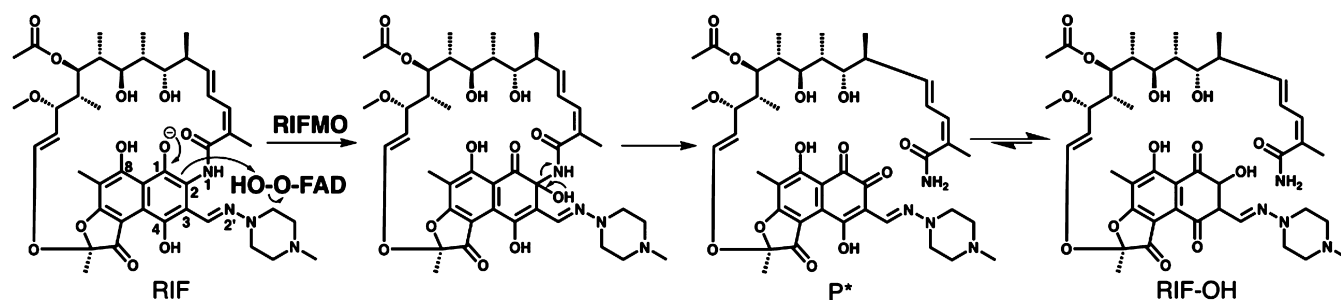
for both samples (Figure S1). The ¹H NMR spectra of RIF-OH from our previous work⁴ helped us identify signals of amide protons (NH) in the aromatic region, which were split into two peaks at 7.07 (1H) and 7.23 (1H) ppm. The integration of both peaks is one, clearly suggesting the presence of two distinct NH protons in RIF-OH (Figure S2). To confirm the location of these two NH protons, ¹H–¹³C HMBC experiments were conducted. From the two-dimensional NMR spectra, strong correlations between NH and C15, as well as between NH and C16, were observed, while there is no correlation displayed between NH and C2, indicating these two protons were both primary amide protons located the end of the chain system (Figure S3). The observation of two magnetically inequivalent protons displaying two distinct chemical shifts is consistent with attachment to an amide group.

In addition, we made chemical shift assignments for ¹H NMR of P* (Figure S2C). Interestingly, the ¹H NMR spectra of P* and RIF-OH are very similar, differing only in hydroxyl protons located at 3.0–4.0 ppm. The P* ¹H NMR spectrum also shows two peaks corresponding to the single NH protons at 7.07 (1H) and 7.24 (1H) ppm, indicating that the C2–N1 bond is also cleaved in P*.

We also acquired ¹³C NMR spectra of RIF-OH to look for a change at C2. Inspection of the ¹³C NMR data revealed major differences between RIF and RIF-OH located in C1–C10, where the naphthoquinol was converted to naphthoquinone (Table S2). For RIF-OH, the chemical shift of C2 was at 176.3 ppm, indicating C2 is an oxygen-bearing carbon, while for RIF, nitrogen-bearing C2 is assigned to 115.5 ppm, which was consistent with previously reported C NMR data of RIF.³ The shift of the carbon signal of C2 from 115.5 to 176.3 ppm served as a direct evidence of cleavage of the bond between C2 and N1. The rest of the signals involved in the naphthoquinone system all have shifts affected by the conversion from naphthoquinol to naphthoquinone. In particular, in the structure of RIF, both C1 and C4 were connected to the hydroxyl group; however, in RIF-OH, both carbons were converted from hydrogenated carbons to carbonyl, as suggested by the observation of the signals downfield-shifted from 148.6 to 189.5 ppm for C1 and from 145.4 to 189.2 ppm for C4. Overall, the NMR analysis indicates that P* and RIF-OH are linearized RIFMO reaction products, consistent with the reaction described by Koteva et al. for the *S. venezuelae* enzyme.⁵

Taken together, our crystal structure, mass spectra, and NMR data are consistent with the mechanism of Koteva et al.⁵ (Scheme 1). During the reaction, the C1 hydroxyl is deprotonated to facilitate nucleophilic attack at the distal hydroxyl of the reactive RIFMO-C4a-hydroperoxyflavin. The

Scheme 1. Mechanism of RIFMO⁵



C2 hydroxyl of the tetrahedral intermediate thus generated is subject to deamination of the ansa amide from naphthoquinone and results in cleavage of the C2–N1 bond. This generates a semiquinone P* with a carbonyl at C2, which subsequently equilibrates to the final product RIF-OH. Deprotonation of the C1 hydroxyl in the initial step of the mechanism can be facilitated by the hydrogen bond ring system incorporating C1 and C8¹¹ and the donation of a hydrogen bond from the N5 atom of the reduced FAD to O1 of RIF (Figure 2B). Many rifamycin derivatives have the conserved naphthoquinone C1–C4 moiety. Therefore, other rifamycins that are capable of generating the C1 anion could be substrates for RIFMO.

The linearization reaction is consistent with the structure of RIFMO complexed with the RIF, which we recently reported.¹⁰ In the RIFMO–RIF complex, the FAD C4a atom is only 4.7 Å from the new proposed site of hydroxylation on RIF, C2 (Figure 2B). Thus, the structure is consistent with transfer of a hydroxyl group from the RIFMO-C4a-hydroperoxyflavin to C2 of RIF. In contrast, the originally proposed site of hydroxylation, N2', is 7.7 Å from C4a and shielded by an active site loop. Thus, N2' is poorly positioned to react with C4a-hydroperoxyflavin. We previously invoked a conformational change in the protein and/or substrate to rationalize the apparent discrepancy between the structure and the mechanism. The linearization reaction solves this conundrum.

The linearization reaction also provides a plausible and direct explanation for how RIFMO compromises the antibiotic activity of RIF. The binding site for RIF on RNA polymerase is complementary to the oval shape of the macrocycle, and the ansa-bridge makes extensive contacts with the enzyme (Figure 3).¹² For example, the ansa-bridge contacts 12 residues of RNA

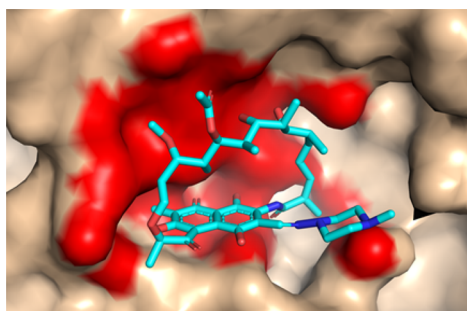


Figure 3. RIF-binding site of RNA polymerase (PDB entry 1I6V). The red surface indicates atoms that contact RIF (4 Å cutoff).

polymerase (4 Å cutoff). Of the 42 atoms of RNA polymerase that contact RIF, 36 contact the ansa-bridge. This binding pose is consistent with studies showing that the two hydroxyl groups of the ansa-bridge are critical for the activity of RIF;¹³ note these two hydroxyls (O21 and O23) are directed toward the polymerase (Figure 3). It seems unlikely that the linearized RIF observed here could adopt the oval conformation of the macrocycle required to make such an intimate and specific association with RNA polymerase. To do so would require a substantial entropy penalty, which would reduce the binding affinity. Furthermore, the originally proposed site of hydroxylation, N2', does not contact RNA polymerase, and it seems unlikely that the addition of a hydroxyl group at this position would substantially decrease the binding affinity (Figure 3). In summary, linearization of the ansa-bridge by RIFMO is a more plausible mechanism of inactivation of RIF than hydroxylation of N2'.

In conclusion, we presented the first direct structural evidence showing the product of the RIFMO reaction. The structure confirms that RIFMO is not an N-hydroxylating enzyme as initially thought. Rather, it is a C-hydroxylating monooxygenase that cleaves the ansa-bridge of rifampicin, compromising the binding affinity of the drug for RNA polymerase.

■ ASSOCIATED CONTENT

● Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.biochem.8b00190.

Experimental Procedures, Tables S1 and S2, and Figures S1–S3 (PDF)

■ AUTHOR INFORMATION

Corresponding Authors

*E-mail: psobrado@vt.edu.

*E-mail: tannerjj@missouri.edu.

ORCID

Yumin Dai: 0000-0002-9228-3671

Pablo Sobrado: 0000-0003-1494-5382

John J. Tanner: 0000-0001-8314-113X

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Notes

The authors declare no competing financial interest.

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