A Flavin-dependent Monoxygenase from *Mycobacterium tuberculosis* Involved in Cholesterol Catabolism

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*Mycobacterium tuberculosis* (Mt) and *Rhodococcus jostii* RHA1 have similar cholesterol catabolic pathways. This pathway contributes to the pathogenicity of *Mt*. The *hsaAB* cholesterol catabolic genes have been predicted to encode the oxygenase and reductase, respectively, of a flavin-dependent mono-oxygenase that hydroxylates 3-hydroxy-9,10-secoandrost-1,3,5(10)-triene-9,17-dione (3-HSA) to a catechol. An *hsaA* deletion mutant of RHA1 did not grow on cholesterol but transformed the latter to 3-HSA and related metabolites in which each of the two keto groups was reduced: 3,9-dihydroxy-9,10-secoandrost-1,3,5(10)-triene-17-one (3,9-DHSA) and 3,17-dihydroxy-9,10-secoandrost-1,3,5(10)-triene-9-one (3,17-DHSA). Purified 3-hydroxy-9,10-secoandrost-1,3,5(10)-triene-9,17-dione 4-hydroxylase (*HsaAB*) from *Mt* had higher specificity for 3-HSA than for 3,17-DHSA (apparent \(K_{m}/K_{cat} = 1000 \pm 100 \text{ M}^{-1} \text{s}^{-1} \) versus \(700 \pm 100 \text{ M}^{-1} \text{s}^{-1} \)). However, 3,9-DHSA was a poorer substrate than 3-hydroxybiphenyl (apparent \(K_{m}/K_{cat} = 80 \pm 40 \text{ M}^{-1} \text{s}^{-1} \)). In the presence of 3-HSA the *K_{m}_app* for \(O_2\) was \(100 \pm 10 \text{ \mu M}\). The crystal structure of HsaA to 2.5-Å resolution revealed that the enzyme has the same fold, flavin-binding site, and catalytic residues as \(p\)-hydroxyphenyl acetate hydroxylase. However, HsaA has a much larger phenol-binding site, consistent with the enzyme’s substrate specificity. In addition, a second crystal form of HsaA revealed that a C-terminal flap (Val<sup>367</sup>–Val<sup>394</sup>) could adopt two conformations differing by a rigid body rotation of 25° around Arg<sup>369</sup>. This rotation appears to gate the likely flavin entrance to the active site. In docking studies with 3-HSA and flavin, the closed conformation provided a rationale for the enzyme’s substrate specificity. Overall, the structural and functional data establish the physiological role of HsaAB and provide a basis to further investigate an important class of monoxygenases as well as the bacterial catabolism of steroids.

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*The atomic coordinates and structure factors (codes 1AFE and 3AFF) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).*

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6 The abbreviations used are: *Mt*, *M. tuberculosis*; *HsaAB*, 3-hydroxy-9,10-secoandrost-1,3,5(10)-triene-9,17-dione 4-hydroxylase; *HsaA*, 3-hydroxy-9,10-secoandrost-1,3,5(10)-triene-9,17-dione 4,17-dioxygenase; *HsaD*, 3,9-dihydroxy-9,10-secoandrost-1,3,5(10)-triene-17-one; *HsaH*, 3,17-dihydroxy-9,10-secoandrost-1,3,5(10)-triene-9-one; *HsaL*, 3,17,20-trihydroxy-9,10-secoandrost-1,3,5(10)-triene-17-one; *HsaR*, 3,9-dihydroxy-9,10-secoandrost-1,3,5(10)-triene-17-one; 3,9,17-DHSA, 3,9,17-trihydroxy-9,10-secoandrost-1,3,5(10)-triene-17-one; 3,17,20-DHSA, 3,17,20-trihydroxy-9,10-secoandrost-1,3,5(10)-triene-17-one; 3,9,17-THSA, 3,9,17-trihydroxy-9,10-secoandrost-1,3,5(10)-triene-17-one; 3,17,20-THSA, 3,17,20-trihydroxy-9,10-secoandrost-1,3,5(10)-triene-17-one; 3-HSA, 3-hydroxy-9,10-secoandrost-1,3,5(10)-triene-9,17-dione (3,4-DHSA) (Fig. 1). (5) The catechol is then subject to meta-

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cleavage and C–C bond hydrolysis by HsaC (5, 6) and HsdD (18), respectively. In Mtb and RHA1, the hsaACDB genes occur in a single operon, and their respective products share 74–82% amino acid sequence identity. In transposon mutagenesis studies, hsaA (3570c) was identified as one of 126 genes necessary for survival of Mtb in macrophages under conditions that model the immune response (4).

Flavin-dependent monooxygenases (FDMs) constitute an important class of enzymes that utilize a reduced flavin to activate O₂ and hydroxylate a variety of organic substrates. Two broad classes are recognized based on whether the flavin is reduced and used to hydroxylate the substrate at a single active site (19) or whether flavin reduction and organic substrate hydroxylation occur at different sites, in two separate polypeptides. In the latter, referred to as two-component (TC-) FDMs, a reductase utilizes NADH to reduce the flavin, which is then transferred to the oxygenase (20). The oxygenase generally has a preference for FADH₂ or FMNH₂. Examples for the former include 4-hydroxyphenyl acetate 3-monooxygenase (HpaB) of Escherichia coli (21) and 2,4,5-trichlorophenol monooxygenase from Burkholderia cepacia (22). FMN-dependent systems are involved in the synthesis of antibiotics such as actinorhodin in Streptomyces coelicolor (23), the bacterial degradation of polyaminocarboxylates such as nitrolotriacetic acid (24), and the desulfurization of fossil fuels by rhodococci (25). pHPAH (EC 1.14.13.3), a well characterized FMN-utilizing enzyme from Acinetobacter baumannii, catalyzes the hydroxylation of p-hydroxyphenyl acetate (HPA) to 3,4-dihydroxyphenyl acetate (PDB entry 2JBT) (26). The reductase component, C₁, requires HPA for effective flavin reduction (27), and the oxygenase component, C₂, binds FMNH₂ prior to HPA (28). HsaAB has been predicted to be a TC-FDM based on the ~32% amino acid sequence identity that it shares with C₂ and C₁ of pHPAH.

Herein we describe the characterization of HsaAB of Mtb. A ΔhsaA mutant of RHA1 was used to investigate the enzyme’s role in cholesterol catabolism and to generate substrates. To substantiate the role of the Mtb enzyme, each component was heterologously produced, purified, and used to reconstitute the enzyme’s activity in vitro. The enzyme’s specificity for a number of steroid metabolites was investigated. Finally, the crystal structure of HsaA was determined and compared with those of related oxygenases. The results are discussed with respect to TC-FDMs and the bacterial catabolism of steroids.

**MATERIALS AND METHODS**

**Chemicals and Reagents**—Chemicals were purchased from the following suppliers: 3-hydroxybiphenyl (3-HB, IVA) from ASDI (Newark, DE); 1,4-androstadiene-3,17-dione from Steraloids, Inc. (Newport, RI); BSTFA + TMCS from Supelco Analytical (Bellefonte, PA); riboflavin and FMN from Sigma; and FAD from MP Biomedicals Inc. (Solon, OH). 2,3-Dihydroxybiphenyl was a gift from Dr. Victor Snieckus. Restriction enzymes and Expand High Fidelity PCR System were purchased from New England Biolabs (Ipswich, MA) and Roche Applied Science (Laval, Quebec), respectively. Oligonucleotides were from Integrated DNA Technology. Crystallization screening solutions were purchased from Hampton Research, Emerald BioSystems, and Jena Bioscience. All other reagents were of HPLC or analytical grade.

**Bacterial Strains and Culture Conditions**—Escherichia coli strains were grown in Luria-Bertani (LB) medium at 37 °C, 200 rpm. *E. coli* DH5α was used to propagate DNA. To produce HsaA, *E. coli* GJ1158 (29) containing pT7HB was grown at 30 °C in low salt LB medium supplemented with 100 μg ml⁻¹ ampicillin. Expression of hsaB was induced when the culture attained an A₆0₀ of 0.6 by adding NaCl to a final concentration of 0.3 M. Cells were further incubated for 16 h, harvested by centrifugation, washed twice with 20 mM sodium phosphate (pH 8.0) containing 10% glycerol, then frozen at −80 °C until use. For metabolite analyses, *R. jostii* RHA1 ΔhsaA mutant, RHA017 (30), was grown in W minimal medium (31) supplemented with 0.5 mM cholesterol as described previously (6). To produce HsaA, RHA017 containing pTipHA was grown at 30 °C in LB supplemented with 25 μg ml⁻¹ chloramphenicol. Expression of hsaA was induced when the culture attained an A₆0₀ of 0.8 by adding thiostrepton to a final concentration of 50 μM. Cells were further incubated for 21 h prior to harvesting and freezing as described above.

**Heterologous Expression of HsaAB**—DNA was propagated, digested, ligated, and transformed using standard protocols (32). Plasmids were electroporated into RHA017 (30) and *E. coli* using a MicroPulser from Bio-Rad (Hercules, CA) with Bio-Rad 0.1-cm GenePulser Cuvettes. The hsaA (Rv3570c) and hsaB (Rv3567c) genes were amplified from *Mtb* H37Rv.
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genomic DNA by PCR using the Expand High Fidelity and the following three-phase thermo-cycler program: 95 °C for 5 min; 25 cycles of 95 °C for 30 s, 57 °C for 30 s, and 72 °C for 1.5 min; and a final phase at 72 °C for 7 min. The hsaA gene was amplified using a forward primer (GCAATAGCATATG-CATCACCATCCATCACTGAGAATGCGGCGTCC-ATTCAACAGTATG), which introduced an Ndel site, six codons for histidine, and a Factor Xa recognition site, and a reverse primer (GCCAAGCTTGTAGGTCTTCTGCCGACCATAG), which introduced a HindIII site. The ampli-

con was first cloned into pET41b+ (Novogen, Oakville, Ontario) as an Ndel/HindIII fragment, its nucleotide sequence was verified, and then subcloned in pTipQC2 (33) using the same restriction sites, yielding pTipHA. The hsaB gene was amplified using oligonucleotides (CTAGCTAGCCGTCAGATGATCAGCTCCAGCGAAGCTTCGTCT-GCTCGGCCACTAG) that introduced NheI and HindIII

—Production of metabolites in culture supernatant was monitored using GC-MS as described below. Samples were analyzed directly or after BSTFA derivatization. When the metabolite concentration was judged to be maximal, the culture supernatant was collected by centrifugation at 10,000 × g for 30 min at 4 °C. The supernatant was passed through a 0.2-μm filter and analyzed via HPLC using the method of Faeder and Siegel (35). Solutions of riboflavin, FMN, and FAD were used as standards.

—Reconstitution of HsaB—Flavin-free HsaB was produced anaerobically in the glove box by incubating a 500-μl solution of 86 μM HsaB and 10 mM NADH in 20 mM HEPES, pH 7.6, for 5 min, then immediately passing the sample over a 0.5-× 5-cm column of Biogel P6 (Bio-Rad) equilibrated with 20 mM HEPES, pH 7.6. HsaB was reconstituted by aerobically incubating the apoenzyme with 2 mM flavin for 1 h. Excess flavin was removed via ultrafiltration.

—Analytical Methods—TLC was performed using silica gel 60 F254 (Merck). Compounds were detected at 254 nm after first spraying plates with 50% sulfuric acid in ethanol and heating them for 5 min at 110 °C. Preparative column chromatography was carried out on silica gel 60 (mesh size 40–63 μm). NMR spectra were recorded using a Varian Unity 500-MHz and an Inova 600-MHz (Varian Inc., Mississauga, Ontario, Canada) spectrometer. Chemical shifts (δ) are reported in ppm relative to CHCl3 (1H: δ = 7.24) and CDCl3 (13C: δ = 77.0) as internal standards. GC-MS was carried out using an HP 6890 series GC system fitted with an HP 5973 mass-selective detector and a 30-m × 250-μm HP-5MS Agilent column. The operating conditions were: TGC (injector), 280 °C; TMS (ion source), 230 °C; oven time program (T0 min), 104 °C; T2 min, 104 °C; T14.4 min, 200 °C, 290 °C (heating rate 15 °C/min); and T29.4 min, 290 °C. HPLC analysis was performed using a Waters 2695 Separations HPLC module equipped with a Waters 2996 photodiode array detector and a 250 × 4.60 mm C18 Prodigy 10 μm ODS-Prep column (Phenomenex, Torrance, CA). SDS-PAGE was performed using a Bio-Rad MiniPROTEAN III apparatus with a 10% or 12% resolving gel. Gels were stained with Coomassie Blue using standard protocols. Protein concentrations were determined using the Micro BCA™ protein assay kit (Pierce) and the Bradford method (36) using bovine serum albumin as a standard.

—Enzyme Activity—HsaAB activity was measured spectrophotometrically by following the hydroxylation of 3-HB in a coupled assay with BphC (37) at 25 ± 0.5 °C. Reactions were performed in 125 μl of 20 mM potassium phosphate, pH 7.0,
HsaA was thawed and stored under N₂ in a sealed vial on ice. Components were incubated for 30 s, then the reaction was initiated by adding NADH to a final concentration of 600 μM and was monitored at 434 nm, the absorbance maximum of 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid (ε₂₃₄ = 16.9 mM⁻¹ cm⁻¹). Stock solutions were prepared fresh daily. Absorbance was monitored using a Varian Cary 5000 spectrophotometer controlled by WinUV software. One unit of activity was defined as the amount of enzyme required to consume 1 μmol of substrate per minute.

Steady-state kinetic parameters were evaluated using standard conditions except that for 3-HSA and 3,17-DHSA, 11.7 μM HsaC was used instead of BphC. Substrate concentrations were varied over the following ranges: 90–1600 μM for 3-HB and 30–1000 μM for each of 3-HSA and 3,17-DHSA. The molar absorptivities of 4,9-DSHA and 4,9-DSDHA were 2.41 and 2.40 M⁻¹ cm⁻¹, respectively, at 392 nm (pH 7.0). In these studies, HsaA was thawed and stored under N₂ in a sealed vial on ice. Aliquots were withdrawn using a gas-tight syringe. Kinetic parameters were evaluated by fitting the Michaelis-Menten equation to the data using the least-squares fitting and dynamic weighting options of LEONORA (38).

The steady-state kinetic parameters for O₂ were determined using a Clarke-type electrode (Hansatech Instruments Ltd., Norfolk, England) interfaced to a computer equipped with the ASTRA software (Wyatt Technology). Molecular weights of HsaA fractions were calculated using the weighting options of LEONORA (38).

Steady-state kinetic parameters were evaluated using the structure of substrate-free HsaA. 1 μl of 11 mg/ml protein was mixed with 1 μl of crystallization solution (0.1 M Tris-HCl, pH 7.0, 20% (v/v) polyethylene glycol-2000 MME). Thin plates were obtained within 3 days. Crystals were harvested directly from the mother liquor and were flash frozen in liquid nitrogen in the anaerobic chamber prior to data collection.

X-ray Data Collection and Structure Determination—X-ray data were collected under cryogenic conditions using a rotating anode x-ray generator (CuKα, radiation λ = 1.542 Å) and synchrotron radiation at the Canadian Light Source (beamline CMCF1, λ = 1.00 Å). Data were processed using XDS (39) or HKL2000 (40). The initial model and phase information were obtained from aerobically grown crystals by molecular replacement using the structure of substrate-free pHPAH (PDB entry 2JBS) and the program EPMR (41). Crystals grew in the space group I222 with two copies of HsaA in the crystallographic asymmetric unit. Model building and structure refinement were performed using CNS (42) and REFMAC (43). Anaerobically, crystals grew in space group P2₁ with four molecules of HsaA in the asymmetric unit. The structure was solved using the structure of aerobically grown HsaA as the starting model and MOLREP (43), and was refined as described above.

Data collection and refinement are summarized in Table 3. Electron density maps were calculated using the CCP4 package (44). Structural figures were made with the program PyMOL (45). The coordinates for the two Mtb HsaA conformations were deposited in the Brookhaven Protein Data bank with accession codes 3AFE and 3AFF.

Molecular Docking Simulation and Modeling—3-HSA was designed using PRODRG (46), energy was minimized using the Dreiding force field (47), and PM6 semi-empirical charges were calculated using MOPAC2007 (48). After removing water molecules from the HsaA⁰ and HsaA⁴ models, docking simulations were initially performed using PATCHDOCK (49). Significant clusters that placed the ligand molecule in the correct orientation with respect to the cofactor were picked and run through Autodock version 4.0 (50) with active site residues held rigid and the initial torsion angles of 3-HSA randomly set.

Solutions of the best free energy were minimized with strong restraints (Force 200) until gradient norm convergence to 1.0, over the entire system (heavy atoms only) with the exception of a sphere of 8 Å around the active site. Residues within this sphere were unrestrained to allow for minor side-chain re-orientations. The cofactor was kept rigid during all calculations and was positioned based on the closely similar binding site of pHPAH. Structural figures and graphics were rendered using PyMOL (45).

RESULTS

Phenotype of the hsaA Deletion Mutant—Bioinformatic analyses indicate that HsaAB of the actinomycete cholesterol catabolic pathway is a TC-FDM that transforms 3-HSA to 3,4-DHSA (5). To test this prediction, we investigated the phenotype of RHA017, an hsaA deletion mutant of RHA1 (30).
Consistent with the enzyme playing a central role in cholesterol catabolism, the deletion strain did not grow on cholesterol but grew normally on pyruvate (data not shown). When pyruvate-grown cells of RHA017 were incubated in the presence of cholesterol, four major metabolites accumulated in the medium (Fig. 2A). Purification of the three major ones afforded 3-HSA and two keto-reduced versions thereof: 3,9-DHSA and 3,17-DHSA. These were characterized using GC-MS and 1H-, 13C-, (H,H)-COSY-, HSQC-, and HMBC-NMR as summarized in the supplemental materials. The fourth metabolite was identified as 3,9,17-THSA using GC-MS.

Purification of HsaAB—HsaA produced in E. coli and purified aerobically had a very low specific activity. Production of the protein in RHA017 using pTipHA increased the specific activity by an order of magnitude; anaerobic purification further increased this 2-fold. This approach typically yielded 25 mg of purified HsaA per liter of culture with an apparent purity of \( >95\% \) as judged from SDS-PAGE and had absorption maxima at 268, 370, and 448 nm consistent with the presence of oxidized flavin (supplemental Fig. 1A). HPLC analyses of the acid-extracted flavin revealed two species with retention times and spectra corresponding to FMN and FAD, respectively (supplemental Fig. 1B). In subsequent experiments, HsaB was reconstituted with either FMN or FAD as solely bound cofactor.

HsaAB Catalyzes \( \omega \)-Hydroxylation—As noted above, RHA017 cultures incubated with cholesterol accumulated 3-HSA, 3,9-DHSA, 3,17-DHSA, and 3,9,17-THSA. When the culture supernatant was incubated for 30 min at room temperature with 9 \( \mu M \) HsaA, 4 \( \mu M \) HsaB-FMN, and 600 \( \mu M \) NADH, 3-HSA and 3,17-DHSA were removed from the mixture and two new compounds were detected by GC-MS (Fig. 2B). Mass spectrometry and NMR spectroscopy identified these compounds as 3,4-DHSA and 3,4,17-THSA (supplemental materials). Under similar conditions, HsaAB transformed 3-HB to 2,3-dihydroxybiphenyl, as confirmed by GC-MS (supplemental materials). These results demonstrate that HsaAB of \( Mtb \) is able to catalyze the \( \omega \)-hydroxylation of a range of \( p \)-substituted phenols to generate the corresponding catechols.

Steady-state Kinetic Analyses—To evaluate the specificity of HsaAB for different substrates, a coupled spectrophotometric assay was developed in which the catechol produced by the HsaAB reaction was cleaved by an extradiol dioxygenase (HsaC or BphC) to yield the yellow-colored \( \text{meta} \)-cleavage product. Dioxygenase concentrations were adjusted so as not to be rate-limiting. The concentration of HsaA was 2.5-fold higher than that of HsaB to maximize coupling between these components (see below). In all studies, the inferred initial rates of hydroxylation displayed Michaelis-Menten kinetics (Fig. 3). The evaluated kinetic parameters are considered to be apparent as they are dependent on the ratio of HsaA and HsaB as well as the concentration of \( O_2 \).

The coupled assay was performed with 3-HSA to evaluate the ability of HsaAB to utilize different flavins. In the presence of 4 \( \mu M \) exogenously added FMN or FAD, no increase in enzyme activity was observed. By contrast, 4 \( \mu M \) riboflavin inhibited the activity by \(~50\%\). To further evaluate the ability of HsaAB to utilize FMN and FAD, the reductase was reconstituted with
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TABLE 2
Crystallographic properties, X-ray diffraction data, phasing, and refinement statistics for HsaA from M. tuberculosis

<table>
<thead>
<tr>
<th>Diffraction data</th>
<th>HsaA (aerobic)</th>
<th>HsaA (anaerobic)</th>
</tr>
</thead>
<tbody>
<tr>
<td>X-ray source</td>
<td>CMCF1</td>
<td>CuKα</td>
</tr>
<tr>
<td>Wavelength (Å)</td>
<td>1.000</td>
<td>1.5418</td>
</tr>
<tr>
<td>Space group</td>
<td>P22</td>
<td>P2_1</td>
</tr>
<tr>
<td>Unit cell (Å, deg)</td>
<td>a = 68.74, b = 173.96, c = 178.07</td>
<td>a = 91.0, b = 94.0, c = 98.1</td>
</tr>
<tr>
<td>Resolution range (Å)</td>
<td>40–200</td>
<td>50–240</td>
</tr>
<tr>
<td>Highest shell (Å)</td>
<td>2.0–2.05</td>
<td>2.4–2.40</td>
</tr>
<tr>
<td>Unique reflections</td>
<td>72,634 (5,414)</td>
<td>64,589 (3,082)</td>
</tr>
<tr>
<td>Bond lengths (Å)</td>
<td>0.020</td>
<td>0.020</td>
</tr>
<tr>
<td>Bond angles (deg)</td>
<td>1.77</td>
<td>1.847</td>
</tr>
</tbody>
</table>

Refined model

| Resolution range (Å)    | 20–2.0        | 50–2.4          |
| No. reflections         | 68,987        | 64,589          |
| R_factor/Rfree (%)      | 18/22         | 20/27           |
| Mean B-values (Å²)      | 31.24         | 35.24           |
| R.m.s.d.                |               |                 |

Rates of O2 consumption resulted in quantitative H2O2 production as the highest resolution shell for each flavin as sole cofactor. For assays containing HsaB-FMN and HsaB-FAD, however, the apparent Km values were ~3-fold greater in the presence of HsaB-FMN. All subsequent kinetic studies were performed using HsaB-FMN and without supplementary flavin.

The coupled assay was then used to evaluate the specificity of HsaB for various p-substituted phenols. Of the purified steroid metabolites, the mycobacterial enzyme had slightly higher apparent specificity (kcat/Km) for 3-HSA versus 3,17-DHSA (Table 1). By contrast, the apparent specificity for 3-HB was an order of magnitude lower. Nevertheless, the activity of 3,9-DHSA was too low to evaluate kinetic parameters. However, in an assay using 205 μM 3,9-DHSA and 25.8 μM HsaA, GC-MS analyses demonstrated that the phenol was transformed to 3,4,9-THSA and that this reaction occurred with one-third the efficiency of the 3-HB transformation.

An oxygraph assay was developed to investigate the reactivity of HsaB with O2. In developing this assay, it was noted that O2 was consumed in the absence of HsaA. This HsaB-dependent O2 consumption resulted in quantitative H2O2 production as indicated by the addition of catalase (results not shown). To minimize uncoupled O2 consumption, assays were performed using 10-fold less HsaB than in the spectrophotometric assay. The remaining H2O2 production was corrected for by measuring the rate of O2 consumption before (vO2) and after (v+c) the addition of catalase. The calculated rate of O2 consumption due to hydroxylation, vHO2, was within 5% of that determined using the spectrophotometric assay and the same concentration of HsaB. Using the oxygraph assay and saturating amounts of 3-HSA, the apparent Km was 100 ± 10 μM (Table 2).

Overall Structure—The refined structure of HsaA purified aerobically from E. coli contains two peptide chains (residues

![FIGURE 3](http://www.jbc.org/)
FIGURE 4. Crystal structure of HsaA from *M. tuberculosis*. A, ribbon representation of the HsaA<sup>0</sup> protomer. The central β-barrel domain of this conserved fold is colored dark gray, and the flanking N- and C-terminal α-helix bundles are colored red and orange, respectively. B, ribbon representation of the HsaA<sup>C</sup> tetramer viewed along the 2-fold symmetry axis. Subunits are colored differentially in shades of violet, cyan, yellow, and green. C, ribbon representation of the HsaA<sup>C</sup> octamer complex. The four subunits that occur above the plane of view are colored with different darker shades. D, expanded view of the interactions between three subunits within the octamer. The C-terminal flaps (Val<sup>367</sup>–Val<sup>394</sup>) of two subunits, colored in two hues of red, align in an anti-parallel fashion. This interface is in close proximity to the hairpin loop (Gly<sup>261</sup>–Val<sup>281</sup>) of a third subunit (dark green). This loop, which is not visible in the electron density representation of the HsaAC octamer complex. The four subunits below the view plane are colored with lighter shades. D, expanded view of the interactions between three subunits within the octamer. The C-terminal flaps (Val<sup>367</sup>–Val<sup>394</sup>) of two subunits, colored in two hues of red, align in an anti-parallel fashion. This interface is in close proximity to the hairpin loop (Gly<sup>261</sup>–Val<sup>281</sup>) of a third subunit (dark green). This loop, which is not visible in the electron density representation of the HsaAC octamer complex. The four subunits below the view plane are colored with lighter shades.

However, the former crystallized as an octamer while the latter crystallized as a tetramer. In HsaA<sup>C</sup>, the two subunits of the asymmetric unit are nearly identical as reflected by an r.m.s.d. between the Ca atoms of less than 0.32 Å. Each protomer forms an extended contact interface with symmetry-related subunits along the crystallographic and non-crystallographic 2-fold axes to form a doughnut-shaped octameric assembly that is 90–100 Å wide with a central solvent-accessible pore of 40–45 Å in diameter (Fig. 4C). Calculations performed using the PISA server indicated that the buried surface in each molecule is 1,138 Å<sup>2</sup> and predicting that the octamer is highly stable. In HsaA<sup>O</sup>, the four protomers of the asymmetric unit have an r.m.s.d. of 0.48 Å over the 369 backbone Ca atoms. In comparison to the octameric HsaA<sup>C</sup>, the tetrameric HsaA<sup>O</sup> has a larger average buried surface area per protomer of 2,616 Å<sup>2</sup> (Fig. 4B).

Static light scattering analysis indicated that HsaA prepared aerobically from the *E. coli* host exists as a mixture of dimers and octamers in solution while HsaA prepared anaerobically from the rhodococcal host exists as a tetramer (supplemental Fig. 2). Conformational States of the C-terminal Flap—Comparison of the tetrameric HsaA<sup>O</sup> and octameric HsaA<sup>C</sup> revealed different contacts between HsaA protomers in the crystal lattice, as reflected by the difference in buried surface area described above. In addition, two regions of the molecule displayed conformational differences that likely further contribute to the difference in crystal packing: an apical hairpin turn (Gly<sup>261</sup>–Val<sup>281</sup>) and a C-terminal flap (Val<sup>367</sup>–Val<sup>394</sup>). In HsaA<sup>O</sup> (P<sub>2<sub>1</sub></sub>), the C-terminal helix bundles (Fig. 4A) are clustered toward the center of the tetramer (Fig. 4A), forming a coiled-coiled complex, with the β-barrel projecting outward in the tetrahedral arrangement (Fig. 4B). At the center of the tetramer, a helix-turn-helix motif (Glu<sup>317</sup>–Gly<sup>365</sup>) composed of residues immediately preceding those of the C-terminal flap, stacks and aligns anti-parallel to the equivalent motif in the adjacent protomer. Moreover, the same cluster of residues from the two protomers comprises the major direct noncovalent interactions at the interface: Phe<sup>341</sup>, Leu<sup>349</sup>, Trp<sup>359</sup>, and His<sup>363</sup>. A second helix bundle motif, involving Phe<sup>278</sup>–Ala<sup>343</sup> and the C-terminal flap, associates with equivalent motifs between dimers to form the HsaA<sup>C</sup> tetramer. This second interface includes two equivalent hydrogen bonds involving Arg<sup>328</sup> and Arg<sup>332</sup> of one molecule and Glu<sup>285</sup> and Asp<sup>289</sup> of an adjacent one. The C-terminal flap also contributes to this second interface, mainly through hydro-
phobic interactions involving Tyr$^{377}$–Phe$^{385}$ and Lys$^{280}$–Ala$^{284}$. More specifically, the side chain of Val$^{378}$ and the backbone of Gly$^{381}$ interact with the aliphatic side-chain atoms of Lys$^{280}$. The ε-amine group of the latter forms a hydrogen bond with the side-chain amide oxygen of Asn$^{382}$. Finally, the side chains of Ile$^{283}$ and Ala$^{284}$ provide a hydrophobic platform for Phe$^{380}$ and Phe$^{385}$ on the C-terminal flap. Overall, the apical hairpin turn is stabilized by the C-terminal flap in the HsaA$^{\mathrm{O}}$ tetramer.

By contrast, the intersubunit interactions in the HsaA$^{\mathrm{C}}$ octamer are more intricate (Fig. 4C), with each protomer interacting with three others. At one end, each protomer interacts with its neighbor through their respective helix-turn-helix motifs in much the same way as in HsaA$^{\mathrm{O}}$. At the other end, contact with a third protomer involves hydrophobic interactions between C-terminal flaps at the entrance of the active site with residues of adjacent protomers, Ile$^{376}$, Tyr$^{374}$, Phe$^{377}$, and Phe$^{382}$ involved. In comparison to its conformation in HsaA$^{\mathrm{O}}$, the flap in HsaA$^{\mathrm{C}}$ differs by a 25° rigid body rotation around Arg$^{366}$ (Fig. 5C) enabling the described intersubunit association and effectively occluding the entrance to the substrate-binding pocket of HsaA. Finally, our structures suggest that the interface between the C-terminal flaps is spatially positioned to potentially interact with the apical hairpin turn (disordered in our HsaA$^{\mathrm{C}}$ structures) of an adjacent protomer (Fig. 4D).

The two conformers also differ in the degree of flexibility of their C-terminal segments, with the $B$-factors increasing toward the C-terminal flap residues, reaching an average value of 50.6 Å$^2$ for the backbone Cos of Pro$^{388}$–Val$^{394}$. This value is ~2-fold higher than that of the rest of the protein. By contrast, the $B$-factors of the C-terminal flap in HsaA$^{\mathrm{C}}$ are comparable to those of the surrounding residues (~27.6 Å$^2$) and are similar throughout the flap.

We considered whether oxidation of a Cys residue might influence the conformation of the C-terminal flap of HsaA. Inspection of the two structures revealed that Cys$^{63}$, Cys$^{79}$, and Cys$^{145}$ all have low solvent accessibilities and are remote (~20 Å) from the C-terminal flap. Inspection of the electron density revealed no evidence for oxidation. Thus, 2Fo − Fc and Fc − Fo maps revealed no additional electron density around the Cys thiol in the structure of the aerobically prepared enzyme. Moreover, water molecules are ordered around the cysteines in a similar fashion in both HsaA$^{\mathrm{C}}$ and HsaA$^{\mathrm{O}}$. Overall, our data indicate that oxidation of a Cys residue does not contribute to the different conformation of the C-terminal flap.

The Flavin Binding (F-)Subsite—The structures of HsaA contain a deep cavity that presumably sequesters the substrates: reduced flavin, O$_2$, and 3-HSA. This cavity is located at the interface between the C-terminal helix bundle and the β-barrel. Superposition of the structure of the pHPAH-flavin-pHPA complex facilitates the description of the substrate-binding pocket of HsaA (Fig. 6A). Notably, the pocket comprises two adjacent subsites: the H-subsite binds the phenol, and the slightly more solvent-accessible F-subsite binds the reduced flavin. The F-subsite of HsaA$^{\mathrm{O}}$ is remarkably similar to that of pHPAH with all first shell residues being conserved (i.e. Trp$^{84}$, Trp$^{141}$, Ser$^{143}$, Trp$^{182}$, Leu$^{187}$, and His$^{368}$). Based on this superimposed model (Fig. 6A), the isoalloxazine ring stacks between the indole rings of Trp$^{84}$ and Trp$^{141}$, and is surrounded by the hydrophobic side chains of Trp$^{182}$ and Leu$^{187}$. Ser$^{143}$ forms a hydrogen bond with flavin N5. Similarly to what was observed in pHPAH, the imidazole group of His$^{368}$ is on the re-face of the isoalloxazine ring within 4 Å of N1, compatible with stabilization of a deprotonated N1 in the bound FMNH$^-$. Finally, the entrance to the F-subsite is lined with charged residues and is partially occluded by an adjacent protomer in the tetramer, as observed in pHPAH. However, although residues contacting the isoalloxazine moiety are largely conserved, side chains that stabilize the phosphate group, located on a C-terminal helix, are not (i.e. Asn$^{372}$).

The F-subsite in the HsaA$^{\mathrm{C}}$ crystal form is very similar, but differs in two respects. First, the entrance to the subsite is occluded by the C-terminal flap. Second, the flavin binding site is partially occupied by Trp$^{139}$ and His$^{368}$.

The 3-HSA Binding (H-)Subsite—In contrast to the F-subsite, the H-subsite is buried in the core of the protein and is predominantly lined with hydrophobic residues: Trp$^{94}$, Pro$^{378}$, Ile$^{241}$, Tyr$^{210}$, His$^{237}$, and Trp$^{231}$. These residues are conserved in HsaA of R. jostii RHA1 but not in pHPAH. The size of the HsaA H-subsite versus that of pHPAH is consistent with the former accommodating a larger phenolic substrate.

To investigate the structural basis of binding of the phenolic substrate, we modeled 3-HSA into the H-subsite of HsaA$^{\mathrm{O}}$ and HsaA$^{\mathrm{C}}$ using molecular docking and energy minimization methods. The high structural similarity of the F-subsites of HsaA$^{\mathrm{O}}$, HsaA$^{\mathrm{C}}$, and pHPAH-flavin-pHPA allowed for positioning of flavin in the binding site. In both forms of HsaA, the His$^{368}$ and Trp$^{141}$ side chains were the only ones of the F-subsite that required minor reorientation to accommodate the fla-
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The latter molecule was kept rigid during docking and energy minimization calculations. Positive clustering of docked solutions was readily observed using either HsaA or HsaAC, with the best positional conformation placing the substrate in close proximity to the flavin molecule. A solution where the C4 atom of 3-HSA is within 4.2 Å to the C4a atom of the cofactor was chosen as the strongest candidate for further energy minimization. In docking 3-HSA, a number of aromatic side chains interacting with the bicycloalkanone moiety underwent rearrangement during the docking and energy minimization process.

In the modeled HsaAC-flavin-3-HSA complex, the phenol ring of 3-HSA is orientated toward the flavin and the bicycloalkanone moiety toward the entrance to the active site, stacking against Pro370 (Fig. 6B). The modeled complex shares at least three important features with the pHPAH-flavin-pHPA complex. First, the phenol ring is orthogonal to the flavin. Second, conserved Ser118 in HsaA appears to interact with the isoalloxazine ring (Fig. 6C). More specifically, the distance between flavin C4a and phenol C4 is 4.9 Å, compatible with the formation of a C4a-hydroperoxyflavin intermediate on the face of the isoalloxazine ring (Fig. 6C). In the modeled complex, the probable O2-binding cavity is slightly larger than in the HsaAC model do not occur in the modeled HsaAC-flavin-3-HSA ternary complex (Fig. 6F). First, Asp372 and Glu374 participate in an H-bond with His368, the small acidic patch is in close proximity to Arg366, the pivotal atom of 3-HSA is within 4.2 Å to the C4a atom of the cofactor is 18 Å3 due to the presence of less bulky residues (e.g. Phe270 in pHPAH is Ile241 in HsaA). Finally, the unassigned electron density in HsaAC occupies a space corresponding to a portion of the isoalloxazine ring of the flavin and the phenol ring of the 3-HSA (Fig. 6D).

Two notable features of the modeled HsaAC-3-HSA interactions involve C9 and C17 of the substrate (Fig. 6, B and F). The C9 carbonyl is orientated toward two acidic residues: Asp372 and Glu374. Asp372 participates in an H-bond with His368, the residue proposed to abstract a proton from the flavin N1. This small acidic patch is in close proximity to Arg366, the pivotal point of the C-terminal flavin, which has distinct conformations in HsaA and HsaAC. For its part, the C17 carbonyl is orientated toward a short hydrophobic channel lying between the helix bundle and the β-barrel domains. This channel is ~9 Å long and is lined with the hydrophobic side chains of Tyr377, Tyr210, Phe380, Phe385, and Leu187.

Several interactions between 3-HSA and the enzyme that are present in the HsaAC model do not occur in the modeled HsaAC-flavin-3-HSA complex (Fig. 6F). First, Asp372 and Glu374 do not stabilize the C9 carbonyl, facing instead the bulk solvent. Second, only Tyr207 is within hydrogen bonding distance of the C17 carbonyl: the interaction with Tyr374 is absent. Finally, Pro370 and Pro119 both pack closely with the bicycloalkanone moiety in the HsaAC model, while only Pro119 is present in the HsaA model. Overall, these differences underscore the possibility that conformational changes in the C-terminal flap may be important for binding and orientating the substrates.

**DISCUSSION**

The current study establishes that the role of mycobacterial and rhodococcal HsaAB is to transform 3-HSA to 3,4-DHSA in the catabolism of cholesterol. When incubated in the presence of cholesterol, a ΔhsaA mutant of RHA1 accumulated the enzyme’s predicted substrate, 3-HSA, together with related metabolites in which the keto groups on the bicycloalkanone moiety are reduced. Consistent with its predicted role, reconstituted HsaAB from *Mtb* had the highest specificity for 3-HSA. Moreover, the crystal structure of HsaA revealed a fold that is typical of TC-FDM oxygenases and a substrate-binding pocket that is well adapted to the steroid metabolite. The function of HsaAB is consistent with transposon mutagenesis studies indicating that hsaA (3570c) is necessary for survival of *Mtb* in macrophages, but hsaB (3567c) is not (4), because it is likely that flavin-reducing function of HsaB could be performed by another enzyme.

The metabolite analyses of the ΔhsaA mutant suggest that at least in RHA1, side-chain degradation largely precedes ring degradation. More specifically, the identified metabolites represented either the end (C17 ketone) or penultimate (C17 hydroxy) product of side-chain degradation. This is consistent with the recent finding that cytochrome P450*125* initiates cholesterol degradation in this strain by hydroxylating a terminal carbon (11). Nevertheless, the Δhsac mutant of RHA1 accumulated three major catecholic metabolites possessing the ketone, hydroxyl as well a propionate group at C17, consistent with some simultaneous degradation of the side chain and rings. The different metabolites in the two mutants may reflect the relative specificities of the side chain-degrading enzymes for the phenolic versus catecholic steroid metabolites. Finally, the significance of the 9-hydroxy metabolites, 3,9-DHSA and 3,9,17-THSA, is unclear. Their relative lack of reactivity with HsaAB suggests that they may have arisen adventitiously in the mutant. The current studies do not rule out the possibility that the physiological substrate of HsaAB is 3-HSA with a partially degraded side chain.

Although the activity of HsaAB is less than that of other characterized TC-FDMs, it is consistent with that of other cholesterol catabolic enzymes of *Mtb*. For example, the apparent specificity (*k*cat/*K*<sub>m</sub>) of a bacterial EDTA monoxygenase for MgEDTA<sup>−</sup> is 430-fold greater than that of HsaAB (51). Simi
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larily, the apparent specificity of alkanesulfonate monoxygenase for alkanesulfonates was up to 112-fold greater (25). Nevertheless, the relative activity of HsaAB is very similar to that of its flanking enzymes in the cholesterol catabolic pathway, KshA (17) and HsaC (6): the apparent $k_{\text{cat}}/K_m$ of these enzymes are also two or three orders of magnitude lesser than those of their homologues. It is unclear whether this is characteristic of non-mycobacterial steroid-degrading oxygenases. Although the $K_{\text{mO2}}$ values of other TC-FDMs have not been reported, that of HsaAB ($100 \pm 10 \, \mu M$) is remarkably similar to HsaC ($90 \pm 20 \, \mu M$). Finally, the relative $k_{\text{cat}}/K_m$ values of HsaAB and HsaC for 3-HSA ($1000 \pm 100 \, \text{mM} \, \text{s}^{-1}$) and 3,4-DHSA ($15000 \pm 2000 \, \text{mM} \, \text{s}^{-1}$), respectively, would help minimize intracellular concentrations of the latter, which is toxic to $Mtb$ (6).

HsaA is similar to $p$HPAH oxygenase (52) in that both utilize FMNH$_2$ and FADH$_2$ with similar efficiencies and that exogenously added flavin does not increase the rate of product formation by either enzyme. Nevertheless, the structure of HsaA suggests that the F-subsite recognizes the isosalloxyline ring of FMNH$_2$ or FADH$_2$ and does not accommodate the entire FADH$_2$ molecule: the adenyl moiety of the latter would protrude into bulk solvent (supplemental Fig. 3). In nitroalkane oxidase from $Fusarium oxysporum$ (PDB entry 2COU), the protruding moiety mediates subunit interactions. Other bacterial TC-FDMs that transform aromatic compounds preferentially utilize one of the flavins: FMNH$_2$ in the case of pristinamycin II$_A$ synthase (53) and FADH$_2$ in the case of chlorophenol-4-hydroxylase (54), pyrrole-2-carboxylate monoxygenase (55), and $p$-nitrophenol hydroxylase (56).

Although the closed conformation of the C-terminal flap correlates with the presence of an active site ligand in HsaA, it is unclear how the expression host ($E. coli$ versus Rhodococcus) correlates with the presence of an active site ligand in HsaA, it is unclear whether this is characteristic of non-mycobacterial steroid-degrading oxygenases. Nevertheless, transposon mutagenesis studies indicate that HsaAB activity is critical for survival in macrophages (4). Further studies of HsaAB should help establish the role of cholesterol catabolism in the pathogenesis of $Mtb$ as well as the catalytic mechanism of TC-FDMs. Indeed, characterization of the ligand bound to HsaA could be useful in both of these respects.

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Supplemental Figure 1.

A

![UV spectrum of FAD (red), FMN (blue)](image)

B

![HPLC chromatogram](image)

UV (A, UV Spectrum of FAD (red), FMN (blue)) and HPLC (B, HPLC Chromatogramm) analysis of HsaB-bound flavins after extraction from the purified protein.
Supplemental Figure 2. A

A: The aerobic preparation of HsaA\textsuperscript{C} exists in two oligomerization states in solution as detected by static light scattering. The octamer state of HsaA\textsuperscript{C} is identified with a molecular weight of 410kD, corresponding the size of 8 to 9 copies of monomer. In addition, a second peak of molecular weight of 85kD (dimer) suggests an equilibration between dimer and octamer in solution. The gel filtration chromatography used is Superdex200 HR 10/30 column.
B: The tetrameric state of HsaA$^O$ is confirmed by static light scattering, which identified that the solution structure of HsaA$^O$ has a molecular weight of 172 kD. This corresponds to 4 copies of HsaA monomer of 43kD.
Supplemental Figure 3.

Models of the HsaA:FAD complexes. (A) Two protomers of the octameric HsaA$^C$:FAD and (B) the tetrameric HsaA$^O$:FAD. The positioning of FAD was based on the respective HsaA:FMN models. The C-terminal flap is highlighted in red. For clarity, a single molecule of FAD is shown.
Analytical data of metabolites.

**3-hydroxy-9,10-seconandrost-1,3,5(10)-triene-9,17-dione (3-HSA)**

\[ R_f(n\text{-hexane/ethyl acetate} = 2:3) = 0.75. \ \varepsilon_{281\text{nm}, \text{pH} 7.0} = 2.33 \text{ mM}^{-1} \text{ cm}^{-1} . \]

GCMS \( R_t \) = 15.35 min. MS (70 eV, EI); \( m/z \): 300 (16.5%), 206 (6.5%), 193 (2.5%), 154 (11.5%), 134 (100%), 121 (17%), 107 (9.5%), 91 (7.5%), 77 (8%), 55 (8.5%).

\(^1\text{H} \text{NMR} (500 \text{ MHz, CDCl}_3): \delta = 6.99 (d, ^3J = 8.0 \text{ Hz}, 1H, 1), 6.65 (d, ^3J = 2.6 \text{ Hz}, 1H, 4), 6.57 (dd, ^3J = 8.0, 2.6 \text{ Hz}, 1H, 2), 4.54 (s, 1H, OH), 2.71 ("dt", ^3J = 12.5, 5.3 \text{ Hz}, 1H, 6\alpha), 2.57 (dd, ^2J = 19.7, ^3J = 9.1 \text{ Hz}, 1H, 16\alpha), 2.53-2.41 (m, 4H, 6\beta, 8, 11\alpha), 2.25 (s, 3H, 19), 2.21 (dd, ^2J = 19.4, ^3J = 9.1 \text{ Hz}, 1H, 16\gamma), 2.09-1.97 (m, 2H, 12\alpha, 15\alpha), 1.93-1.82 (m, 2H, 14, 7\alpha), 1.77-1.63 (m, 3H, 7\gamma, 12\gamma, 15\gamma), 1.16 (s, 3H, 18).

\(^{13}\text{C} \text{NMR} (125.8 \text{ MHz, CDCl}_3): \delta = 218.0 (17), 210.5 (9), 153.7 (3), 142.0 (5), 131.2 (1), 128.1 (10), 115.7 (4), 112.7 (2), 49.7 (8), 49.3 (14), 47.6 (13), 37.5 (11), 36.1 (16), 31.0 (6), 30.4 (12), 26.7 (7), 22.5 (15), 18.4 (19), 13.5 (18).

**3-tms-HSA** GCMS \( R_t = 15.06 \text{ min.} \) MS (70 eV, EI); \( m/z \): 372 (20%), 206 (100%), 191 (16%), 179 (5%), 163 (3.5%), 149 (1.5%), 73 (15.5%), 55 (4%).

**3,17-dihydroxy-9,10-seconandrost-1,3,5(10)-triene-9-one (3,17-DHSA)**

\[ R_f(n\text{-hexane/ethyl acetate} = 2:3) = 0.5. \ \varepsilon_{280\text{nm}, \text{pH} 7.0} = 1.92 \text{ mM}^{-1} \text{ cm}^{-1} . \]

\(^1\text{H} \text{NMR} (500 \text{ MHz, CDCl}_3): \delta = 6.91 (d, ^3J = 8.0 \text{ Hz}, 1H, 1), 6.64 (d, ^3J = 2.8 \text{ Hz}, 1H, 4), 6.56 (dd, ^3J = 8.0, 2.8 \text{ Hz}, 1H, 2), 2.99 ("dd", ^3J = 19.8, 8.2 \text{ Hz}, 1H), 2.85-2.73 (m, 1H), 2.72-2.63 (m, 1H), 2.57-2.37 (m, 4H), 2.23 (s, 3H, 19), 1.90-1.76 (m, 2H), 1.73-1.63 (m, 1H), 1.62-1.41 (m, 6H), 1.20 (s, 3H, 18).

\(^{13}\text{C} \text{NMR} (125.8 \text{ MHz, CDCl}_3): \delta = 211.6 (9), 153.6 (3), 142.1 (5), 131.1 (1), 128.1 (10), 115.6 (4), 112.6 (2), 79.6 (17), 53.5 (8), 49.5 (14), 47.0 (13), 37.9 (11), 36.1 (16), 35.3 (12), 30.9 (6), 27.9 (7), 25.6 (15), 18.4 (19), 15.1 (18).
3,17-tms-DHSA GCMS $R_t = 15.59$ min. MS (70 eV, EI); $m/z$: 446 (12.5%), 206 (100%), 194 (11.5%), 191 (10%), 179 (2.5%), 163 (3.5%), 135 (2.5%), 115 (2%), 91 (1.5%), 73 (20%), 55 (2%).

3,9-dihydroxy-9,10-seconandrost-1,3,5(10)-triene-17-one (3,9-DHSA)

$R_f$ (n-hexane/ethyl acetate = 2:3) = 0.66.

$^1$H NMR (500 MHz, CDCl$_3$): $\delta = 6.94$ (d, $J = 8.3$ Hz, 1H, 1), 6.66 (d, $^3J = 2.5$ Hz, 1H, 4), 6.57 (dd, $J = 8.3$, 2.5 Hz, 1H, 2), 6.4 (br s, OH(Ar)), 3.40 (“dt”, $J = 10.4$, 4.7 Hz, 1H, 9), 2.62-2.43 (m, 3H, 6xy, 16x), 2.19 (s, 3H,19), 2.17-2.08 (“dt”, $J = 19.3$, 9.1 Hz, 1H,16y), 2.01-1.89 (m, 2H, 11x, 15x), 1.82-1.69 (m, 3H, 7xy, 12x), 1.68-1.55 (m, 3H, 8, 11y, 15y), 1.44 (“dt”, $J = 12.3$, 5.7 Hz, 1H, 14), 1.30 (“dt”, $J = 13.3$, 4.7 Hz, 1H, 12y), 0.91 (s, 3H, 18).

$^{13}$C NMR (125.8 MHz, CDCl$_3$): $\delta = 220.4$ (17), 154.2 (3), 142.1(5), 131.0 (1), 127.3 (10), 115.6 (4), 112.7(2), 74.2 (9), 48.1 (13), 47.8 (14), 42.6 (8), 36.1 (16), 31.2 (11), 29.9 (6), 29.5 (12), 29.3 (7), 22.0 (15), 18.4 (19), 13.7 (18).

3,9-tms-DHSA GCMS $R_t = 15.44$ min. MS (70 eV, EI); $m/z$: 446 (35%), 431 (4%), 356 (7.5%), 246 (6.5%), 206 (100%), 193 (26.5%), 191 (12.5%), 169 (17.5%), 163 (6%), 129 (25%), 101 (4%), 73 (45%).

3,9,17-tms-THSA (tms derivative of 3,9,17-THSA: 3,9,17-trihydroxy-9,10-seconandrost-1,3,5(10)-triene) GCMS $R_t = 15.70$ min. MS (70 eV, EI); $m/z$: 520 (11%), 505 (3%), 430 (3%), 207 (22%), 206 (100%), 194 (11%), 193 (18%), 191 (8%), 169 (3%), 147 (6%), 129 (7%), 75 (8%), 73 (34%).
Analytical data of HsaAB transformation products.

3,4-dihydroxy-9,10-seconandrost-1,3,5(10)-triene-9,17-dione (3,4-DHSA) GCMS $R_t = 16.52$ min. MS (70 eV, EI); $m/z$: 316 (61.5%), 298 (2.5%), 179 (34%), 161 (4.5%), 150 (62.5%), 138 (100%), 123 (8%), 109 (7.5%), 91 (11.5%), 79 (8.5%), 55 (11.5%).

3,4-tms-DHSA GCMS $R_t = 15.91$ min. MS (70 eV, EI); $m/z$: 460 (69%), 445 (4%), 294 (50%), 281 (9%), 267 (6.5%), 251 (67.5%), 207 (10.5%), 193 (47.5%), 179 (6%), 147 (6%), 129 (8.5%), 73 (100%).

The retention time and fragmentation pattern of the HsaA-catalyzed transformation product of 3-HSA (IA) corresponded to those of 3,4-DHSA (IB) generated by incubating a culture of the ΔhsaC mutant of *R. jostii* RHA1 (1) with cholesterol (2). This showed in the Proton NMR 2 signals ($\delta = 6.57$ (d, $^3J = 8.4$ Hz, 1H, 1), 6.47 (d, $^3J = 8.4$ Hz, 1H, 2)) corresponding to the aromatic protons.

3,4,17-tms-THSA (tms derivative of 3,4,17-THSA: 3,4,17-trihydroxy-9,10-seconandrost-1,3,5(10)-triene-9-one) GCMS $R_t = 16.37$ min. MS (70 eV, EI); $m/z$: 534 (57%), 519 (2.5%), 429 (7%), 325 (57.5%), 294 (86.5%), 282 (9.5%), 281 (9.5%), 193 (29%), 147 (5.5%), 129 (5%), 73 (100%).

3,4,9-tms-THSA (tms derivative of 3,4,9-THSA: 3,4,9-trihydroxy-9,10-seconandrost-1,3,5(10)-triene-17-one) GCMS $R_t = 16.18$ min. MS (70 eV, EI); $m/z$: 534 (65%), 444 (3%), 429 (3%), 355 (4.5%), 294 (26%), 281 (27.5%), 267 (6%), 193 (24%), 169 (10.5%), 147 (6.5%), 129 (21.5%), 73 (100%).

A Flavin-dependent Monoxygenase from *Mycobacterium tuberculosis* Involved in Cholesterol Catabolism

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