Structural and functional characterization of a ketosteroid transcriptional regulator of *Mycobacterium tuberculosis* *

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*Running title: Characterization of KstR2 from *Mycobacterium tuberculosis

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Background: KstR2 regulates cholesterol catabolic genes in *Mycobacterium tuberculosis*. Results: Dimeric KstR2<sub>Mtb</sub> binds two molecules of HIP-CoA with high affinity. Each binding site spans the subunits and includes residues conserved in TetR family repressors (TFRs) that bind CoA thioesters. Conclusion: HIP-CoA binding to KstR2<sub>Mtb</sub> induces a conformation that abrogates DNA-binding. Significance: The study identifies molecular determinants of cholesterol catabolism and CoA binding in TFRs.

SUMMARY

Catabolism of host cholesterol is critical to *Mycobacterium tuberculosis*’s virulence and is a potential target for novel therapeutics. KstR2, a TetR family repressor (TFR), regulates the expression of 15 genes encoding enzymes that catabolize the last half of the cholesterol molecule, represented by 3αa-H-4α(3'-propanoate)-7αβ-methylhexahydro-1,5-indanedione (HIP). Binding of KstR2 to its operator sequences is relieved upon binding of HIP-CoA. A 1.6 Å resolution crystal structure of the KstR2<sub>Mtb</sub>-HIP-CoA complex reveals that the KstR2<sub>Mtb</sub> dimer accommodates two molecules of HIP-CoA. Each ligand binds in an elongated cleft spanning the dimerization interface such that the HIP and CoA moieties interact with different KstR2<sub>Mtb</sub> protomers. In isothermal titration calorimetry studies, the dimer bound two equivalents of HIP-CoA with high affinity ($K_d = 80±10$ nM) but bound neither HIP nor CoASH. Substitution of Arg-162 or Trp-166, residues that interact respectively with the diphosphate and HIP moieties of HIP-CoA, dramatically decreased KstR2<sub>Mtb</sub>’s affinity for HIP-CoA but not for its operator sequence. The R162M variant’s decreased affinity for HIP-CoA (ΔΔ$G = 13$ kJ mol<sup>-1</sup>) is consistent with the loss of three hydrogen bonds as indicated in the structural data. A 24-bp operator sequence bound two dimers of KstR2. Structural comparisons with a ligand-free rhodococcal homologue and a DNA-bound homologue suggest that HIP-CoA induces conformational changes of the DNA-binding domains of the dimer that preclude their proper positioning in the major groove of DNA. The results provide insight into KstR2-mediated regulation of expression of steroid catabolic genes and the determinants of ligand binding in TFRs.
INTRODUCTION

The ability to degrade host cholesterol is critical to the virulence of Mycobacterium tuberculosis, the causative agent of tuberculosis (1–3). The precise role of cholesterol catabolism in pathogenesis is unknown, although M. tuberculosis’s ability to grow on this steroid as a sole organic substrate suggests that it is a nutritional source during infection. It has also been suggested that cholesterol catabolites play a role in host-pathogen signaling (4). Interestingly, virulent strains of M. tuberculosis induce cholesterol synthesis in macrophages (5) and the pathogen sequesters the steroid from cholesterol-rich granulomas (6). The importance of cholesterol catabolism for virulence and its potential as a target for urgently needed novel therapeutics is highlighted by the recent isolation of compounds that inhibit the intracellular growth of M. tuberculosis by targeting various cholesterol catabolic enzymes (VanderVen et al., submitted).

The cholesterol catabolic pathway of M. tuberculosis was first discovered in Rhodococcus jostii RHA1 and appears to be almost ubiquitous in mycolic-acid producing Actinobacteria (7). The pathway is encoded by a cluster of ~80 genes and degradation is organized according to the structure of the four-ringed steroid molecule: side chain, rings A/B and rings C/D (1). Degradation of the side chain and rings A/B, respectively, occur concurrently to yield 2-hydroxy-hexa-2,4-dienoate (HHD) and 3αα-H-4α(3′-propanoate)-7αβ-methyl-hexahydro-1,5-indanedione (HIP), the latter of which retains steroid rings C/D (1,8,9) (Fig. 1A). HIP catabolism is largely uncharacterized, but is initiated by FadD3, an acyl-CoA synthetase that transforms the substrate to HIP-CoA (10,11). Our recent results indicate that the KstR2 regulon, which includes fadD3, encodes HIP catabolic enzymes (12).

KstR2 (Rv3557c) is one of two TetR family repressors (TFRs) involved in regulating the cholesterol catabolic genes in mycolic-acid-producing Actinobacteria (13). The other one, KstR (Rv3574), is involved in regulating the transcription of genes involved in cholesterol uptake and degradation of the side chain and rings A/B (13,14). KstR2 regulates the expression of 15 genes (Fig. 1B), including fadD3 and ipdAB. IpdAB is required for virulence in Rhodococcus equi and an ipdAB deletion strain has been patented as a live vaccine (13,15). KstR2 binds to three ~14 bp inverted palindromic operator sequences, or KstR2 boxes, located at the intergenic regions of the regulon (13). Casabon et al. recently demonstrated that binding of KstR2Mb to its operator sequences is relieved by HIP-CoA (12), the product of the FadD3-catalyzed reaction (10). In contrast, HIP, CoASH and a variety of cholesterol metabolites did not relieve KstR2 binding to DNA.

TFRs are named after a founding member involved in regulating tetracycline resistance (16) and is one of the most widely distributed families of transcriptional regulators in bacteria (17-19). Despite significant sequence variation, the proteins form a conserved L-shaped α-helical structure featuring an N-terminal DNA-binding domain (DBD) and a larger C-terminal effector-binding domain (EBD) (17). The DBD contains a helix-turn-helix motif involved in binding to operator DNA. TetR protomers associate into homodimers or higher oligomers that recognize palindromic sequences in the operator DNA. More specifically, an α-helix of the TetR DBD called the recognition helix forms specific electrostatic and aromatic contacts in the major groove of the DNA of the operator sequence (17). The vast majority of TFRs are repressors in the absence of their effector, while the binding of the effector triggers a conformational change that shifts the position of the recognition helix, resulting in release of operator DNA by the regulator. Despite a generally conserved structure and mechanism of action, the specific position of the ligand-binding pocket in TFRs and its chemical composition vary dramatically, resulting in specific responses to a vast assortment of small molecules. This variation also makes it difficult to predict the chemical nature of the cognate ligand, necessitating the characterization of individual family members.

Herein, we used a combination of isothermal titration calorimetry (ITC), electrophoretic mobility assays (EMSA), X-ray crystallography and directed mutagenesis to characterize the molecular function of KstR2 from M. tuberculosis, KstR2Mb. The data define interactions between KstR2 and its effector, HIP-CoA, and provide
insights into the function of this regulator in the bacterial catabolism of steroids as well as into TFRs in general.

MATERIALS AND METHODS

Chemicals and Reagents - ATP, CoASH, and cholesterol (>99%) were purchased from Sigma-Aldrich. NdeI and HindIII Fast Digest restriction enzymes were purchased from Thermo Fisher Scientific Inc. T7 DNA Ligase and DpnI were purchased from New England Biolabs. Oligonucleotides were ordered from Integrated DNA Technologies. FadD3 and poly-His-tagged tobacco etch virus protease (TEV Pro) were produced as previously described (10,20). Water for buffers was purified using a Barnstead Nanopure Diamond™ system to a resistivity of at least 18 MΩ. Reagents were of HPLC or analytical grade.

DNA manipulation – Plasmid DNA was manipulated and propagated using standard procedures (21). Oligonucleotide-directed mutagenesis was performed using the Quickchange™ PCR protocol with slight modifications. Briefly, a single 5’ phosphorylated mutagenic DNA primer was annealed to pETKstR2 carrying a gene encoding poly-His tagged (Ht-)KstR2 Mtb (12), then amplified using Phusion DNA Polymerase. T7 DNA ligase was added to the reaction mixture to form single stranded mutagenized plasmid DNA. Template DNA was removed using DpnI and the remaining ssDNA was electroporated into *Escherichia coli* NovaBlue. The R162M and W166L variants were producing using primers with the following respective nucleotide sequences: 5’-pGTCTACCGATTCATCATGACACCACCTGGGTG-3’ and 5’-pCATCCGTGACACCACCCTCGTGCGGT GCGCTGG-3’. The nucleotide sequences of variant kstR2 were confirmed.

Purification of KstR2 - Wild-type and variant KstR2<sub>Mtb</sub> were produced using *E. coli* Rosetta 2(DE3)pLysS carrying the appropriate derivative of pETKstR2 as previously described (12). The proteins were purified as previously described (12) with the following modification. The affinity-purified Ht-KstR2<sub>Mtb</sub> was dialyzed overnight against cleavage buffer (25 mM HEPES, pH 7.5, 50 mM KCl, 1 mM DTT, and 0.5 mM EDTA). The affinity tag was removed by incubating ~100 mg of Ht-KstR2<sub>Mtb</sub> with 0.5 mg TEV<sup>Pro</sup> in 10 ml cleavage buffer overnight at 4°C. Complete digestion was confirmed by SDS-PAGE analysis. TEV<sup>Pro</sup>-digested KstR2<sub>Mtb</sub> was loaded onto Mono-Q 10/100 HR (GE Healthcare) and eluted as previously described (12). Proteins were exchanged into 25 mM HEPES, pH 7.5, 50 mM KCl, concentrated to ~20 mg ml<sup>-1</sup> and flash frozen in liquid nitrogen as beads. Typically, 50 mg of protein were purified per 1 l culture. Protein concentrations were measured using the bicinchoninic acid (BCA) protein assay with bovine serum albumin as a standard.

Preparation of HIP and HIP-CoA - HIP was obtained using a ΔfadD3 mutant of RHA1 as previously described (12). HIP-CoA was produced by incubating 2 mM HIP with 2.25 mM ATP, 2.25 mM CoASH, 5 mM MgCl<sub>2</sub> and 5 μM FadD3 in 800 μl 25 mM HEPES, pH 7.5, 50 mM KCl for 30 min. HIP-CoA was purified at room temperature by high-performance liquid chromatography (HPLC) using a Luna 3 μm PFP(2) 50 × 4.6 mm column (Phenomenex) in 100 mM ammonium acetate, pH 4.5 at 1 ml min<sup>-1</sup> over a 20 ml linear gradient of 0-90% methanol. HIP-CoA containing fractions were pooled and methanol was removed under nitrogen. HIP-CoA was purified to >95% purity and its identity was confirmed by ESI-MS as described (12). HPLC purified HIP-CoA was desalted using a Strata-X 33u 30 mg solid phase extraction (SPE) column (Phenomenex). The SPE column was equilibrated with 1 ml methanol then 1 ml water. The HIP-CoA solution was passed through the column, washed with 1 ml water, and eluted in 100% methanol. HIP-CoA for co-crystallization was produced as described above but in a final volume of 6 ml (2.9 mg HIP). HPLC-eluted fractions containing high concentrations of HIP-CoA were desalted on the SPE column, dried under nitrogen, suspended in 250 μl water and lyophilized overnight. The residue was suspended in 50 μl water to a final concentration of 62 mM.
Isothermal titration calorimetry (ITC) - ITC experiments were performed using an ITC200 instrument (GE Healthcare) operated at 25°C and a stirring speed of 1000 rpm. Titrations were performed using 25 mM HEPES, pH 7.5, 50 mM KCl. KstR2<sub>Mtb</sub> (20 μM or 40 μM variant) was titrated with 40 × 1 μl injections of HIP-CoA (200 or 400 μM). For HIP and CoASH, 40 μM KstR2<sub>Mtb</sub> was titrated with 20 × 4 μl injections of 400 μM titrant. Injections of buffer into KstR2<sub>Mtb</sub> and variants showed no significant background heats. The data were processed by subtracting the background heats and removing outlier data points. One- and two-site models were fit using Origin 7.0. Experiments were independently repeated at least three times.

Crystallization of KstR2<sub>Mtb</sub>·HIP-CoA - Crystals of the KstR2<sub>Mtb</sub>·HIP-CoA complex were obtained by mixing 2 μl of 48 mg ml<sup>-1</sup> protein with HIP-CoA at final concentration of 1 mM and 2 μl of reservoir solution (0.2 M ammonium sulfate, 0.1 M bis-Tris, pH 5.5 and 25% (w/v) PEG3350) using the hanging drop vapor diffusion method. Crystals appeared at room temperature and were flash frozen in liquid nitrogen after being cryoprotected with paratone oil. X-ray diffraction data was collected at 100 K using a Rigaku HomeLab system featuring Micromax-007 HF rotating copper anode fitted with a Rigaku R-AXIS IV++ image plate detector. Diffraction data was processed and reduced using the HKL-3000 software package (22). The crystal structure was solved by molecular replacement using MrBump from the CCP4 software package (23) with the structure of KstR2<sub>RHA1</sub> (Ro04598) from R. jostii RHA1 (PDB 2IBD) as a search query. Structure refinement was carried out using Phenix.refine (24) and Coot (25). Geometry was verified using Phenix.refine, Coot and the RCSB PDB Validation server. The final asymmetric unit (AU) contains one copy of the KstR2<sub>Mtb</sub> protein chain encompassing residues 3-198. The presence of one copy of HIP-CoA in the AU was verified using simulated annealing (Cartesian) omit maps using Phenix.refine with default parameters, followed by model building into residual positive F<sub>c</sub>-F<sub>c</sub> density and occupancy refinement of HIP-CoA.

Structural analysis - The PDBEPISTA server was used to analyze inter-protomeric and protein-ligand interactions (26). The DaliLite and PDBEFold servers were utilized for structure comparisons (27,28). Electrostatic surfaces were analyzed using Chimera (29). Binding cavity properties were analyzed using the CASTp server (30).

Electrophoretic mobility shift assays (EMSA) - A dsDNA probe of the KstR2<sub>Mtb</sub> operator sequence located in the intergenic region of rv3557c and rv3558 was prepared by heating complementary ssDNA oligomers to 95°C and annealing at room temperature in 20 mM Tris-HCl, pH 8.0, 10 mM MgCl<sub>2</sub>, and 75 mM NaCl as previously described (12). DNA probes were labelled with DIG-11-ddUTP using the second generation DIG gel shift kit from Roche according to the manufacturer’s protocol. Binding assays contained 0-2 pmol KstR2<sub>Mtb</sub> (WT or variant), 0.04 pmol DIG-labelled DNA probe and 0-10 nmol HIP-CoA in 20 μl 20 mM HEPES, pH 7.6, 10 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 1 mM DTT, 0.2% (w/v) Tween 20, 30 mM KCl, and 1 mM EDTA. Assays were incubated for 30 min at 37°C then loaded onto 9% polyacrylamide gels containing 0.5× TBE. Gels were run for 45 min at 105 V then blotted onto positively charged Hybond-N+ nylon membranes (GE Healthcare). DNA was viewed using anti-DIG-alkaline phosphatase and chemiluminescent substrate, CSPD, as described by the manufacturer (Roche). Sequences of DNA probes were 5’-GGTAGC-GCGTACCAAGCAAGTGCTTGCTTA-3’ and 5’-GCTACCTAAGCAAGCACTTGCTTGGTGACG-3’.

Size exclusion chromatography – The oligomeric state of KstR2 was analyzed using size exclusion chromatography multi-angular light scattering (SEC-MALS). Twenty five μl of 80 μM KstR2<sub>Mtb</sub> was injected onto a HPLC 1260 Infinity LC (Agilent Technologies) coupled to a Superdex 200 5/150 column (GE Healthcare). A second sample was incubated at 37°C for 30 min with 20 μM of a 24-bp DNA fragment representing the KstR2 operator from the intergenic region of rv3549c/rv3550 (13). SEC-MALS was operated at 0.2 ml/min in 25 mM HEPES, 50 mM KCl, pH 7.5. Data were collected using a miniDAWN TREOS multi-angle static light scattering device.
and an Optilab T-rEX refractive index detector (Wyatt Technologies). The molecular weight of complexes was determined using the ASTRA6 program (Wyatt Technologies). The nucleotide sequences of the oligonucleotides used to generate the DNA fragment were 5’-ACCTAAGCAAGC- ACTTGCTTGGTA-3’ and its complement. Oligonucleotides were HPLC-purified by the manufacturer (Integrated DNA Technologies) and annealed as described above in 25 mM HEPES, 50 mM KCl, pH 7.5.

RESULTS

KstR2Mtx binds HIP-CoA with high affinity - We have previously shown that HIP-CoA is the chemical effector of KstR2Mtx, relieving the binding of the repressor to its operator DNA upon interaction with this molecule (12). We performed ITC to better analyze the interaction of KstR2Mtx with its effector. The binding was exothermic and driven by enthalpy with an unfavorable entropic contribution (Fig. 2, Table 2). The one-site equation best fit the binding isotherms. No cooperativity was detected and attempts to model the data using a two-site equation yielded poor fits. Replicate titration curves were centered at a mole ratio of 0.89 consistent with a one-to-one stoichiometry between the KstR2Mtx protomer and HIP-CoA. Under the experimental conditions, the $K_d$ was 80 ± 10 nM (25 mM HEPES, pH 7.5, 50 mM KCl).

We then used ITC to test whether KstR2Mtx binds either HIP or CoASH. Titrations of 400 μM HIP or CoASH into 40 μM KstR2Mtx (Fig. 2B and C, respectively) gave heats that were slightly above background. However, neither compound yielded a titration curve. Increasing the concentrations to 1 mM titrant and 100 μM KstR2Mtx generated a proportional increase in measured heats for both compounds and no titration of CoASH. Titrations at 1 mM HIP were unreliable due to precipitation of KstR2Mtx during titration.

Structure of KstR2Mtx·HIP-CoA reveals an effector binding cleft spanning the two protomers of the dimer - To further understand the molecular function of KstR2Mtx, we crystallized KstR2Mtx in the presence of HIP-CoA and solved the structure of the complex. The asymmetric unit of the KstR2Mtx·HIP-CoA structure contained a single protomer of KstR2Mtx associated with one molecule of HIP-CoA (Fig. 3) in agreement with our ITC analysis. Structure determination statistics are presented in Table 1.

Similarly to previously characterized TFRs, the KstR2Mtx protomer adopted an all α-helical L-shaped fold covering approximately 25 × 38 × 57 Å (Fig. 3A). The short axis of the protomer comprises the N-terminal DBD (residues 6 to 54) with most α-helices in this domain arranged perpendicular to the long axis of the protein representing the C-terminal EBD (residues 55-198) (Fig. 3A). The two domains are connected by a kinked α-helix (α6) with one face of this helix interacting with the DBD and the other with the EBD.

The KstR2Mtx protomer formed an extended interface of 1687 Å$^2$ with an adjacent KstR2Mtx protomer related to the first by a crystallographic two-fold symmetry axis. This arrangement likely represents the biological dimer, the typical minimal oligomeric state of TFRs. Dimerization of KstR2Mtx is mediated by contacts between 24 residues, 15 of which are hydrophobic, belonging to α-helices α8 and α9 of the C-terminal domain of each protomer.

The electron density corresponding to the HIP-CoA molecule (Fig. 3B) occupies a large extended cavity (2637 Å$^2$ in surface area) that spans the two KstR2Mtx protomers and that sequesters over half of the ligand molecule from the solvent. This cavity, whose shape and chemical nature closely complement that of the ligand, is composed of two elements: a positively charged pocket lined by helices α8, α9, and their connecting loop in one protomer that binds the adenosine moiety; and a deep hydrophobic pocket defined by helices α4', α5', α6', α7' and α8' in the second protomer that binds the HIP moiety (' identifies elements of the second protomer). Thus, each HIP-CoA molecule binds across the KstR2Mtx dimer interface (Fig. 3), and the two binding clefts are independent of each other. Indeed, the two ligands approach no closer than 7.6 Å.
A total of 23 amino acids from each KstR2<sub>Mtb</sub> protomer interact with the HIP·CoA molecule. The adenine moiety of the ligand is anchored primarily through interactions with residues belonging to the loop connecting helices α8 and α9. The diphosphates of the CoA moiety are stabilized by four hydrogen bonds, including three to the loop connecting helices α8 and α9. The cycloalkanone rings of the HIP moiety form stacking interactions with the aromatic side chains of Trp-166 (from α8) and Tyr-108 (from α6) that make up the deep hydrophobic pocket. In addition, the HIP moiety forms many hydrophobic interactions with side chains of residues that line the pocket: Phe-65', Leu-66', Leu-69', Phe-70', Tyr-73' and Val-105'. Finally, the 5-carbonyl oxygen forms a hydrogen bond with the side chain of Gln-109'. The high number of protein-ligand contacts and the close complementarity between the chemical environment of KstR2<sub>Mtb</sub>’s binding cleft and the specific chemical groups of the ligand suggests that KstR2<sub>Mtb</sub> is highly specific toward HIP·CoA. This is in line with the biophysical characterization of KstR2<sub>Mtb</sub> interactions with this ligand presented above.

**Binding of HIP·CoA alters the conformation of KstR2** - Effector-binding typically triggers conformational changes in TFRs. To evaluate whether HIP·CoA binding triggers similar changes in KstR2<sub>Mtb</sub>, we sought to structurally characterize the ligand-free form of the regulator. Attempts to obtain crystals of KstR2<sub>Mtb</sub> in the absence of HIP·CoA were unsuccessful. However, a structure of the ligand-free form of KstR2<sub>RHA1</sub> from *R. jostii* RHA1 is available (PDB 2IBD). KstR2<sub>RHA1</sub> shares 59% amino acid sequence identity with KstR2<sub>Mtb</sub> including 19 of the 23 residues that interact with HIP·CoA. A superposition of the KstR2<sub>Mtb</sub>·HIP·CoA complex and KstR2<sub>RHA1</sub> structures yielded an RMSD of 1.5 Å over 186 matching Cα atoms (Fig. 5A) facilitating the identification of potential DNA-binding secondary structure elements in KstR2<sub>Mtb</sub>. As is characteristic of TFRs, the SlmA dimer formed symmetric contacts in adjacent major grooves of its operator DNA. The closest suitable match retrieved by our PDB search was SlmA from *Vibrio cholera* (PDB: 4GCT (31)). SlmA and KstR2<sub>Mtb</sub> protomers superimposed with RMSD 2.8 Å over 179 matching Cα atoms (Fig. 5A) facilitating the identification of potential DNA-binding secondary structure elements in KstR2<sub>Mtb</sub>. As is characteristic of TFRs, the SlmA dimer formed symmetric contacts in adjacent major grooves of its operator DNA (i.e., Val-33, Ser-44 and Tyr-48). Importantly, the relative position of the recognition helices differed dramatically in the operator-bound SlmA and ligand-bound KstR2<sub>Mtb</sub> dimers (Fig. 5B): in KstR2<sub>Mtb</sub>·HIP·CoA, this helix is positioned further away from the DNA major groove. Overall, these analyses suggest that the conformation of the DBD domain in KstR2<sub>Mtb</sub>·HIP·CoA is not compatible with binding to its operator. This further suggests that HIP·CoA regulates the DNA-binding activity of KstR2 in the same manner as that established for other TFRs where effector-binding of induces
conformational changes that result in relieving the binding of the TFR to its operator DNA.

**Functional validation of KstR2\textsubscript{Mb}-HIP-CoA interactions** - To functionally validate the KstR2\textsubscript{Mb}-HIP-CoA structural model, two key HIP-CoA binding residues were individually substituted using directed mutagenesis and the resulting KstR2\textsubscript{Mb} variants were characterized using ITC and EMSA. More specifically, the structural data indicate that Arg-162 and Trp-166 form important interactions with HIP-CoA (Fig. 3B) and that their substitution with methionine and leucine, respectively, should disrupt the binding of KstR2\textsubscript{Mb} to its effector but not to its operator DNA.

Isotherms showed that both KstR2 variants were significantly impaired with respect to HIP-CoA binding. The R162M variant bound HIP-CoA with an affinity ~200 times lower than WT (Table 2). Like WT, HIP-CoA binding to the R162M variant was enthalpically driven with an unfavorable entropic contribution (Table 2). Unlike WT, the isotherm of the R162M variant showed a shallow titration curve consistent with the variant not being saturated at a 3-fold molar excess of HIP-CoA (Fig. 6A). The one-site equation fit poorly to the isotherm, yielding a stoichiometry of N=1.77. The W166L variant showed no titration with HIP-CoA: the generated heats were equal to background (Fig. 6B).

Using EMSA, both the R162M and W166L variants bound to the KstR2 operator sequence with comparable affinity as WT KstR2\textsubscript{Mb} (Fig. 7). More specifically, WT and variants formed DNA:protein complexes at a mole ratio of 1:1 and no protein-free DNA probe was detected at a ratio of 1:50 DNA:protein. Consistent with previous results, the binding of DNA by WT KstR2\textsubscript{Mb} was relieved in the presence of 50 μM HIP-CoA. Consistent with the ITC results, 500 μM HIP-CoA was required to detectable relieve binding to DNA by R162M. Moreover, the W166L-DNA complex was not detectably disrupted even at high concentrations of HIP-CoA.

*A KstR2 operator sequence binds two KstR2\textsubscript{Mb} dimers* - The oligomeric state of KstR2\textsubscript{Mb} was investigated using SEC-MALS. KstR2\textsubscript{Mb}, eluted as a single peak ($t_R = 10.2$ min; 25 mM HEPES, 50 mM KCl, pH 7.5) with a molecular weight of 42.9 ± 0.2 kDa determined using the Rayleigh ratio (Fig. 8). This is within 10% of the predicted mass of the KstR2\textsubscript{Mb} dimer (46 kDa). The small discrepancy between the two may be partly due to the elongated structure of KstR2\textsubscript{Mb}; the ASTRA6 software calculates molecular weight from molecular radius using spherical structures. To investigate the oligomeric state of KstR2\textsubscript{Mb} bound to its operator, a DNA fragment containing a 14-bp KstR2 box flanked by 5 bp on either side was synthesized. A sample of KstR2\textsubscript{Mb} incubated with this 24-bp fragment yielded a single protein-containing peak ($t_R = 8.5$ min) with a molecular weight of 97 ± 1 kDa. This is within 10% of the molecular weight predicted for a complex of two KstR2\textsubscript{Mb} dimers and one DNA fragment (106 kDa). The DNA fragment alone (14.7 kDa) eluted at 10 min and caused negligible light scattering (data not shown).

**DISCUSSION**

This study provides the first molecular insights into the KstR2-mediated regulation of the expression of steroid catabolic genes in *M. tuberculosis* and other Actinobacteria. The structural and titration data establish that the KstR2 dimer binds 2 molecules of HIP-CoA. Each HIP-CoA molecule binds in a deep cleft that spans the KstR2 dimer with the adenosine and HIP moieties bound by separate protomers. The extensive electrostatic and hydrophobic interactions that mediate the binding of HIP-CoA to KstR2\textsubscript{Mb} are corroborated by the high affinity of the regulator for its effector molecule. The functional significance of the KstR2\textsubscript{Mb}-HIP-CoA structure was further validated by directed mutagenesis, which established that Arg-162 and Trp-166 contribute significantly to the binding of HIP-CoA while minimally affecting the binding of the operator DNA. Finally, comparison of the KstR2\textsubscript{Mb}-HIP-CoA, KstR2\textsubscript{RHA1} and SlmA-DNA structures suggests how effector binding alters the conformation of the regulator to relieve binding of the operator DNA.

The amino acid sequence conservation among KstR2 orthologs in steroid-degrading Actinobacteria further validates the functional importance of the residues identified in the
KstR$_{\text{Mtb}}$-HIP-CoA structure. These orthologs share ~50% amino acid sequence identity with higher conservation among DBD residues predicted to bind the operator DNA and EBD residues that bind HIP-CoA. Specifically, the residues located on helices α2 and 3 in KstR$_{\text{Mtb}}$ are all conserved with the exception of Gly-39. This is consistent with the conserved nucleotide sequence of the operator across Actinobacteria (13). Similarly, 19 of 23 residues that contact the sequence of the operator across Actinobacteria are all conserved with the exception of Gly-39. This is consistent with the conserved nucleotide sequence of the operator across Actinobacteria (13). Similarly, 19 of 23 residues that contact the effector in the KstR$_{\text{Mtb}}$-HIP-CoA complex, located between residues 138 to 195, are conserved in KstR2 orthologs.

The occurrence of a single KstR2 regulon in Actinobacteria in strains that contain several distinct steroid catabolic pathways (7,32) suggests that hydroxylated HIP-CoA can act as the effector of at least some KstR2 orthologs. For example, R. jostii RHA1 possesses at least three distinct pathways that converge on the HIP catabolic pathway, two of which are responsible for cholesterol and bile acid catabolism, respectively (32). The catabolism of bile acids such as cholate and bile acid catabolism, respectively (32). The catabolism of bile acids such as cholate results in the production of 3’OH HIP (10), suggesting that 3’OH HIP-CoA would be an effector of KstR2$_{\text{RHA1}}$. Inspection of the KstR$_{\text{Mtb}}$-HIP-CoA structure indicates that there is sufficient space adjacent to C3’ of the HIP moiety to accommodate a hydroxyl group. Moreover, all of the residues within a radius of 6 Å of C3’ are conserved in KstR$_{\text{RHA1}}$(Fig 3B). By contrast, the binding of 7β-OH HIP-CoA is predicted to be sterically hindered by Tyr-108 (Tyr-112 in KstR$_{\text{RHA1}}$). This is consistent with the finding that C12 hydroxyl groups of bile acids (corresponding to C7 of HIP) are removed prior to rings C/D degradation in Actinobacteria (10,11). By contrast, 7β-OH HIP is produced in steroid-degrading Gram negative bacteria such as Pseudomonas putida DOC21 (33). However, the HIP catabolic genes in these strains appear to be regulated by a LuxR-type transcriptional repressor (33,34).

The affinity of KstR$_{\text{Mtb}}$ for its effector is in line with what has been reported for other TFRs that bind CoA thioester effectors to regulate catabolic genes. Thus, PaaR and FadR from Thermus thermophilus HB8 are involved in the catabolism of phenylacetate (PAA) and fatty acids, respectively, and bind PAA-CoA and lauroyl-CoA with $K_d$ values of 24 and 90 nM, respectively (35-37). The regulation of HIP, PAA and fatty acid catabolism share a common logic since catabolism is initiated by an acyl-CoA synthetase (ligase) whose product is the effector for the corresponding TFR in the pathway. TFRs that bind smaller molecules typically have dissociation constants in the 1-10 μM range (18,38). The tighter binding of the CoA thioesters is consistent with the increased number of protein-ligand interactions afforded by the CoA moiety. An interesting exception is DesT, which binds palmitoyl-CoA with a $K_d$ value of ~3 μM (39). However, DesT regulates the unsaturated:saturated ratio of acyl chains in lipid bilayers and binds the acyl-CoA in a fundamentally different way than the other TFRs.

Comparison of the structures of FadRs from Thermus thermophilus (3ANP, 3ANG; (36)) and Bacillus subtilis (1V10; (40)) in complex with fatty acyl-CoA are strikingly similar to the KstR$_{\text{Mtb}}$-HIP-CoA complex: in each, the ligand is bound in a cleft that spans the two protomers with the fatty acid and adenosine moieties bound to separate chains. Conserved residues include Arg-159 and Arg-173 (KstR2 numbering) that interact with the adenine and Arg-162’ which hydrogen bonds with the diphosphate moiety of CoA. Nevertheless, the adenine ring in the FadR-fatty acyl-CoA complexes is flipped 180° with respect to that in the KstR$_{\text{Mtb}}$-HIP-CoA complex and oriented perpendicular to the dimer’s rotational axis. By contrast, the fatty acyl-CoA binds in a different way in the DesT complexes, with the CoA moiety at the top of the EBD and the acyl chain extending down a channel, parallel to the α-helices of the EBD (39). Consistent with the different binding mode, none of the Arg residues are conserved in DesT. The three conserved Arg residues are also not conserved in TFRs that bind smaller effectors, such as QacR of Staphylococcus aureus (PDB 1JT6), Pseudomonas putida TtgR (PDB 2UXI), and Streptomyces coelicolor ActR (PDB 3B6A).

Conservation of the CoA-binding residues in the KstR2 and FadR may be extended to other TFRs to gain insight into their respective effectors. Comparison of sequence topology maps of 48 structures of TFRs identified in Yu et al. (2010)
indicate that 13 contain at least two of the three conserved basic residues in orientations permissive to CoA binding (18). Of note, Fad35R of M. tuberculosis (PDB 4G12) regulates the expression of fad35, which encodes an acetyl-CoA synthetase involved in fatty acid degradation (41). Although Fad35R binds tetracycline, other evidence suggested that a fatty acyl-CoA could be the physiological effector (41). The presence of Lys-184 on the α8-α9 loop, Arg-166 on the apical end of helix α8, and His-170 in the middle of helix α8 supports this hypothesis. T. thermophilus HB8 FfmR (PDB 3VPR), which is predicted to regulate PAA or fatty acid catabolism possess basic residues on the α8-α9 loop and middle of helix α8 (Arg-163 and Arg-149, respectively) but lacks a basic residue at the top of helix α8. Although the respective effectors of these 13 TFRs have yet to be identified, it appears that a significant subset of TFRs bind CoA thioesters.

Our model predicts that the effector of M. tuberculosis KstR (PDB 3MNL) is also a CoA thioester. KstR\textsubscript{Mtb} and KstR\textsubscript{Mtb} share only 18% amino acid sequence identity. However, the conserved residues include four that mediate CoA binding in KstR2 (Arg-162, Asp-163, Trp-171 and Arg-173) and two that mediate steroid binding (Trp-166 and Phe-70). Although the effector of Kst\textsubscript{Mtb} has yet to be identified, the possibility that it is a CoA thioester is consistent with cholesterol catabolism. More particularly, C27 of the steroid’s alkyl side chain is oxidized to a carboxylate by Cyp125 or 142 (4) and is then thioesterified by FadD19 (42). It is unclear whether these transformations occur prior to oxidation of the 3β-hydroxyl. Nevertheless, the role of a cholesterol-26-oyl-CoA as the effector of Kst\textsubscript{Mtb} would mirror the regulatory logic of the KstR2 regulon in M. tuberculosis in that the catabolic genes are induced by the first CoA thioester produced. Interestingly, 3-oxo-4-cholesten-26-oic acid was identified as the effector of KstR of M. smegmatis (43). It seems unlikely that the two KstR orthologs have different effectors considering that they share 87% amino acid sequence identity, including the four residues predicted to interact with the CoA moiety. Indeed, Garcia-Fernandez et al. recognized the possibility that the physiological effector of KstR is a CoA thioester but were unable to test this hypothesis (43).

The proposed mechanism of response of KstR\textsubscript{Mtb} to HIP-CoA is similar to what has been proposed for other TFRs where the binding of a small molecule effector induces conformational changes that abrogate the regulator’s interactions with its operator DNA (17,18). In the absence of a KstR\textsubscript{Mtb}-DNA structure, the comparative analysis between the KstR\textsubscript{Mtb}-HIP-CoA and SlmA-DNA complexes unveiled differences in the relative conformation of DBD domains in the TFRs that, in case of KstR\textsubscript{Mtb}, are likely triggered by HIP-CoA binding. More specifically, HIP-CoA binding to KstR\textsubscript{Mtb} repositions helices α4 and α6 causing a net 15° outward rotation of DBD helix α1, displacing helices α2 and α3 compared to corresponding elements in SlmA-DNA complex (Fig 4). Interestingly, Tyr-48 and His-50 on DBD helix α3 in KstR\textsubscript{Mtb}-HIP-CoA are rotated outward by ~10 Å as compared to the ligand-free structure of KstR\textsubscript{RHA1} (Fig 4). These residues are highly conserved in TFRs and, in E. coli TetR, directly interact with the bases in the major groove of the DNA (18). Lastly, Lys-54, which is also highly conserved in TFRs (18), is displaced by ~7 Å in KstR\textsubscript{Mtb}-HIP-CoA compared to KstR\textsubscript{RHA1}. The effect of HIP-CoA on KstR2 is also remarkably similar to the displacement of helix α4 and outward rotation of helix α3 observed in apo- vs. ligand bound B. subtilis FadR (35). In TetR, effector binding thermodynamically stabilizes the DBD in a conformation that is incompatible with DNA binding, preventing the DBD from assuming a conformation that is competent for DNA binding (44). The inability of KstR\textsubscript{Mtb}-HIP-CoA to bind DNA likely has the same mechanistic origin.

The binding of two KstR\textsubscript{Mtb} dimers to its operator was somewhat unexpected considering that the KstR box of 14 bp (13) is too short to accommodate four recognition helices in the major groove. Typically the TFR operators that bind two dimers are at least 22 bp in length (31,45-47). By contrast, TFRs that bind operator sequences of less than 17 bp bind as a single dimer (46). It is possible that the KstR2 box extends beyond the 14 bp identified by Kendall et al. (13). Alternatively, the KstR2 dimers may bind opposite sides of the DNA helix, as in the SlmA-DNA complex (PDB: 4CGT). In such a scenario, the first KstR dimer induces a conformational change in the DNA that facilitates the binding of the second dimer to a
non-canonical sequence. Intriguingly, KstR2<sub>Mtb</sub> contains the Arg-X-Thr motif that is present in SlmA and induces a kink in the DNA (31). Similar models have been invoked to explain cooperative binding in each of two other TFRs, QacR and CprB (46,47), although QacR lacks the Arg residue and does not induce a kink in the DNA. Interestingly, single KstR2 boxes occur between divergently transcribed promoters (13) and gel shift assays indicate that KstR2 binds to each of the three boxes with the same stoichiometry (12). The binding of two KstR2 dimers to opposite sides of the DNA helix may enable the repressor to act at both promoters. Additional experiments and structural data are required to determine the precise architecture of the KstR2<sub>Mtb</sub>-operator complexes and how this regulates divergently transcribed promoters.

This first crystal structure of a steroid catabolite-bound TFR provides general insights into the regulation of steroid catabolism in Actinobacteria. Significantly, the catabolism of HIP is poorly elucidated despite the occurrence of the virulence factors such as ipdA in the pathway. Further elucidation of HIP catabolism and its regulation should facilitate the development of novel therapeutics to treat tuberculosis.

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FIGURE LEGENDS

FIGURE 1. Cholesterol catabolism and the KstR2 regulon of *M. tuberculosis*. A. Cholesterol is catabolized via concurrent β-oxidation of the side chain and O2-dependent cleavage of rings A/B yielding 3αα-H-4α(3’-propanoate)-7αβ-methylhexahydro-1,5-indanedione (HIP). HIP is thioesterified to HIP-CoA by FadD3 and subsequently catabolized by enzymes encoded by the KstR2 regulon. B. The KstR2 regulon of *M. tuberculosis*. Genes in black are up-regulated in the presence of cholesterol. Grey boxes indicate KstR2 binding sites.

FIGURE 2. Representative isotherms of potential KstR2*mb* ligands. A. Titration of 20 μM KstR2*mb* with aliquots of 200 μM HIP-CoA. B. Corresponding isotherm with the best fit single binding site model. C. Titration of 40 μM KstR2*mb* with aliquots of 400 μM CoASH. D. Titration of 40 μM KstR2*mb* with aliquots of 400 μM HIP. Titrations were performed using 25 mM HEPES 50 mM KCl, pH 7.5 at 25°C.

FIGURE 3. Crystal structure of KstR2*mb*-HIP-CoA complex. A. Overall structure. Chain A from the crystal’s AU is colored dark blue and chain B, produced by crystallographic symmetry, is colored light blue. HIP-CoA is shown in pink sticks. B. Details of interactions between KstR2*mb* and HIP-CoA. The shown electron density is the Fc-Fc simulated annealing map calculated in the absence of HIP-CoA. Dashes indicate hydrogen bonds; black circle, the non-crystallographic symmetry axis; and dashed arrow, the closest approach between the dimer-bound ligands.
FIGURE 4. Conformational differences between KstR2<sub>Mtb</sub>·HIP-CoA and ligand-free KstR2<sub>RHA1</sub>. KstR2<sub>Mtb</sub>·HIP-CoA and ligand-free KstR2<sub>RHA1</sub> are blue and brown, respectively. Chains A and B are dark and light shades, respectively. HIP-CoA is shown as pink sticks. A. Overall comparison of the two conformations. Helices α2 and α3 are the putative DNA binding elements (helix-turn-helix motif). Inset shows a detail of the helix-turn-helix motif and the positional difference of a representative residue (KstR2<sub>Mtb</sub> Leu-43' / KstR2<sub>RHA1</sub> Leu-47'). B. Localized conformational changes upon HIP-CoA binding. Single chains of the KstR2 dimer are shown for clarity. The positions of helices α1, α4, α6 and α8 differ between ligand-bound and ligand-free KstR2 structures.

FIGURE 5. Comparison of KstR2 structures with a TFR·DNA complex. Superposition of KstR2<sub>Mtb</sub>·HIP-CoA (blue), KstR2<sub>RHA1</sub> (red) and SlmA (green, PDB 4GCT) bound to its operator DNA. Superposition performed on subunit chains colored white, which adopt the same conformation. The recognition helices (α2), which adopt different positions, are shown as cylinders.

FIGURE 6. Isotherms of KstR2<sub>Mtb</sub> variants. A. and B. show representative titrations of 400 μM R162M and W166L, respectively, with aliquots of 40 μM HIP-CoA (25 mM HEPES, 50 mM KCl, pH 7.5 at 25°C). The bottom panel shows the corresponding isotherms with the best fit of the single binding site model.

FIGURE 7. EMSA of KstR<sub>Mtb</sub> and variants. Each lane contains 2 nM DIG-labelled DNA probe and the indicated amount KstR2<sub>Mtb</sub> and HIP-CoA. Additional experimental details are in Materials and Methods.

FIGURE 8. SEC-MALS of KstR2<sub>Mtb</sub>. Rayleigh ratios of 80 μM KstR2<sub>Mtb</sub> with (dotted line) or without (solid line) 20 μM DNA (a 24-bp duplex containing a KstR2 box) resolved using a Superdex 200 5/150 column. Calculated molecular weights from the corresponding peaks are shown on the right axis. Results are representative of two independent experiments.
Table 1. X-ray crystallographic statistics for KstR2<sub>Mtb</sub>-HIP-CoA complex

| PDB Code | 4W97 |
|----------|------|

**Data collection**
- **Space group**: C2
- **Cell dimensions**: 72.5, 90.5, 49.8 Å, 129.7°
- **Resolution, Å**: 25.00 – 1.60
- **R<sub>merge</sub>**<sup>a</sup>, 0.032 (0.545)<sup>b</sup>
- **I / σ(I)**: 48.68 (3.78)
- **Completeness, %**: 100 (99.9)
- **Redundancy**: 4.4 (4.2)

**Refinement**
- **Resolution, Å**: 24.85 – 1.60
- **No. of reflections**: 31023, 1650
  - working, test
- **R-factor/free R-factor**<sup>c</sup>: 16.0/19.9 (23.7/29.4)
- **No. of refined atoms**:
  - Protein: 1626
  - Substrate: 64
  - Solvent: 4
  - Water: 310
- **B-factors, Å<sup>2</sup>**:
  - Protein: 28.9
  - Substrate: 37.9
  - Solvent: 41.2
  - Water: 43.5
- **r.m.s.d.**:
  - Bond lengths, Å: 0.02
  - Bond angles, °: 2.14

---

<sup>a</sup> <i>R<sub>merge</sub></i> = \( \frac{\sum_{hkl} \sum_j |I_{hkl,j} - \langle I_{hkl}\rangle|}{\sum_{hkl} \sum_j I_{hkl,j}} \)

<sup>b</sup> Values in parentheses refer to highest resolution shells of 1.63-1.60 Å for data collection and 1.64-1.60 Å for refinement.

<sup>c</sup> R-factor = \( \frac{\sum_{hkl} |F_{hkl}^o - F_{hkl}^c|}{\sum_{hkl} F_{hkl}^o} \). Free R-factor calculated with 5% reflections set aside.
Table 2. Thermodynamic parameters of KstR2<sub>Mtb</sub> binding HIP-CoA.

| KstR2 variant | Ligand     | N   | $K_d$  | $\Delta H$       | $\Delta S$       |
|--------------|------------|-----|--------|-------------------|-------------------|
|              |            | μM  | kJ mol<sup>-1</sup> | J K<sup>-1</sup>mol<sup>-1</sup> |
| WT           | HIP        | NB<sup>a</sup> | -     | -                 | -                 |
| WT           | CoASH      | NB  | -      | -                 | -                 |
| WT           | HIP-CoA    | 0.89 (0.01) | 0.08 (0.01) | -69.4 (0.5)       | -56               |
| R162M        | HIP-CoA    | 1.77 (0.02) | 16 (2) | -43.0 (0.9)       | -66               |
| W166L        | HIP-CoA    | NB  | -      | -                 | -                 |

<sup>a</sup> No binding detected.
Fig. 6
Fig. 7

| KstR2 (nM) | 0 | 1 | 5 | 10 | 50 | 50 | 50 | 50 | 50 |
|-------------|---|---|---|----|----|----|----|----|----|
| HIP-CoA (μM) | 0 | 0 | 0 | 0 | 0 | 1 | 10 | 50 | 500 |

WT

R162M

W166L
Structural and functional characterization of a ketosteroid transcriptional regulator of *Mycobacterium tuberculosis*

Adam M. Crowe, Peter J. Stogios, Israël Casabon, Elena Evdokimova, Alexei Savchenko and Lindsay D. Eltis

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