

Enzymatic β -oxidation of the cholesterol side chain in *Mycobacterium tuberculosis* bifurcates stereospecifically at hydration of 3-oxo-cholest-4,22-dien-24-oyl-CoA

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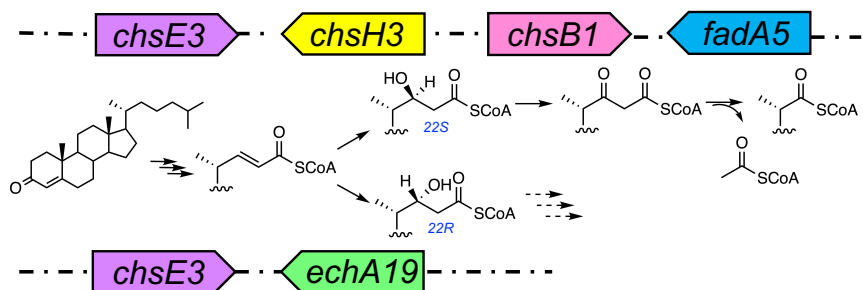
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Abstract

The unique ability of *Mycobacterium tuberculosis* (Mtb) to utilize host lipids such as cholesterol for survival, persistence, and virulence has made the metabolic pathway of cholesterol an area of great interest for therapeutics development, and bioproduction of valuable sterol intermediates. Herein, we identify and characterize two genes from the Cho-region of the Mtb genome, *chsH3* (Rv3538) and *chsB1* (Rv3502c). Their protein products catalyze two sequential stereospecific hydration and dehydrogenation steps in the β -oxidation of the cholesterol side chain. ChsH3 favors the *22S* hydration of 3-oxo-cholest-4,22-dien-24-oyl-CoA in contrast to the previously reported EchA19 (Rv3516) which catalyzes formation of the (*22R*)-hydroxy-3-oxo-cholest-4-en-24-oyl-CoA from the same enoyl-CoA substrate. ChsB1 is stereospecific and catalyzes dehydrogenation of the ChsH3 product, but not the EchA19 product. The X-ray crystallographic structure of the ChsB1 apo-protein was determined at a resolution of 2.03 Å and the holo-enzyme with bound NAD⁺ cofactor at 2.21 Å. The homodimeric structure is representative of a classical NAD⁺ utilizing short-chain type alcohol dehydrogenase/reductase, including a Rossmann-fold motif, but exhibits a unique substrate binding site architecture that is of greater length and width than its homologous counterparts, likely to accommodate the bulky steroid substrate. Intriguingly, Mtb utilizes MaoC-like hydratases in sterol side-chain catabolism in contrast to fatty acid β -oxidation in other species that utilize the evolutionarily distinct crotonase family of hydratases.

TOC graphic:



Mycobacterium tuberculosis (Mtb) infects one fourth of the world population and is the etiologic agent that causes Tuberculosis (TB). When the host is co-infected with HIV, TB causes severe morbidity.¹ Progress over the last 5 years for reduced TB incidence and deaths as part of the World Health Organization's End TB Strategy for 2030 in conjunction with the United Nations Sustainable Development Goals (SDGs) has been moderate.¹ Furthermore, the effects of the COVID-19 pandemic are expected to erase this modest progress with an anticipated increase of 1 million deaths per year from TB over the next five years.¹ As part of multi-factorial solutions to the TB pandemic, shorter and simpler treatments for TB disease are required.

Mtb utilizes an array of carbon sources in the host for survival and persistence,²⁻³ including cholesterol whose metabolism plays an essential role in Mtb's virulence and pathogenesis.⁴ During the last 15 years, many cholesterol metabolism associated genes that are crucial for the survival and virulence of Mtb have been identified and characterized.⁵ Different strategies have been developed to selectively target cholesterol metabolism in Mtb,⁶⁻⁷ and these cholesterol metabolism associated genes and their protein products have proven vulnerable to small-molecule inhibition for potential TB therapeutic intervention.⁸⁻⁹

Mtb does not synthesize cholesterol but maintains the ability to import and utilize host derived cholesterol during infection from, for example, co-localized macrophages.³⁻⁴ Mtb engages at least three distinct pathways for cholesterol catabolism.¹⁰ The metabolites of cholesterol degradation, i.e., acetyl-CoA, propionyl-CoA, succinyl-CoA, and pyruvate, are further incorporated into metabolic pathways for energy production and lipid biosynthesis in Mtb.¹¹⁻¹³

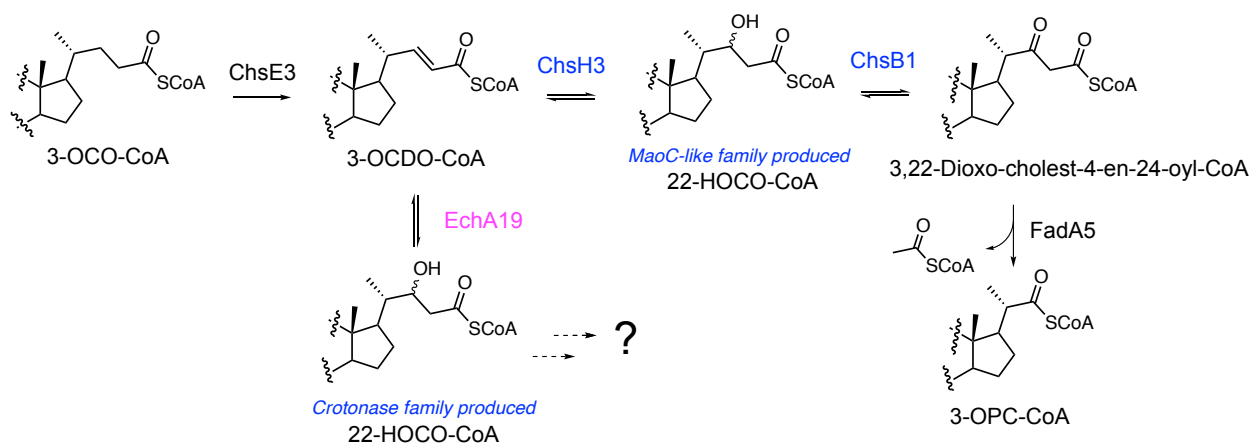
In Mtb and other steroid utilizing actinomycetes, the alkyl side chain of cholesterol is largely degraded through a series of β -oxidation reactions. These processes are analogous to fatty acid

degradation in mammals and bacteria. In a typical fatty acid β -oxidation cycle, a fatty acyl-CoA thioester first undergoes β -dehydrogenation catalyzed by an acyl-CoA dehydrogenase. Then, an enoyl-CoA hydratase catalyzes the hydration of the 2-enoyl-CoA resulting in a 3-hydroxyacyl-CoA ester. Next, oxidation of the hydroxyacyl-CoA is catalyzed by a hydroxyacyl-CoA dehydrogenase to produce a 3-ketoacyl-CoA ester. Last, the 3-ketoacyl-CoA undergoes retro-Claisen cleavage catalyzed by a β -ketothiolase (FadA) to release an acetyl-CoA and regenerate a two-carbon shortened acyl-CoA thioester.

The genes which encode enzymes involved in the cholesterol side chain catabolism in Mtb genome are transcriptionally regulated by the repressor KstR1.^{14, 10} In this work we sought to identify and characterize cholesterol side chain degrading enzymes from the KstR1 regulon in Mtb. Among the essential genes required for Mtb's growth on cholesterol in vitro, we investigated the catalytic activity of the gene products of Rv3538 and Rv3502c, which we will refer to as *chsH3* and *chsB1* respectively to emphasize their newly established function in Mtb cholesterol side chain degradation. We employed biophysical, biochemical and steady-state kinetic characterizations to determine the role of ChsH3 and ChsB1 in cholesterol side chain degradation. Furthermore, we characterized the structure of ChsB1 through crystallography to further understand the steroid hydroxyl dehydrogenation process in Mtb.

Our data clearly demonstrate that ChsH3 is a MaoC-like family hydratase catalyzing stereospecific hydration of 3-oxo-chol-4,22-dien-24-oyl-CoA (3-OCDO-CoA), the enoyl-CoA formed in the second cycle of cholesterol side chain β -oxidation (Scheme 1). ChsH3 catalyzes formation of the 22*S* diastereomer of 22-hydroxy-3-oxo-cholest-4-en-24-oyl-CoA (22-HOCO-CoA) whereas the previously reported EchA19, also in the KstR1 regulon, catalyzes formation of the 22*R* diastereomer. Furthermore, ChsB1 is the 3-hydroxy acyl-CoA dehydrogenase

responsible for catalyzing dehydrogenation of the ChsH3 22-HOCO-CoA product, and does not catalyze dehydrogenation of the EchA19 22-HOCO-CoA product. The results presented highlight the unique assembly of β -oxidation enzymes that Mtb utilizes to metabolize sterol side chains. The discovery of stereospecific ChsH3 and EchA19 enzymes that utilize the same substrate indicate a new branch of the cholesterol side chain degradation pathway that may extend beyond core β -oxidation catabolic reactions.



Scheme 1. Proposed reactions in the second β -oxidation cycle of the Mtb cholesterol side chain catabolism.

Results

Through bioinformatic analyses, *chsB1* is the only gene in the KstR1 regulon that is annotated to encode a short-chain type alcohol dehydrogenase/reductase (SDR) or a 17- β -hydroxysteroid dehydrogenase and *chsH3* is the last uncharacterized hydratase encoded in the KstR1 regulon. Given their gene loci, common regulation, and bioinformatic annotations, we hypothesized that ChsB1 and ChsH3 are involved in the sterol side chain degradation in Mtb. Therefore, we undertook biophysical, biochemical, and structural characterizations of these two proteins to elucidate the specific steps in Mtb sterol metabolism catalyzed by these enzymes.

ChsH3 is a dimeric enoyl-CoA hydratase and preferentially catalyzes the hydration of the five-carbon sterol intermediate 3-OCDO-CoA.

Construct *pchsH3* (Table S1) was expressed heterologously in *R. jostii* RHA1 to provide ChsH3 which was isolated by ion affinity chromatography (IMAC) and further purified by gel filtration (Figure S1). To determine the oligomeric state of the purified ChsH3 in solution state, we performed analytical ultracentrifugation (AUC) sedimentation velocity analysis. The molecular weight determined by AUC indicated that ChsH3 forms a homodimer in solution (Figure S2).

We then assayed ChsH3 for hydratase activity using different substrates: *trans*-2-octenoyl-CoA, 3-oxo-pregna-4,17-diene-20-carboxyl-CoA (3-OPDC-CoA), 3-OCDO-CoA, and 3-oxo-cholest-4,24-dien-26-oyl-CoA (3-OCDS-CoA) (Figure 1A). The latter three cholesterol metabolites are the enoyl-CoA species from the three separate β -oxidation cycles in the proposed cholesterol side chain degradation pathway. The hydration reactions were monitored spectrophotometrically, and the formation of the hydrated products was further confirmed by matrix assisted laser desorption ionization (MALDI) mass spectrometry. ChsH3 was able to bind and catalyze the hydration of bulky steroid enoyl-CoA esters, as well as octenoyl-CoA which is a medium-length fatty acyl ester. The highest activity was observed in the reactions with 3-OCDO-CoA (Figure 1B). ChsH3 also displayed high catalytic activity with octenoyl-CoA, and modest activity with 3-OCDS-CoA (Figure 1B).

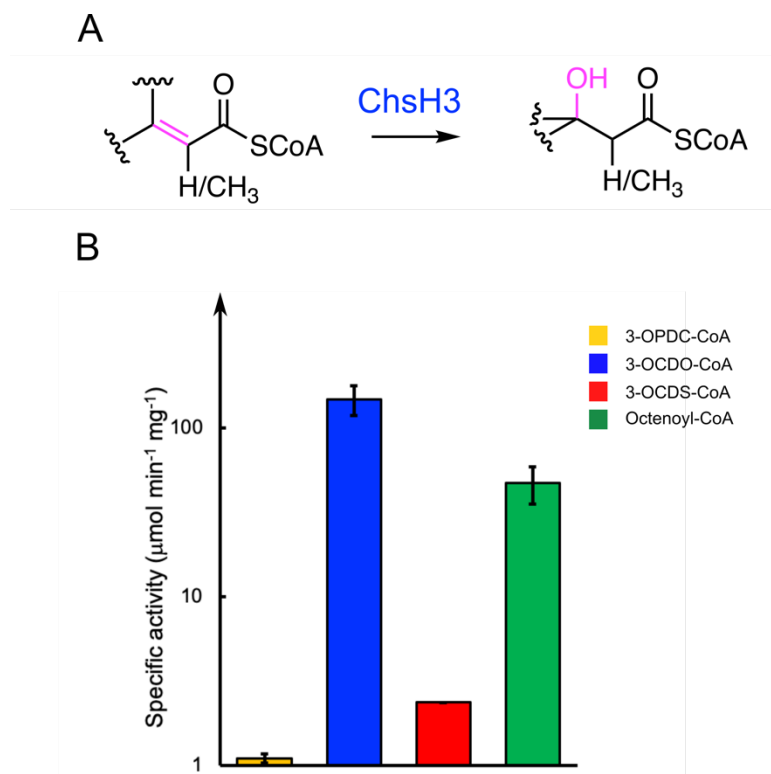


Figure 1. Specific activities of ChsH3 with enoyl-CoA substrates. A. Reaction scheme for hydration catalyzed by ChsH3. B. 3-OPDC-CoA, 3-OCDO-CoA, 3-OCDS-CoA are the three-carbon, five-carbon, and eight-carbon enoyl-CoAs generated during cholesterol side-chain degradation. ChsH3 was assayed with 3-OPDC-CoA, 3-OCDO-CoA, 3-OCDS-CoA, and octenoyl-CoA at 50 μM. The specific activity was measured as the initial velocity of the first 10% of the hydration reaction.

We then determined the steady state kinetic rate constants for ChsH3 with 3-OCDO-CoA, octenoyl-CoA, and 3-OCDS-CoA as substrates. ChsH3 catalyzed the hydration of 3-OCDO-CoA most efficiently with an apparent second order rate constant k_{cat}/K_m that is 15 times higher than for 3-OCDS-CoA and 2 times higher than for octenoyl-CoA. Thus, by examining the substrate specificity of ChsH3 with the enoyl-CoA thioesters of the cholesterol side chain degradation intermediates and fatty enoyl-CoA, we established a role for ChsH3 in the second β-oxidation cycle of cholesterol side chain catabolism in Mtb.

Table 1. Steady-state kinetic parameters for ChsH3 with enoyl-CoA thioesters

Substrate	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\text{M}^{-1} \text{s}^{-1}$)
3-OCDO-CoA	55 ± 5	113 ± 4	$(2.0 \pm 0.2) \times 10^6$
Octenoyl-CoA	112 ± 12	164 ± 8	$(1.5 \pm 0.2) \times 10^6$
3-OCDS-CoA	12 ± 6	1.5 ± 0.7	$(1.2 \pm 0.1) \times 10^5$

ChsH3 is a MaoC-like stereospecific enoyl-CoA hydratase.

Amino acid sequence analysis of ChsH3 yields high similarity to the PLN-2864 enoyl-CoA hydratase superfamily. This superfamily of proteins consists of a C-terminal hot-dog fold, a characteristic of the MaoC domain which is known to preferentially catalyze the hydration of 2-enoyl-CoA esters in a stereospecific manner.¹⁵ In addition, sequence alignment of ChsH3 with other MaoC-like hydratases across species revealed the conserved active site residues Asp189 and His194 are present in ChsH3 (Figure S3).

We previously reported that EchA19 is an enoyl-CoA hydratase that is regulated by the KstR1 repressor and has been implicated in the cholesterol side chain catabolism in Mtb, where it preferentially catalyzes the hydration of 3-OCDO-CoA among other sterol enoyl-CoA esters.¹⁶ EchA19, structurally unlike ChsH3, possesses a canonical crotonase-like structure and is a member of the crotonase (PRK-07799) superfamily, which is known to catalyze the hydration of enoyl-CoAs with an opposite stereochemistry to the MaoC-like family.

To further investigate the role of ChsH3 and EchA19 in the cholesterol side chain degradation pathway and the validity of their functional assignments, we characterized and compared the enzymatic products of the MaoC-like ChsH3 with the products of the crotonase EchA19. Analysis of the purified products of both enzymatic reactions by MALDI mass spectroscopy confirmed that both EchA19 and ChsH3 performed the hydration reaction on 3-

OCDO-CoA resulting in a product that was 18 Da greater than the starting material, 1136.37 (m-H/z) from 1118.36 (m-H/z) (Figure 2B).

The purified products from the individual reactions were lyophilized and subsequently subjected to nuclear magnetic resonance (NMR) spectroscopic analysis. ^1H -NMR experiments were carried out in deuterium oxide. Analysis of spectra from 3-OCDO-CoA and 22-HOCO-CoA from EchA19 and ChsH3 showed the disappearance of olefinic peaks from the δ 5-6.5 ppm region and the appearance of three new protons in the δ 2.5-4.5 ppm region which correspond to protons on C22 and C23 (Figure 2A), β and α to the thioester, respectively (Figure 2C). Comparison of the ^1H -NMR spectra of the resulting 22-HOCO-CoA from ChsH3 and EchA19 confirmed that the two molecules were in fact configurationally different.

A 2D ^1H - ^1H correlation spectroscopy (COSY) experiment was then carried out in order to assign the relevant peaks, and J coupling constants were determined to assign stereochemical configuration. The configuration at the C20 position (γ -carbon) is *S* in cholesterol and the conformation between C17 and C20 is essentially locked due to a high rotational barrier (Figure 2A). Thus, the dihedral angle between the γ and β protons that determines the J coupling constant is not rotationally averaged, and the J coupling may be used to determine the absolute configuration of each diastereomer. The γ and β J coupling constants were determined to be 1.3 ± 1 Hz for the ChsH3 product and 10.2 ± 1 Hz for the EchA19 product. Staggered γ - β Newman projections for the lowest energy conformer for each stereoisomer reveal a larger dihedral angle for the γ - β protons in the 22*R*-product, near 180° , vs 60° for the 22*S*-product. In accordance with the Karplus equation the 22*R*-product is predicted to have a larger J coupling constant (Figure 2D), whereas the 22*S*-product is anticipated to have a smaller J coupling constant. Comparison to the ^1H -NMR spectra of the enzymatic products revealed that ChsH3 produces (22*S*)-HOCO-CoA

and EchA19 produces (22*R*)-HOCO-CoA. These findings confirmed the stereochemical assignment of each enzymatic product from ChsH3 and EchA19 and suggested different roles of each enzyme in the metabolism of the cholesterol side chain in Mtb.

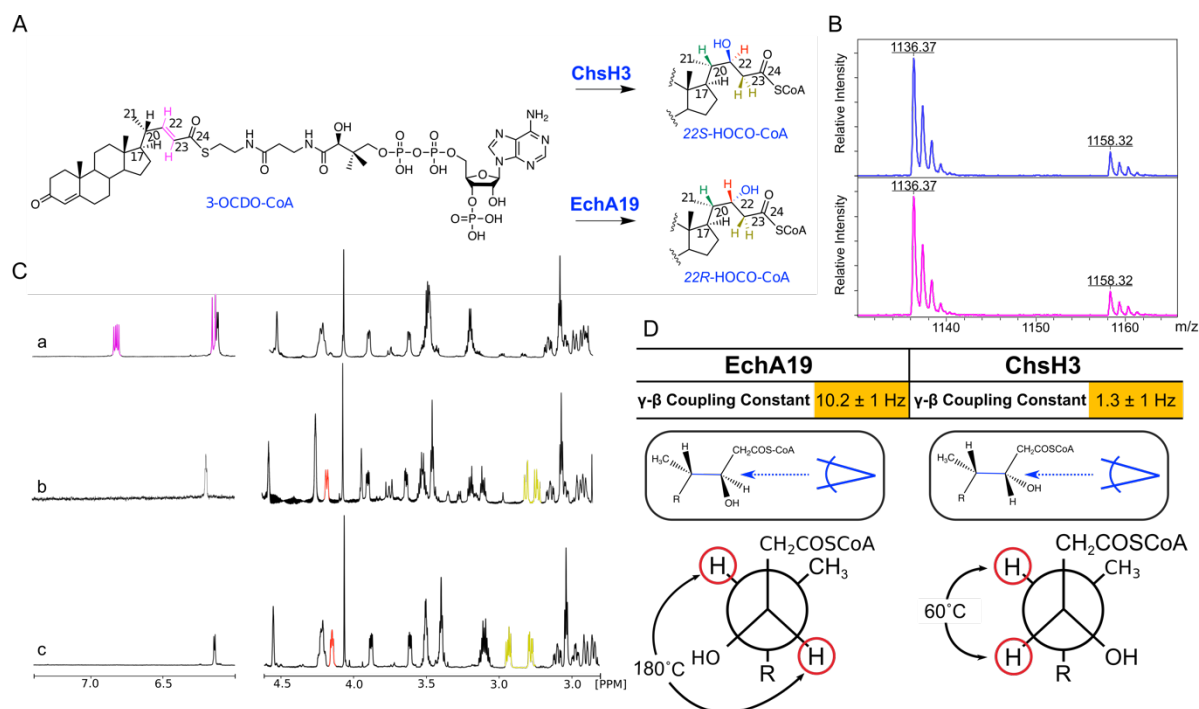


Figure 2. Analytical chemical characterizations of the hydration products from 3-OCDO-CoA catalyzed by ChsH3 or EchA19. A. ChsH3 and EchA19 produce hydration products with different stereochemistry. B. MALDI spectra of isolated hydration products of ChsH3 (blue) and EchA19 (pink) with 3-OCDO-CoA. $[M-H]^- = 1136.37$ m/z, $[M+Na-2H]^- = 1158.32$ m/z. C. 1H -NMR spectra of isolated (5 mM) (a) 3-OCDO-CoA, (b) (22*R*)-HOCO-CoA, and (c) (22*S*)-HOCO-CoA. Olefinic protons are colored pink, C22 proton is colored red, and C23 protons are labeled in yellow. D. J -coupling constants between the C20 and C22 protons were used to estimate H20-C20-C22-H22 dihedral angle and to determine the absolute configuration of the products of the EchA19 and ChsH3 hydration reactions. Staggered, lowest energy conformer Newman projections along the C20-C22 bond are depicted.

The identical substrate specificity but opposite stereospecificity of ChsH3 and EchA19 prompted us to assess whether these two proteins also exist across steroid utilizing actinobacteria. Through BLAST we queried the protein sequences of ChsH3 and EchA19 against the nonredundant protein sequence database. We found that all the organisms belonging to Corynebacterineae under Actinobacteria, which possess homologues of ChsH3 with identities

over 60%, have homologues of EchA19 with protein identities over 60% present as well (Figure S4), or vice versa. This observation indicates that the existence of stereochemistry-driven divergence in the steroid degradation pathway is likely to be conserved across the actinobacteria.

ChsB1 is a stereospecific hydroxyacyl-CoA dehydrogenase that preferentially catalyzes the dehydrogenation of a hydroxyl cholesterol CoA ester metabolic intermediate.

Construct *pchsB1* (Table S1) was expressed heterologously in *R. jostii* RHA1 to provide ChsB1 which was isolated by IMAC and further purified by gel filtration (Figure S1). AUC sedimentation velocity analysis was performed to determine the oligomeric state of ChsB1 in solution. The result indicated a homodimeric quaternary structure of ChsB1 in solution (Figure S2).

Protein sequence analysis of ChsB1 revealed that ChsB1 shared homologies with the hydroxyacyl-CoA dehydrogenases in the multifunctional enzymes type-2 (MFE-2) family from fungi and mammals. Typically, these enzymes are hydroxyacyl-CoA dehydrogenases (HAD) with stereospecificity for the fatty acyl *R* diastereomer. To characterize the biochemical function of ChsB1, the recombinant protein was assayed for dehydrogenase activity using NAD⁺ as the cofactor. Each 22-HOCO-CoA diastereomer, isolated from ChsH3 and EchA19 reactions, was individually tested as the substrate. The reactions were monitored spectrophotometrically. The formation of NADH at 340 nm was only observed when (22*S*)-HOCO-CoA from the ChsH3 catalyzed reaction was used as the substrate (Figure 3A). This reactivity was also confirmed by the mass shift of -2 Da in the MALDI mass spectrum of (22*S*)-HOCO-CoA, while the EchA19 product, (22*R*)-HOCO-CoA, was not oxidized (Figure 3B). When NADP⁺ was assayed as the

cofactor with (22*S*)-HOCO-CoA at 50 μ M, minimal activity was observed for ChsB1, indicating ChsB1 utilizes NAD⁺ as its cofactor.

As ChsB1 was also annotated as a 17 β -hydroxysteroid dehydrogenase, we then spectroscopically examined its activity against 17 β -hydroxyandrost-4-en-3-one and 17 α -hydroxyandrost-4-en-3-one. The concomitant formation of NADH was only observed with 17 β -hydroxyandrost-4-en-3-one but not 17 α -hydroxyandrost-4-en-3-one.

In order to understand the substrate specificity, we determined the apparent steady-state rate constants for ChsB1 with (22*S*)-HOCO-CoA, (3*R*)-hydroxyoctanoyl-CoA, and 17 β -hydroxyandrost-4-en-3-one by varying one substrate at a fixed saturating concentration of the second substrate. ChsB1 showed a high substrate specificity towards (22*S*)-HOCO-CoA with an apparent second order rate constant k_{cat}/K_m that is 100 times higher than for (3*R*)-hydroxyoctanoyl-CoA. Although ChsB1 was able to catalyze the dehydrogenation of 17 β -hydroxyandrost-4-en-3-one, the k_{cat}/K_m is more than 3 orders of magnitude lower than for (22*S*)-HOCO-CoA (Table 2).

To further characterize the reactivity of ChsB1, we assayed the reverse 3-keto reductase activity using NADH as the cofactor. ChsB1 was able to catalyze the reverse 3-keto reduction of 3,22-dioxo-cholest-4-en-24-oyl-CoA. However, the forward dehydrogenation is strongly favored over the reverse reduction reaction. As compared spectrophotometrically, for the reverse reaction, the apparent k_{cat}/K_m is $(6.3 \pm 0.1) \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$ for 3,22-dioxo-cholest-4-en-24-oyl-CoA and $(4.1 \pm 0.1) \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$ for NADH. The calculated K_{eq} is $(1.1 \pm 0.1) \times 10^2$ indicating the oxidation product is thermodynamically favored.

With such a high catalytic efficiency, it is evident that ChsB1 functions as a sterol hydroxyacyl-CoA dehydrogenase in cholesterol side chain degradation in Mtb.

We next determined the bi bi reaction kinetic mechanism of ChsB1 by measuring initial velocities at varying concentrations of (22*S*)-HOCO-CoA and NAD⁺. The steady-state kinetics of ChsB1 with (22*S*)-HOCO-CoA and NAD⁺ followed a compulsory ordered bi-bi mechanism as determined by the best global fit of the initial velocities to the steady state bi-bi kinetic model assessed by coefficient of determination (R^2) values. A binary complex of ChsB1 and NAD⁺ is formed first, followed by the ternary complex formation upon binding of (22*S*)-HOCO-CoA. Catalysis then occurs via hydride transfer from the steroid substrate to the cofactor, after which, the keto ester dissociates from the ternary complex and NADH is released from the enzyme.¹⁷ The kinetic parameters obtained from global fitting are similar to the apparent kinetic parameters obtained by varying one substrate at a fixed saturating concentration of the second substrate (Table 2).

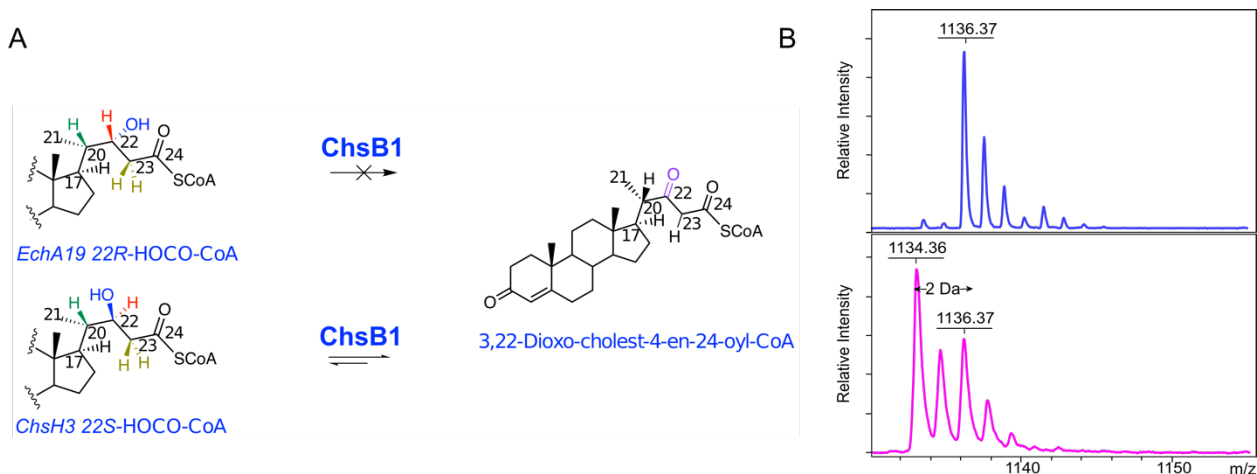


Figure 3. The ChsB1 dehydrogenation reaction and compatibility with diastereomeric substrates. A. Stereospecificity of the ChsB1-catalyzed dehydrogenation reaction. B. MALDI spectra of the ChsB1 catalyzed dehydrogenation reactions using ChsH3-produced (pink) and EchA19-produced (blue) 22-HOCO-CoA as the substrate, $[M-H]^- = 1136.37$ m/z.

Table 2. Steady-state kinetic parameters for ChsB1

Substrate	K_m (μM)		k_{cat} (s^{-1})	k_{cat}/K_m ($\text{M}^{-1} \text{s}^{-1}$)
	Hydroxyl substrate	NAD^+		
(22 <i>S</i>)-HOCO-CoA	6.2 ± 1	47 ± 3	9.0 ± 0.5	$(1.5 \pm 0.2) \times 10^6$
(3 <i>R</i>)-Hydroxyoctanoyl-CoA	169 ± 21	162 ± 18	2.5 ± 0.1	$(1.5 \pm 0.2) \times 10^4$
17 β -hydroxyandrost-4-en-3-one	369 ± 75	99 ± 9	0.30 ± 0.03	$(8.1 \pm 2) \times 10^2$

ChsB1 crystallizes as a homodimer with a classic Rossmann-fold motif but with a larger substrate binding cavity than homologues.

Needle-like ChsB1 crystals were obtained using the hanging drop vapor diffusion method. The structure was solved using the molecular replacement method and refined to a resolution of 2.03 Å (Table 3). The apo-structure of ChsB1 revealed a homodimeric structure within a single asymmetric unit (Figure 4A). Ample electron density allowed the structure to be built out and 539/634 (85%) amino acids of the dimeric structure were modeled. The substrate binding loop, residues 217 to 230, which is known to be variable among HADs in order to accommodate a wide range of substrates accepted by SDR proteins, was the only intra-protein section that was not explicitly modeled due to a high degree of flexibility of this loop, while the remaining absent residues were N- and C-terminal.

The overall architecture of ChsB1 resembles its SDR homologues across a variety of organisms including yeast, fungus and mammal, as evidenced by the two-tandem repeat β/α Rossmann fold in which a β -sheet is situated in-between two sets of α -helices – typical of nucleoside cofactor binding proteins (Figure 4B). Interestingly, the Rossmann fold of ChsB1 and homologous HADs are different than the typical Rossmann motif, which maintains only six parallel β -strands in the distinct order of $\beta 3$ - $\beta 2$ - $\beta 1$ - $\beta 4$ - $\beta 5$ - $\beta 6$ (Figure 4B). ChsB1 and homologues consist of a central β -sheet with two additional C-terminal β -strands, $\beta 7$ and $\beta 8$, and

one additional $\beta 9/\alpha 11$ repeat motif (Figure 4B). The $\beta 9/\alpha 11$ repeat motif is extended outward and $\beta 9$ is incorporated into the β -sheet of the second monomer of the dimeric structure while $\alpha 11$ interacts with $\alpha 10$ of the second monomer via van der Waals interactions between $\alpha 11$ -(2) Ser243 and $\alpha 10$ -(1) Pro289, and $\alpha 11$ -(2) Phe247 and $\alpha 10$ -(1) Thr293 and Leu296 (Figure S5). This conserved structural characteristic of SDR family HADs contributes to the overall quaternary structure by providing an almost knot-like interface between individual monomers. The two largest helices, $\alpha 7$ and $\alpha 9$, which are parallel, form the dimeric interfacial region and consist of the largest surface area contact between monomers (Figure 4B). The orientation of the two monomers in relation to one another can be described by a mirror plane reflection followed by 180° rotation, resulting in the two sets of $\alpha 7$ and $\alpha 9$ helices having an antiparallel interaction. The geometric transformations of the monomers place the active sites on opposite sides of the structure, enabling catalytic activity on two faces.

Table 3. Data collection and refinement statistics

	ChsB1	ChsB1:NAD ⁺
Data collection		
Space group	<i>P 21 21 21</i>	<i>C2</i>
Unit cell dimensions		
<i>a</i> , <i>b</i> , <i>c</i> (Å)	44.85, 80.13, 163.53	84.66, 84.23, 47.34
α , β , γ (°)	90.00, 90.00, 90.00	90.00, 116.19, 90.00
Diffraction limits (Å)	2.00, 2.74, 2.52	
Eigenvector-1	1.00, 0.00, 0.00	
Eigenvector-2	0.00, 1.00, 0.00	
Eigenvector-3	0.00, 0.00, 1.00	
Wavelength	0.9793	0.9793
Resolution (Å)	81.77-2.03 (2.31-2.03) ^a	42.48-2.21 (2.25-2.21) ^a
<i>R</i> _{merge} (%)	0.20 (1.13)	0.15 (0.78)
<i>CC</i> _{1/2}	0.991 (0.665)	0.995 (0.739)
<i>I</i> / σI	6.8 (1.8)	9.4 (2.3)
Completeness (%)	93.1 (68.7)	100.0 (100.0)
Redundancy	5.5 (6.1)	6.9 (5.6)

Refinement		
Resolution (Å)	81.77-2.03	42.12-2.21
No. reflections	122908	103397
$R_{\text{work}} / R_{\text{free}}$	21.20/24.99	16.70/20.89
Ramachandran analysis		
Favored (%)	95.66	96.38
Allowed (%)	4.34	3.62
Outliers (%)	0	0
PDB ID	7LG9	7LGB

^aValues in parentheses are for the highest-resolution shell

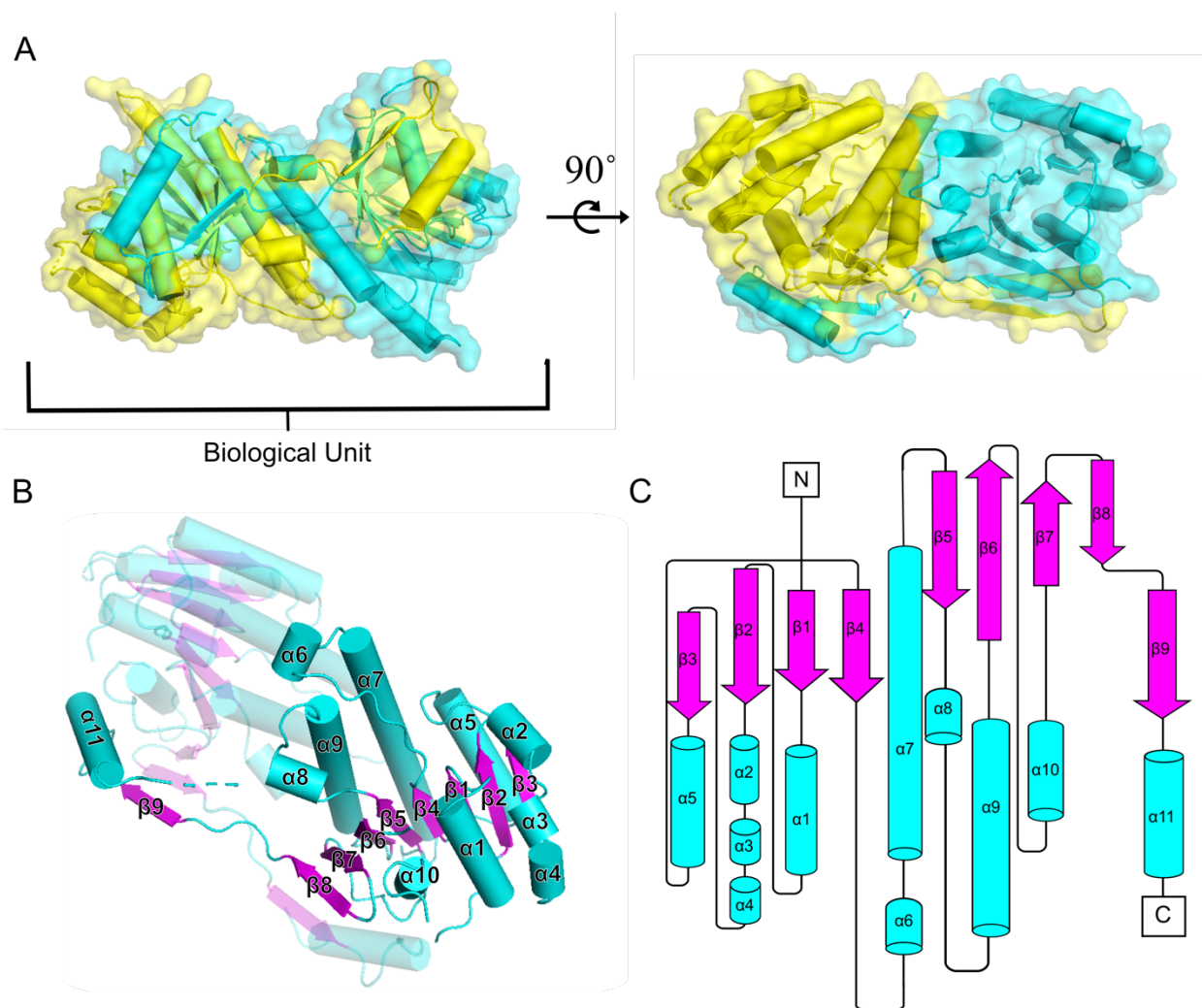


Figure 4. Overall atomic presentation of ChsB1. A. The biologically functional unit is a homodimer with two active sites. B. Each monomer is shown as cylindrical cartoons and colored by secondary structures with the identities of chains labeled. C. Topology of one monomer is presented in the same color coding in panel B. Secondary structure was assigned using Pro-Origami.¹⁸

The characteristic catalytic triad of SDRs, [His132]-Ser168-Tyr181-Lys185, is conserved in ChsB1 (Figure 5) and is spatially adjacent to the Gly-X-Gly-X-X-Ala NAD⁺ cofactor binding motif¹⁹ found in the loop between α 1 and β 1 (Figure 4B and 4C). Sequence alignment shows high conservation of residues across the main structural features, β -strands and α -helices, among ChsB1 and its HAD homologues (Figure 5 and S6). The greatest source of amino acid identity variability exists in the substrate binding loop and the final motifs, β 9 and α 11 which play a large role in the monomer-monomer interface and greatly contribute to the variability that is observed in the quaternary structure of the HAD homologues (Figure S6). The protein surface forms a large groove around the active sites, of nearly 30 Å, that leads into a slightly deeper cavity, nearly 10 Å deep where the catalytic residues lie. In comparison to its homologues, whose substrate binding cavities vary greatly in their dimensions, the binding groove of ChsB1 is of greater length and is largely exposed – likely to accommodate bulky [sterol-type] substrates. Structural alignment of ChsB1 and its homologues revealed that, although the NAD⁺ binding sites are largely conserved, the length of the substrate binding pocket is linked to the length of the β 8 strand regardless of the identity of the individual residues and their order (Figure S6). Across species, the length of the substrate binding pocket had an inverse relationship with the number of residues in the β 8 strand; ChsB1 β 8 for example contains five residues and had the longest substrate binding pocket length at around 30 Å, while its human homologue (PDB: 1ZBQ) contained nine residues and had a substantially shorter substrate binding pocket length at nearly 23 Å.

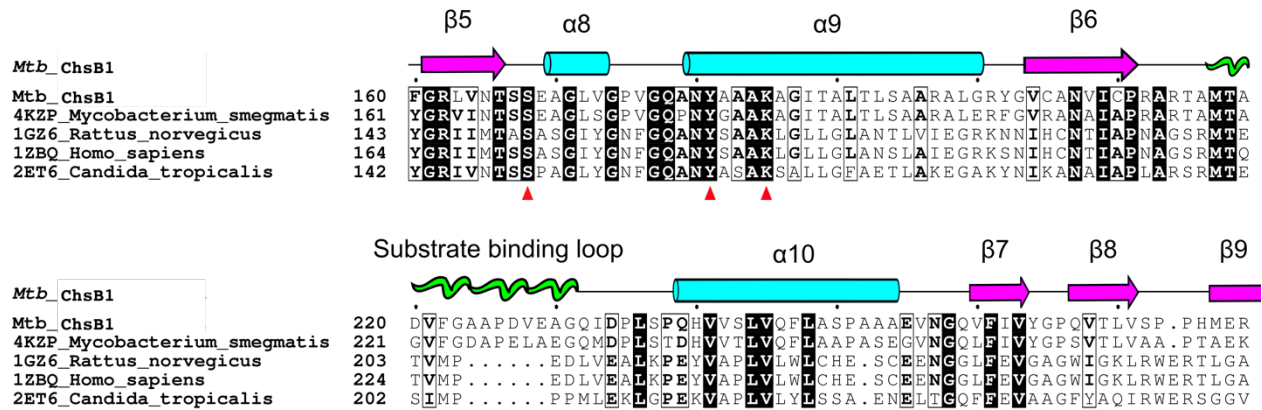


Figure 5. Sequence alignment of ChsB1 (Rv3502c) to homologous 3(R)-hydroxyacyl-CoA dehydrogenase proteins with crystal structures (PDB IDs 1ZBQ Human, 4KZP *M. Smeg*, 1GZ6 *Rattus Norvegicus*, and 2ET6 *C. Tropicalis*). Sequence alignment was performed using Clustal Omega²⁰ online server and visualization tools from Esript 3.0.²¹ Secondary structural motifs were assigned based on the crystal structure of ChsB1 and are shown above the set of sequences with α -helices shown as cylinders and β -strands shown as arrows. Active site residues and the catalytic Ser-Tyr-Lys triad are annotated with a red triangle below the aligned sequences. The substrate binding loop, which was identified by comparison to homologous proteins²² is additionally demarcated in green.

ChsB1 forms a complex with NAD⁺ cofactor.

Co-crystallization of NAD⁺ and ChsB1 yielded cubic crystals by sitting drop vapor diffusion and diffracted to a resolution of 2.21 Å (Table 3). Molecular replacement was used to solve the co-crystal structure using the apo-protein structure as the template. Each monomer of the homodimer of ChsB1/NAD⁺ represents an asymmetric unit. The missing residues from 218-223 within the substrate binding domain in the apo protein have been successfully modeled in the co-crystal structure. The substrate binding domain forms a short α -helix, α 9' between β 6 and α 10, which wraps on top of the Rossmann fold motif to close the lid for binding NAD⁺ (Figure 6A). Residues 68 to 70 were missing in the holo-protein structure, thus α 4 helix is missing from the structure (Figure 6A). In addition, β 9 in the apo structure is replaced by a flexible loop in the holo-protein structure (Figure 6A).

The NAD⁺ cofactor occupies two conformations in the binding site (Figure 6B), which is likely due to the high flexibility of the molecule caused by the absence of the hydroxyacyl-CoA ester substrate in the binding pocket. Conformation A is the predominant conformation. The binding of NAD⁺ did not result in a large quaternary structural change. The ligand is bound between two surfaces, $\alpha 3$ - $\alpha 1$ - $\alpha 10$ and $\alpha 5$ - $\alpha 7$ - $\alpha 9$, and is in apposition to the $\beta 1$ - $\beta 4$ - $\beta 5$ - $\beta 6$ sheet. The adenine-ribose moieties of the two NAD⁺ conformations are almost identical, while the diphosphate moieties differ from each other - conformation A points toward $\alpha 1$ and conformation B points away. The adenine nucleotides of the two NAD⁺ conformations are in an anti-conformation and the ribose rings are in C₂'-endo puckering conformation. The active triad residues are located close to the nicotinamide moiety of NAD⁺ (Figure 6B). In conformation A, the nicotinamide amide moiety is pointing towards the active sites, while in conformation B the amide is facing away.

Hydrogen bonding interactions (3 Å cutoff) between NAD⁺ and the binding pocket residues were analyzed (Figure 6C). The adenine has strong hydrogen bonding interactions with the side chain of Asp82 and the backbone of Ile83. The nicotinamide ribose moiety in conformation A is in close contact with the catalytic triad residues with Lys185 and Tyr181 forming hydrogen bonds with the O4 and O3 hydroxyl groups and Ser168 forming hydrogen bond with the amino group. In contrast, in conformation B, only is within 3 Å with the O3 hydroxyl groups of the ribose.

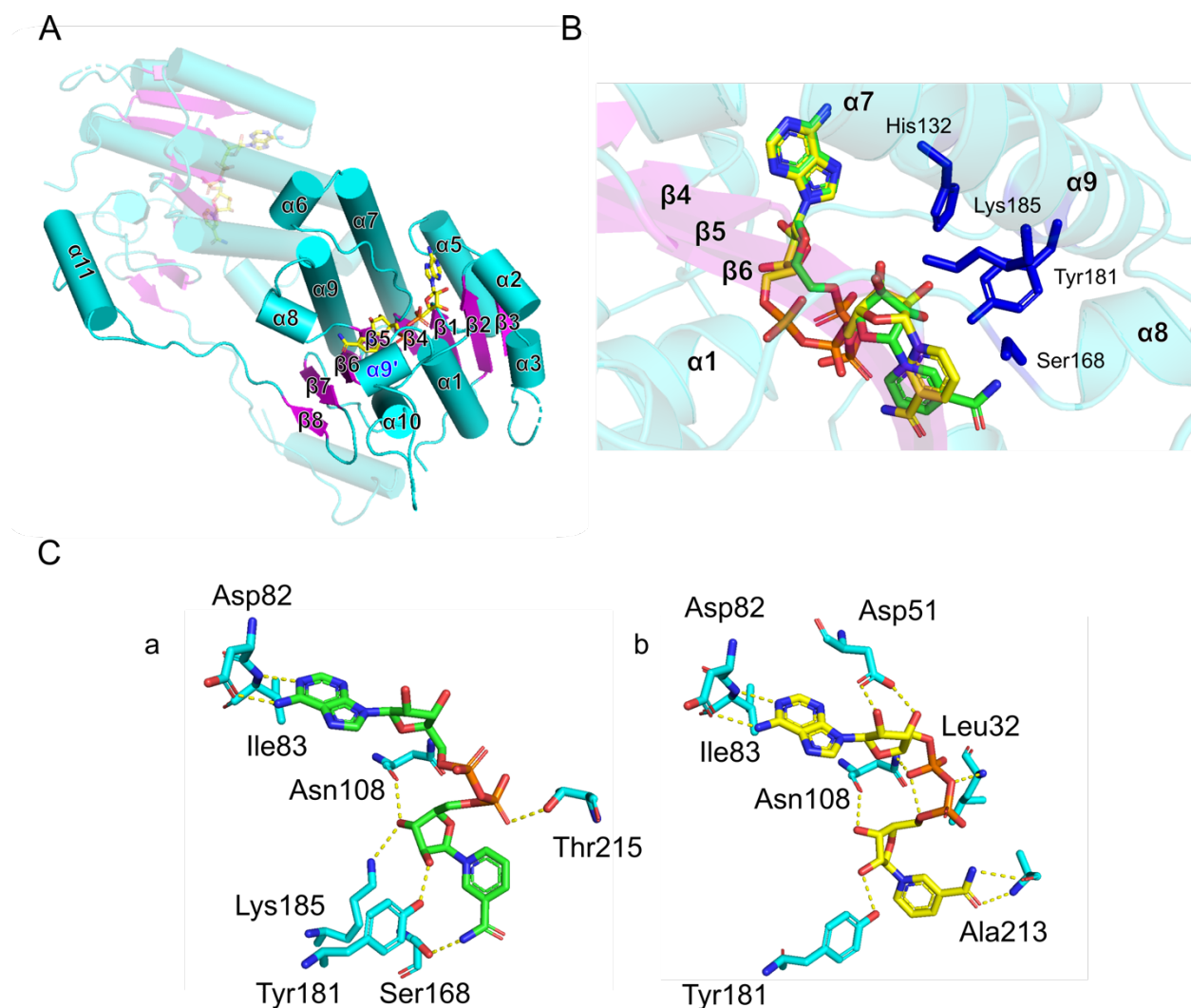


Figure 6. ChsB1 complexed with NAD⁺. A. Each monomer of ChsB1 has one substrate binding site. The homodimer is shown as cylindrical cartoons and colored by secondary structures with the identities of chains labeled. The missing substrate binding domain residue 218-213 in the apo-protein structure is $\alpha 9'$. NAD⁺ is rendered as sticks and colored by atom. B. Two conformations of NAD⁺ were observed. Conformation A (green) is the predominant conformation, and conformation B is yellow. The catalytic triad residues and His132 are shown as blue sticks. C. Hydrogen bonding interactions (3 Å cutoff) between NAD⁺ and ChsB1 are presented. Conformation A of NAD⁺ is labeled green and conformation B is labeled yellow.

Protein sequence analysis by CoABind²³ identified distinct CoA binding residues/motifs in ChsB1. The predicted CoA binding residues mainly reside in the flexible loops between $\beta 4$ and $\alpha 6$, and between $\alpha 8$ and $\alpha 9$ (Figure S7). An acetoacetyl-CoA was modeled into the structure of ChsB1 with NAD⁺ based on a co-crystal structure of a ChsB1 homologue (PDB: 4N5M) (Figure S7). The CoA motif in the ligand matches well with the CoA binding residues predicted

by CoABind. This observation further validates the utilization of a hydroxyacyl-CoA substrate in the reaction catalyzed by ChsB1.

Discussion

The cholesterol degradation pathway in sterol-utilizing actinomycetes bifurcates at multiple stages through catalysis of duplicative chemistry with different stereochemical outcomes. In the terminal C26 oxidation of cholest-4-en-3-one, Cyp142 and Cyp125 both catalyze stereospecific terminal C26 oxidation and their products are diastereomers of cholest-4-en-3-one-27-oic acid with opposite stereochemistry at C25 (Scheme 2).²⁴⁻²⁵

In the first cycle of side chain β -oxidation, ChsE4-ChsE5 is stereospecific and catalyzes the dehydrogenation of (25*S*)-3-OCS-CoA.²⁶⁻²⁷ A methyl acyl-CoA racemase, MCR, catalyzes epimerization of the C25 diastereomers of 3-OCS-CoA which allows metabolic flux of both diastereomers into the cholesterol catabolic pathway. Alternative fates of the (25*R*)-3-OCS-CoA are unknown.

In the second β -oxidation cycle (Scheme 2), ChsE3 catalyzes dehydrogenation of 3-oxo-cholest-4-en-24-oyl-CoA (3-OCO-CoA) to 3-OCDO-CoA (Scheme 2).²⁶ Here we demonstrate that ChsH3 catalyzes the hydration of 3-OCDO-CoA to form (22*S*)-HOCO-CoA. ChsB1 catalyzes the oxidation of the (22*S*)-HOCO-CoA to form a ketoacyl-CoA ester. FadA5 catalyzes the retro-Claisen cleavage of the five-carbon ketoacyl-CoA ester intermediate, 3,22-dioxo-cholest-4-en-24-oyl-CoA, to produce a three-carbon acyl-CoA ester cholesterol metabolite, 3-oxo-4-pregnene-2-carboxyl-CoA, and an acetyl-CoA.²⁸

Previously, we demonstrated that EchA19 preferentially catalyzes the hydration of 3-OCDO-CoA compared to the other enoyl-CoA intermediates of cholesterol side chain catabolism.¹⁶ Here

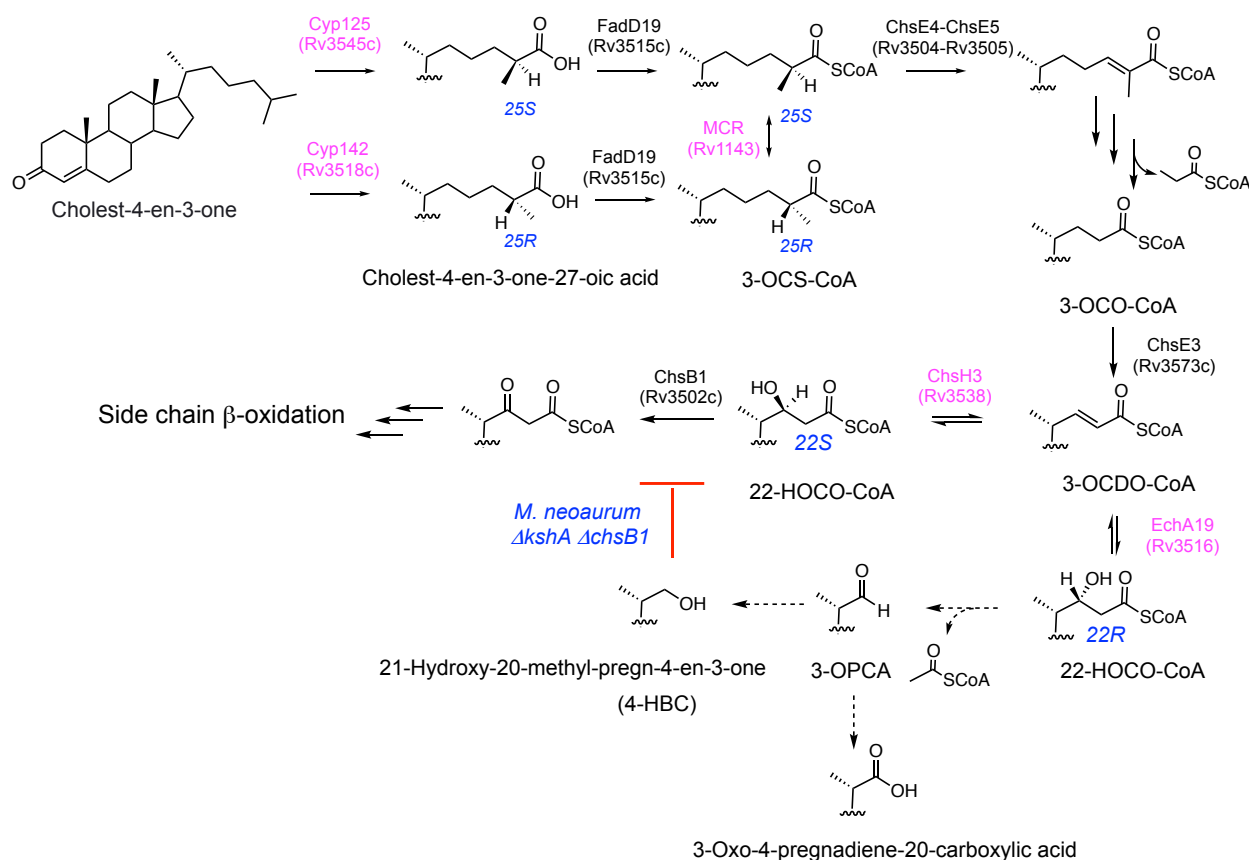
we find that stereochemical outcome of the EchA19-catalyzed hydration is opposite to that of ChsH3, and (22*R*)-HOCO-CoA is produced.

Prior to our work, Xu et al. proposed that the *M. neoaurum* ortholog of Rv3502c is the 3-hydroxyacyl-CoA dehydrogenase in the second β -oxidation cycle of cholesterol side chain degradation, although catalytic activity with the 22-HOCO-CoA substrate was not tested biochemically.²⁹ Deletion of the ChsB1 (Rv3502c) ortholog in mutant strain *M. neoaurum* ATCC 25795 (which also has impaired steroid ring degradation activity) results in accumulation of a three-carbon terminal hydroxyl cholesterol metabolite, 21-hydroxy-20-methylpregn-4-en-3-one (4-HBC), when the mutant is grown on cholesterol.²⁹ Xu et al. proposed that the identity of the accumulated metabolite does not directly correlate to the substrate of the deleted 3-hydroxyacyl-CoA dehydrogenase and that the ChsB1 substrate is rerouted through an alternate metabolic pathway to form 4-HBC upon deletion of *chsB1*.

The 4-HBC metabolite from the *chsB1* knockout strain of *M. neoaurum* ATCC 25795 is likely to be accumulated only when cholesterol ring degradation is deactivated. With equilibrium constants near one, hydratase reactions are usually reversible under physiological substrate concentrations. Upon deletion of *chsB1*, the manifold of (22*S*)-HOCO-CoA conversion to ketoacyl-CoA will be blocked and the flux of 3-OCDO-CoA will proceed through EchA19-mediated hydration of 3-OCDO-CoA. It is possible that the (22*R*)-HOCO-CoA produced by EchA19 could undergo carbon-carbon bond cleavage to form an aldehyde, 3-OPCA (Scheme 2), the formation of which can be coupled to a reductase to form the 21-hydroxy-20-methylpregn-4-en-3-one metabolite observed by Xu et al in *M. neoaurum* ATCC 25795.²⁹ Retro-aldol cleavage to form a ketone product occurs in the last cycle of side chain β -oxidation through Ltp2.³⁰⁻³¹ In addition,

aldolytic reactions have been reported in cholate side chain degradation in *Pseudomonas* sp. Chol1.³²

Alternatively, coupling formation of 3-OPCA with a dehydrogenation to form 3-oxo-4-pregnadiene-20-carboxylic acid (Scheme 2) would aid formation of the thermodynamically unfavorable aldehyde from an aldolase reaction and prevent reaction of the 3-OPCA with nucleophiles in the cell. Subsequent ligation of coenzyme A with 3-oxo-4-pregnadiene-20-carboxylic acid would rescue the metabolite and allow reentry into the cholesterol catabolism pathway. However, such a CoA ligase has not been identified in Mtb. The Mtb genome does not contain an ortholog of *casI*, the 3-oxo-4-pregnadiene-20-carboxylic acid CoA ligase present in actinomycete *R. jostii* RHA1.³³



Scheme 2. Stereochemical bifurcations in Mtb cholesterol metabolism. ChsH3 and EchA19 are hydratases with identical substrate specificities that produce opposite product stereochemistry

at C22. Cyp125 and Cyp142 are cytochrome P450 oxidases with identical substrate specificities that produce opposite product stereochemistry at C25.

chsH3 and *chsB1* are both required for Mtb growth on cholesterol (Table 4),³⁴ indicating the essential functions of these two genes and their gene products in cholesterol catabolism in Mtb. The resulting (22*R*)-HOCO-CoA from EchA19 is not a substrate of ChsB1, and *echA19* is not essential for growth on cholesterol.³⁴ These data suggest a different function than catabolic β -oxidation of cholesterol for this hydratase, a role that has yet to be fully elucidated.

Table 4. Gene essentiality in cholesterol metabolism

	Relative increase in transcription cholesterol/glycerol ¹	Essentiality for growth on cholesterol ²	Essentiality for survival in mice ³
<i>echA19</i>	5.23	nonessential	nonessential
<i>chsH3</i>	3.61	essential	nonessential
<i>chsB1</i>	4.46	essential	essential

¹Transcription up-regulation by cholesterol after 24 h.¹⁴

²Required for in vitro growth in cholesterol media by transposon mapping.³⁴

³Required for growth in C57BL/6J mouse spleen, by transposon site hybridization (TraSH).³⁵

Typically, in bacteria, one hydroxyacyl-CoA diastereomer is used in biosynthesis of polyesters for energy storage and the other in fatty acid degradation for energy production. Genes encoding putative SCP-2 aldolases Ltp3 and Ltp4 are both highly upregulated in Mtb and *R. jostii* RHA1 during growth on cholesterol.^{14, 36} SCP-2 proteins have been shown to have multiple roles in lipid metabolism and intracellular lipid transfer. Typically, the N-terminus of an SCP-2 protein functions as a ketoacyl-CoA thiolase and the C-terminus functions as the sterol carrier domain of the protein that can be proteolytically cleaved and is responsible for intracellular lipid transfer for cell wall or membrane biosynthesis.³⁷⁻³⁸ It is possible that the

EchA19-produced (22*R*)-HOCO-CoA could be transferred and cleaved through these sterol transfer proteins for construction of other lipids in the cell membrane.

The EchA19 catalyzed hydration reaction is reversible, and we previously reported that EchA19 is regulated by succinylation through lysine post translational modification.¹⁶ Since the EchA19 product appears to be the non-productive direction for cholesterol catabolism, a possible role for succinylation might be regulating the flow of cholesterol between catabolism and lipid utilization in the bacterium. ChsH3 and EchA19 are largely conserved in steroid-utilizing actinobacteria indicating the general involvement of both hydroxyacyl-CoA diastereomers in the steroid degradation pathway in actinobacteria.

In this work, we elucidate a second example of stereochemical bifurcation in the cholesterol metabolism pathway in Mtb (Scheme 2). Throughout the cholesterol catabolic pathway, we have identified protein architectures and assemblies that are unique to actinomycetes and Mtb.^{26, 30, 39-42} Importantly, *chsB1* is required for the in vivo survival of Mtb in mice,³⁵ and the structures of ChsB1 elucidated in this work provide insight into binding site cavities that differ from its homologues and will allow discrimination between host and pathogen enzymes by inhibitors in the future. The enzymatic evolutionary adaptations that have emerged in Mtb for the breakdown of cholesterol and the in vivo essentiality of various genes in the pathway highlight the value of this pathway's enzymes for further exploitation in drug discovery.

Experimental section

Materials, strains, media, and general methods. Mtb H37Rv total genomic DNA was obtained from the TB Research Materials Facility at Colorado State University (NIAD NO1-AI40091). Coenzyme A and nicotinamide adenine dinucleotide (NAD, grade I, free acid) were purchased from MilliporeSigma (Burlington, MA). Testosterone was obtained from Steraloids

Inc. (Newport, RI). Thiostrepton (*Streptomyces laurentii*) was purchased from Thermo Fisher Scientific (Ward Hill, MA). Chloramphenicol was from IBI Scientific (Dubuque, IA). Ampicillin was purchased from Roche Diagnostics (Indianapolis, IN). iProof DNA polymerase and IMAC nickel resin were from Bio-Rad (Hercules, CA). Restriction endonucleases, Quick Ligase, and protein ladder were from New England Biolabs (Beverly, MA). Superdex 200 HiLoad 16/60 columns were from GE Healthcare Biosciences Corp. (Piscataway, NJ). ZipTip U-C18 was from Merck Millipore. Oligonucleotides were from Integrated DNA Technologies (Coralville, IA). Sanger DNA sequencing (performed by GENEWIZ LLC) was used to verify the coding sequence of the expression plasmids. All other chemicals were obtained from Fisher Scientific (Hampton, NH), unless otherwise stated. XL1-Blue *E. coli* was obtained from Agilent.

Rhodococcus jostii RHA1 strain is a gift from Lindsay Eltis (Department of Microbiology and Immunology, University of British Columbia). pTipQC1 vector is a gift from Tomohiro Tamura (National Institute of Advanced Industrial Science and Technology, Sapporo, Japan). The LB media is composed of 25 g Luria broth. Buffer A: 20 mM Tris-HCl pH 8.5, 300 mM NaCl, and 10 mM imidazole. Buffer B: 20 mM Tris-HCl pH 8.5, 300 mM NaCl, and 500 mM imidazole. Buffer C: 50 mM Tris-HCl pH 8.5, and 200 mM NaCl. Buffer D: 100 mM HEPES-HCl pH 7.4 and 3 mM EDTA. Buffer E: 100 mM TAPS-HCl pH 8.5 and 150 mM NaCl.

Expression plasmid construction. *chsB1* and *chsH3* were amplified from Mtb H37Rv total genomic DNA by PCR (Table S1). The PCR products were digested with NdeI and HindIII restriction endonucleases and ligated into pTipQC1 vector. Ligated plasmids containing the gene of interest were transformed into competent *E. coli* XL1-Blue cells for amplification and isolation of plasmid DNA. DNA sequencing of the plasmids confirmed that the sequences were correct and that no mutations were introduced during PCR amplification.

Protein preparation using heterologous expression in *R. jostii* RHA1. Construct *pchsB1* (Rv3502c) or *pchsH3* (Rv3538) was used for the expression of ChsB1 or ChsH3 in *R. jostii* RHA1 (Table S1). The appropriate plasmid was transformed into *R. jostii* RHA1 cells through electroporation. Single colonies were selected on LB plates containing 30 µg/mL chloramphenicol and cultured in LB media at 30 °C. Expression was induced at OD₆₀₀ ~ 0.4-0.6 by the addition of 20 µg/mL thiostrepton, and cells were grown at 30 °C for 24 h.

Cells were harvested by centrifugation at 5000 rpm for 20 min at 4 °C and all subsequent steps were conducted at 4 °C. The cells were suspended in Buffer A and lysed by cell disruption (4 passages at 27,000 psi). Cellular debris was removed by ultracentrifugation at 40,000 rpm (185511 g) for 1 h. The supernatant was loaded onto IMAC nickel resin, washed with 10 column volumes of Buffer A, and eluted with Buffer B. Protein solutions were immediately desalted by dialysis with Buffer C.

Isolated proteins in Buffer C were concentrated by ultrafiltration (MWCO 10 kDa) to less than 2 mL and purified by size-exclusion chromatography (SEC) on a Superdex 200 column equilibrated in Buffer C. The column was equilibrated in Buffer C at a flow rate of 1.0 mL/min and monitored at 280 nm. Fractions were collected and analyzed by reducing SDS-PAGE analysis. The fractions containing desired proteins were concentrated by ultrafiltration (MWCO 10 kDa).

Substrate synthesis. 3-Oxo-pregna-4,17-diene-20-carboxyl-CoA (3-OPDC-CoA), 3-OCDO-CoA, 3-OCDS-CoA, and *trans*-2-octenoyl-CoA were synthesized as previously reported.¹⁶ To make (22*R*)-HOCO-CoA or (3*R*)-hydroxyoctanoyl-CoA, 200 µM 3-OCDO-CoA or *trans*-2-octenoyl-CoA was incubated with 1 µM ChsH3 in buffer D in a 10 mL reaction volume at 25 °C for 2 h. The reaction was quenched by adding 100 µL TFA and product was purified by HPLC

on a C18 column using 10 mM ammonium acetate with a linear gradient of acetonitrile from 5-100% over 40 min. (2*S*)-HOCO-CoA was synthesized in a similar manner by using EchA19. The purities of the substrates were accessed by analytical HPLC to be over 99% pure.

The identity of the desired product was confirmed by MALDI mass spectrometry. Matrices for MALDI mass spectrometry were prepared by dissolving 2,5-dihydroxybenzoic acid (20 mg/mL) in a 7:3 (v/v) mixture of 0.1% (v/v) trifluoroacetic acid and acetonitrile. MALDI mass spectra were acquired on a Bruker Microflex operated in the reflectron negative mode. Mass spectral data were analyzed using Bruker flexAnalysis software (version 3.4).

NMR spectroscopy characterization of reaction products from ChsH3 and EchA19.

Reaction products from ChsH3 and EchA19 using 3-OCDO-CoA as the substrate were purified by HPLC on a C18 column using 10 mM ammonium acetate with a linear gradient of acetonitrile from 5-100% over 40 min. The solvents were removed under reduced pressure followed by lyophilization. The resulting purified products were characterized on an 850 MHz Bruker Ascend spectrometer by dissolving the CoA esters in D₂O to a final concentration of 50 μ M. Chemical shifts are reported in ppm (δ) calibrated using residual protic solvent as an internal reference.

Steady state kinetics. *Hydratase assay.* ChsH3 was assayed for hydratase activity with substrates 3-OCDO-CoA, 3-OCDS-CoA, and octenoyl-CoA in buffer D containing 500 pM enzyme (5 nM for 3-OCDS-CoA) at 25 °C. Reactions were initiated by the addition of enzyme and were monitored at 263 nm. Product formation was quantified using $\epsilon_{263\text{nm}} = 6700 \text{ M}^{-1} \text{ cm}^{-1}$, which corresponds to the α,β -unsaturation of the enoyl-CoA substrates. Initial velocities were determined from the first 10% of the reaction. The rates of product formation were fit to the Michaelis-Menten equation to determine k_{cat} and K_M for each substrate. Each initial velocity was determined in triplicate, and at least five different substrate concentrations were examined.

Hydroxy dehydrogenase assay. ChsB1 was assayed for hydroxyl dehydrogenase activity with substrate (22*R*)-HOCO-CoA, (3*R*)-hydroxyoctanoyl-CoA, and testosterone in buffer E containing 1 nM enzyme for 20-HOCO-CoA, 300 nM enzyme for 3-hydroxyoctanoyl-CoA, and 1 μ M for testosterone at 25 °C. For testosterone, the stock solution was prepared in ethanol (50 mM). No oxidation of ethanol was detected in control assays. The final volume of ethanol was kept constant in all assays at 5% (v/v). Assays were initiated by the addition of enzyme and were monitored at 340 nm for the reduction of NAD⁺ to NADH. Product formation was quantified using $\epsilon_{340\text{nm}} = 6300 \text{ M}^{-1} \text{ cm}^{-1}$. Initial velocities were determined from the first 10% of the reaction and were obtained as a function of one of the substrates while keeping the second substrate at a fixed, saturating concentration. k_{cat} and K_M for each substrate were determined by fitting the initial velocities of product formation into the Michaelis-Menten equation. Each initial velocity was determined in triplicate, and at least five different substrate concentrations were examined. To assess the bi bi reaction kinetic mechanism of ChsB1, reaction velocities were measured by varying concentrations of (22*S*)-HOCO-CoA and at different fixed concentrations of NAD⁺. The initial velocities were globally fit into the following equation using GraphPad Prism8.

$$v = \frac{V_m[A][B]}{K_{iA}K_{mB} + K_{mb}[A] + K_{mA}[B] + [A][B]}$$

where K_{mA} and K_{mB} are the Michaelis constants for A and B at saturating concentrations of B and A, respectively, K_{iA} is the dissociation constant for the enzyme and A, V_m is the maximum velocity of the reaction.

Analytical ultracentrifugation. Sedimentation velocity AUC experiments were performed at 20 °C using a Beckman Coulter XL-I analytical ultracentrifuge with an An-60Ti rotor at 325,000 rpm for 12 h with scans performed every 60 s. A double-sector cell, equipped with a 12-

mm Epon centerpiece and sapphire windows, was loaded with 380 and 400 μl of sample and buffer C, respectively. The concentrations that were used in the experiments are 31.8 μM for ChsB1 and 30 μM for ChsH3. Samples were monitored with UV at 280 nm. The data were analyzed with Sedfit version 1501b using a continuous $c(s)$ distribution. Numerical values for the solvent density, viscosity, and the partial specific volume were determined using Sednterp. Residual and $c(s)$ distribution graphs were plotted using GUSI.

Protein crystallization. The ChsB1 apo-protein crystals were obtained by hanging drop vapor diffusion at room temperature. Briefly, 2 μL of a 20 mg mL^{-1} protein was mixed 1:1 with a reservoir solution of 15% w/v polyethylene glycol 4000, 0.2 M ammonium acetate, and 0.1 M sodium citrate pH 5.6 and equilibrated against 500 μL of the reservoir solution. The crystals were then harvested and transferred to a cryoprotectant solution containing the mother liquor and 25% ethylene glycol. For ChsB1 co-crystallization, ChsB1 was mixed with NAD^+ and co-crystals were obtained by sitting drop vapor diffusion at room temperature. 20 mg mL^{-1} protein was pre-incubated with NAD^+ (1:1.2 molar ratio) for 15 min on ice. 200 nL of the mixture was then mixed 1:1 with the mother liquor which contains 0.2 M sodium citrate, 0.1 M HEPES pH 7.5, and 30% v/v 2-methyl-2,4-pentanediol. Crystals were harvested and cryocooled in liquid nitrogen before data collection.

X-ray data collection and structural determination. Diffraction data were collected at the FMX beamline of the National Synchrotron Light Source-II at Brookhaven National Laboratory (Upton, NY) at the selenium absorption edge using a wavelength of 0.9793 Å. Data sets were processed using XDS⁴³ and Aimless⁴⁴ as implemented in the autoPROC pipeline.⁴⁵ Diffraction for the apo-protein crystals was strongly anisotropic and thus was further processed with STARANISO.⁴⁶ Phases were obtained by molecular replacement using Phaser. Search models

for the apo- and holo-protein were created from 4KZP. Model building was carried out in Coot, followed by refinement in Phenix. The geometric quality of the refined model was assessed with MolProbity and the structure validation tools in the Phenix suite. Data collection and refinement statistics are shown in Table 3.

Acknowledgment

The X-ray experiments were conducted using resources of the National Synchrotron Light Source II, a U.S. Department of Energy (DOE) Office of Science User Facility operated for the DOE Office of Science by Brookhaven National Laboratory under contract no. DESC0012704. This research is funded by NIH RO1AI134054 to N.S.S. The authors would like to thank Dr. Kalle Gehring from McGill University for his help with AUC analysis. The authors also thank Dr. Lindsay Eltis from the University of British Columbia for providing *R. jostii* RHA1 and Dr. Tomohiro Tamura from National Institute of Advanced Industrial Science and Technology (AIST), Japan, for providing the pTipQC1 vector.

Accession Codes

Atomic coordinates and structure factors for ChsB1 and ChsB1/NAD⁺ have been deposited in the Protein Data Bank with accession codes 7LG9 and 7LGB. UniProt accession IDs for proteins used in this work: ChsH3, Q6MWW2; EchA19, O53561; ChsB1, O53547.

Abbreviations

AUC	Analytical ultracentrifugation
HAD	Hydroxyacyl-CoA dehydrogenase
4-HBC	21-Hydroxy-20-methyl-preg-4-en-3-one

22-HOCO-CoA	22-Hydroxy-3-oxo-cholest-4-en-24-oyl-CoA
IMAC	Ion affinity chromatography
MALDI	Matrix assisted laser desorption ionization
MCR	Methyl acyl-CoA racemase
Mtb	<i>Mycobacterium tuberculosis</i>
NAD ⁺	Nicotinamide adenine dinucleotide
NADP ⁺	Nicotinamide adenine dinucleotide phosphate
3-OCDO-CoA	3-Oxo-chole-4,22-dien-24-oyl-CoA
3-OCDS-CoA	3-Oxo-cholest-4,24-dien-26-oyl-CoA
3-OPCA	3-Oxo-preg-4-en-21-carbaldehyde
3-OPDC-CoA	3-Oxo-pregna-4,17-diene-20-carboxyl-CoA
SDR	Short-chain type alcohol dehydrogenase/reductase
TB	Tuberculosis

Conflict of interest

The authors declare no conflict of interest for this work.

References

1. Global tuberculosis report 2020. Geneva: World Health Organization; **2020**.
<https://apps.who.int/iris/bitstream/handle/10665/336069/9789240013131-eng.pdf>
(accessed 7 February 2021).
2. de Carvalho, L. P.; Fischer, S. M.; Marrero, J.; Nathan, C.; Ehrt, S.; Rhee, K. Y.,
Metabolomics of *Mycobacterium tuberculosis* reveals compartmentalized co-catabolism
of carbon substrates. *Chem Biol* **2010**, *17* (10), 1122-31.
3. Zimmermann, M.; Kogadeeva, M.; Gengenbacher, M.; McEwen, G.; Mollenkopf, H.
J.; Zamboni, N.; Kaufmann, S. H. E.; Sauer, U., Integration of metabolomics and

transcriptomics reveals a complex diet of *Mycobacterium tuberculosis* during early macrophage infection. *mSystems* **2017**, 2 (4).

4. Pandey, A. K.; Sassetti, C. M., Mycobacterial persistence requires the utilization of host cholesterol. *Proc Natl Acad Sci U S A* **2008**, 105 (11), 4376-80.
5. Wilburn, K. M.; Fieweger, R. A.; VanderVen, B. C., Cholesterol and fatty acids grease the wheels of *Mycobacterium tuberculosis* pathogenesis. *Pathog Dis* **2018**, 76 (2).
6. Yuan, T.; Sampson, N. S., Hit generation in TB drug discovery: from genome to granuloma. *Chem Rev* **2018**, 118 (4), 1887-1916.
7. Bonds, A. C.; Sampson, N. S., More than cholesterol catabolism: regulatory vulnerabilities in *Mycobacterium tuberculosis*. *Curr Opin Chem Biol* **2018**, 44, 39-46.
8. VanderVen, B. C.; Fahey, R. J.; Lee, W.; Liu, Y.; Abramovitch, R. B.; Memmott, C.; Crowe, A. M.; Eltis, L. D.; Perola, E.; Deininger, D. D.; Wang, T.; Locher, C. P.; Russell, D. G., Novel inhibitors of cholesterol degradation in *Mycobacterium tuberculosis* reveal how the bacterium's metabolism is constrained by the intracellular environment. *PLoS Pathog* **2015**, 11 (2), e1004679.
9. Yang, X.; Yuan, T.; Ma, R.; Chacko, K. I.; Smith, M.; Deikus, G.; Sebra, R.; Kasarskis, A.; van Bakel, H.; Franzblau, S. G.; Sampson, N. S., Mce3R stress-resistance pathway is vulnerable to small-molecule targeting that improves tuberculosis drug activities. *ACS Infect Dis* **2019**, 5 (7), 1239-1251.
10. Wipperman, M. F.; Sampson, N. S.; Thomas, S. T., Pathogen roid rage: cholesterol utilization by *Mycobacterium tuberculosis*. *Crit Rev Biochem Mol Biol* **2014**, 49 (4), 269-93.

11. Upton, A. M.; McKinney, J. D., Role of the methylcitrate cycle in propionate metabolism and detoxification in *Mycobacterium smegmatis*. *Microbiology (Reading)* **2007**, *153* (Pt 12), 3973-3982.
12. Savvi, S.; Warner, D. F.; Kana, B. D.; McKinney, J. D.; Mizrahi, V.; Dawes, S. S., Functional characterization of a vitamin B12-dependent methylmalonyl pathway in *Mycobacterium tuberculosis*: implications for propionate metabolism during growth on fatty acids. *J Bacteriol* **2008**, *190* (11), 3886-95.
13. Lee, W.; VanderVen, B. C.; Fahey, R. J.; Russell, D. G., Intracellular *Mycobacterium tuberculosis* exploits host-derived fatty acids to limit metabolic stress. *J Biol Chem* **2013**, *288* (10), 6788-800.
14. Nesbitt, N. M.; Yang, X.; Fontan, P.; Kolesnikova, I.; Smith, I.; Sampson, N. S.; Dubnau, E., A thiolase of *Mycobacterium tuberculosis* is required for virulence and production of androstenedione and androstadienedione from cholesterol. *Infect Immun* **2010**, *78* (1), 275-82.
15. Park, S. J.; Lee, S. Y., Identification and characterization of a new enoyl coenzyme A hydratase involved in biosynthesis of medium-chain-length polyhydroxyalkanoates in recombinant *Escherichia coli*. *J Bacteriol* **2003**, *185* (18), 5391-7.
16. Bonds, A. C.; Yuan, T.; Werman, J. M.; Jang, J.; Lu, R.; Nesbitt, N. M.; Garcia-Diaz, M.; Sampson, N. S., Post-translational succinylation of *Mycobacterium tuberculosis* enoyl-CoA hydratase EchA19 slows catalytic hydration of cholesterol catabolite 3-oxo-cholesterol-4,22-diene-24-oyl-CoA. *ACS Infect Dis* **2020**, *6* (8), 2214-2224.
17. Benach, J.; Atrian, S.; Gonzalez-Duarte, R.; Ladenstein, R., The catalytic reaction and inhibition mechanism of *Drosophila* alcohol dehydrogenase: observation of an enzyme-

- bound NAD-ketone adduct at 1.4 Å resolution by X-ray crystallography. *J Mol Biol* **1999**, 289 (2), 335-55.
18. Stivala, A.; Wybrow, M.; Wirth, A.; Whisstock, J. C.; Stuckey, P. J., Automatic generation of protein structure cartoons with Pro-origami. *Bioinformatics* **2011**, 27 (23), 3315-6.
 19. Rossmann, M. G.; Moras, D.; Olsen, K. W., Chemical and biological evolution of nucleotide-binding protein. *Nature* **1974**, 250 (463), 194-9.
 20. Madeira, F.; Park, Y. M.; Lee, J.; Buso, N.; Gur, T.; Madhusoodanan, N.; Basutkar, P.; Tivey, A. R. N.; Potter, S. C.; Finn, R. D.; Lopez, R., The EMBL-EBI search and sequence analysis tools APIs in 2019. *Nucleic Acids Res* **2019**, 47 (W1), W636-W641.
 21. Robert, X.; Gouet, P., Deciphering key features in protein structures with the new ENDscript server. *Nucleic Acids Res* **2014**, 42 (Web Server issue), W320-4.
 22. Ylianttila, M. S.; Pursiainen, N. V.; Haapalainen, A. M.; Juffer, A. H.; Poirier, Y.; Hiltunen, J. K.; Glumoff, T., Crystal structure of yeast peroxisomal multifunctional enzyme: structural basis for substrate specificity of (3*R*)-hydroxyacyl-CoA dehydrogenase units. *J Mol Biol* **2006**, 358 (5), 1286-95.
 23. Meng, Q.; Peng, Z.; Yang, J., CoABind: a novel algorithm for Coenzyme A (CoA)- and CoA derivatives-binding residues prediction. *Bioinformatics* **2018**, 34 (15), 2598-2604.
 24. Capyk, J. K.; Kalscheuer, R.; Stewart, G. R.; Liu, J.; Kwon, H.; Zhao, R.; Okamoto, S.; Jacobs, W. R., Jr.; Eltis, L. D.; Mohn, W. W., Mycobacterial cytochrome p450 125 (cyp125) catalyzes the terminal hydroxylation of c27 steroids. *J Biol Chem* **2009**, 284 (51), 35534-42.

25. Driscoll, M. D.; McLean, K. J.; Levy, C.; Mast, N.; Pikuleva, I. A.; Lafite, P.; Rigby, S. E.; Leys, D.; Munro, A. W., Structural and biochemical characterization of *Mycobacterium tuberculosis* CYP142: evidence for multiple cholesterol 27-hydroxylase activities in a human pathogen. *J Biol Chem* **2010**, 285 (49), 38270-82.
26. Yang, M.; Lu, R.; Guja, K. E.; Wipperman, M. F.; St Clair, J. R.; Bonds, A. C.; Garcia-Diaz, M.; Sampson, N. S., Unraveling cholesterol catabolism in *Mycobacterium tuberculosis*: ChsE4-ChsE5 alpha2beta2 acyl-CoA dehydrogenase initiates beta-oxidation of 3-oxo-cholest-4-en-26-oyl CoA. *ACS Infect Dis* **2015**, 1 (2), 110-125.
27. Lu, R.; Schmitz, W.; Sampson, N. S., alpha-Methyl acyl CoA racemase provides *Mycobacterium tuberculosis* catabolic access to cholesterol esters. *Biochemistry* **2015**, 54 (37), 5669-72.
28. Schaefer, C. M.; Lu, R.; Nesbitt, N. M.; Schiebel, J.; Sampson, N. S.; Kisker, C., FadA5 a thiolase from *Mycobacterium tuberculosis*: a steroid-binding pocket reveals the potential for drug development against tuberculosis. *Structure* **2015**, 23 (1), 21-33.
29. Xu, L. Q.; Liu, Y. J.; Yao, K.; Liu, H. H.; Tao, X. Y.; Wang, F. Q.; Wei, D. Z., Unraveling and engineering the production of 23,24-bisnorcholesterol steroids in sterol metabolism. *Sci Rep* **2016**, 6, 21928.
30. Yuan, T.; Yang, M.; Gehring, K.; Sampson, N. S., *Mycobacterium tuberculosis* exploits a heterohexameric enoyl-CoA hydratase retro-aldolase complex for cholesterol catabolism. *Biochemistry* **2019**, 58 (41), 4224-4235.
31. Gilbert, S.; Hood, L.; Seah, S. Y. K., Characterization of an aldolase involved in cholesterol side chain degradation in *Mycobacterium tuberculosis*. *J Bacteriol* **2018**, 200 (2).

32. Holert, J.; Jagmann, N.; Philipp, B., The essential function of genes for a hydratase and an aldehyde dehydrogenase for growth of *Pseudomonas sp.* strain Chol1 with the steroid compound cholate indicates an aldolytic reaction step for deacetylation of the side chain. *J Bacteriol* **2013**, *195* (15), 3371-80.
33. Casabon, I.; Swain, K.; Crowe, A. M.; Eltis, L. D.; Mohn, W. W., Actinobacterial acyl coenzyme A synthetases involved in steroid side-chain catabolism. *J Bacteriol* **2014**, *196* (3), 579-87.
34. Griffin, J. E.; Gawronski, J. D.; Dejesus, M. A.; Ioerger, T. R.; Akerley, B. J.; Sassetti, C. M., High-resolution phenotypic profiling defines genes essential for mycobacterial growth and cholesterol catabolism. *PLoS Pathog* **2011**, *7* (9), e1002251.
35. Sassetti, C. M.; Rubin, E. J., Genetic requirements for mycobacterial survival during infection. *Proc Natl Acad Sci U S A* **2003**, *100* (22), 12989-94.
36. Van der Geize, R.; Yam, K.; Heuser, T.; Wilbrink, M. H.; Hara, H.; Anderton, M. C.; Sim, E.; Dijkhuizen, L.; Davies, J. E.; Mohn, W. W.; Eltis, L. D., A gene cluster encoding cholesterol catabolism in a soil actinomycete provides insight into *Mycobacterium tuberculosis* survival in macrophages. *Proc Natl Acad Sci U S A* **2007**, *104* (6), 1947-52.
37. Ishibashi, T.; Bloch, K., Intermembrane transfer of 5 alpha-cholest-7-en-3 beta-ol. Facilitation by supernatant protein (SCP). *J Biol Chem* **1981**, *256* (24), 12962-7.
38. Seedorf, U.; Brysch, P.; Engel, T.; Schrage, K.; Assmann, G., Sterol carrier protein X is peroxisomal 3-oxoacyl coenzyme A thiolase with intrinsic sterol carrier and lipid transfer activity. *J Biol Chem* **1994**, *269* (33), 21277-83.

39. Thomas, S. T.; Sampson, N. S., *Mycobacterium tuberculosis* utilizes a unique heterotetrameric structure for dehydrogenation of the cholesterol side chain. *Biochemistry* **2013**, 52 (17), 2895-904.
40. Wipperman, M. F.; Yang, M.; Thomas, S. T.; Sampson, N. S., Shrinking the FadE proteome of *Mycobacterium tuberculosis*: insights into cholesterol metabolism through identification of an $\alpha_2\beta_2$ heterotetrameric acyl coenzyme A dehydrogenase family. *J Bacteriol* **2013**, 195 (19), 4331-41.
41. Yang, M.; Guja, K. E.; Thomas, S. T.; Garcia-Diaz, M.; Sampson, N. S., A distinct MaoC-like enoyl-CoA hydratase architecture mediates cholesterol catabolism in *Mycobacterium tuberculosis*. *ACS Chem Biol* **2014**, 9 (11), 2632-45.
42. Gadberry, J. E.; Round, J. W.; Yuan, T.; Wipperman, M. F.; Story, K. T.; Crowe, A. M.; Casabon, I.; Liu, J.; Yang, X.; Eltis, L. D.; Sampson, N. S., IpdE1-IpdE2 is a heterotetrameric acyl coenzyme A dehydrogenase that is widely distributed in steroid-degrading bacteria. *Biochemistry* **2020**, 59 (10), 1113-1123.
43. Kabsch, W., XDS. *Acta Crystallogr D Biol Crystallogr* **2010**, 66 (Pt 2), 125-32.
44. Evans, P. R.; Murshudov, G. N., How good are my data and what is the resolution? *Acta Crystallogr D Biol Crystallogr* **2013**, 69 (Pt 7), 1204-14.
45. Vonrhein, C.; Flensburg, C.; Keller, P.; Sharff, A.; Smart, O.; Paciorek, W.; Womack, T.; Bricogne, G., Data processing and analysis with the autoPROC toolbox. *Acta Crystallogr D Biol Crystallogr* **2011**, 67 (Pt 4), 293-302.
46. Tickle, I. J.; Flensburg, C.; Keller, P.; Paciorek, W.; Sharff, A.; Vonrhein, C.; Bricogne, G., *STARANISO*. Cambridge, United Kingdom: Global Phasing Ltd. 2018.

