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α-Methyl Acyl CoA Racemase Provides *Mycobacterium tuberculosis* Catabolic Access to Cholesterol Esters

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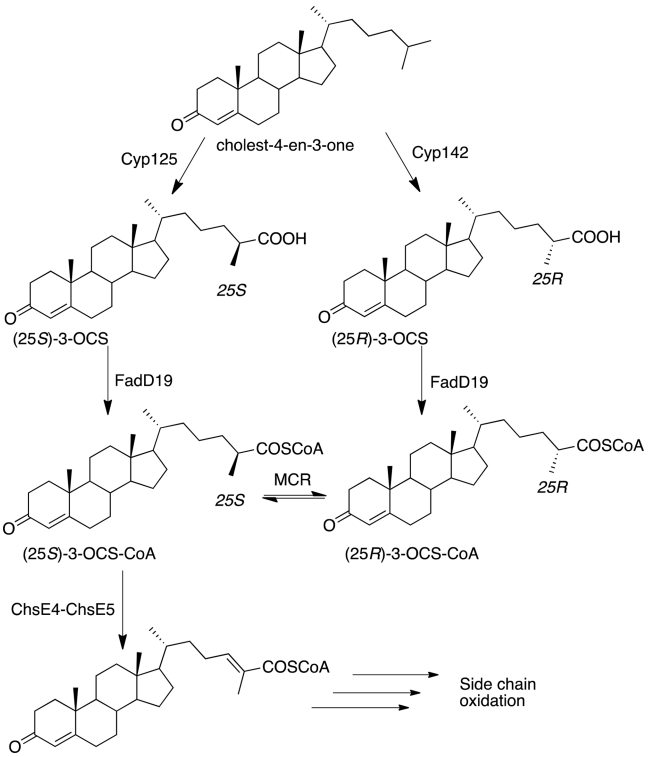
Supporting Information

ABSTRACT: Metabolism of cholesterol by *Mycobacterium tuberculosis* (*Mtb*) contributes to its pathogenesis. We show that ChsE4-ChsE5 (Rv3504/Rv3505) specifically catalyzes dehydrogenation of the (25*S*)-3-oxo-cholest-4-en-26-oyl-CoA diastereomer in cholesterol side chain β-oxidation. Thus, a dichotomy between the supply of both 25*R* and 25*S* metabolic precursors by upstream cytochrome P450s and the substrate stereospecificity of ChsE4-ChsE5 exists. We reconcile the dilemma of 25*R* metabolite production by demonstrating that mycobacterial MCR (Rv1143) can efficiently epimerize C25 diastereomers of 3-oxo-cholest-4-en-26-oyl-CoA. Our data suggest that cholesterol and cholesterol ester precursors can converge into a single catabolic pathway, thus widening the metabolic niche in which *Mtb* survives.

Cholesterol side chain β-oxidation by *Mycobacterium tuberculosis* (*Mtb*) is important for the survival of *Mtb* in the host.[1−3] Degradation of the aliphatic cholesterol side chain by *Mtb* proceeds via a modified fatty acyl β-oxidation pathway.[2,4−9] The expression of enzymes required for side chain catabolism is regulated by a Tet-like repressor KstR1.[10,11]

Fatty acid β-oxidation is a ubiquitous coenzyme A (CoA)-dependent process in living organisms, in which acyl-CoA esters are degraded into acetyl-CoA and/or propionyl-CoA. Before the cholesterol side chain can be degraded by β-oxidation, the terminal C26 methyl group must be oxidized to a carboxylic acid and undergo CoA thioesterification by a fatty acyl-CoA ligase (FadD). Three *Mtb* cytochrome P450s (Cyp), Cyp125, Cyp142, and Cyp124, can catalyze the sequential oxidization of the terminal methyl into an alcohol, aldehyde, and then carboxylic acid; however, only Cyp125 and Cyp142 are utilized for this activity by *Mtb*.[12−15] Interestingly, even though *cyp142* can support the growth of the H37Rv strain on cholesterol in the absence of *cyp125*,[13] the two encoded proteins have different stereospecificities.[13] The reaction of Cyp125 with cholesterol or cholest-4-en-3-one produces exclusively 25*S* product, whereas the reaction of Cyp142 produces the 25*R* product[13] (Scheme 1). FadD19 is an essential enzyme when *Mtb* is grown on cholesterol[16] and is the only fatty CoA ligase that has been identified to esterify the terminal cholesterol carboxylic acid. However, FadD19 is not stereoselective as it accepts both the 25*R* and 25*S* carboxylic acids[6] (Scheme 1). Thus, the metabolic pathway that *Mtb* utilizes to activate cholesterol to its CoA ester provides both the 25*R* and 25*S* diastereomers of 3-oxo-cholest-4-en-26-oyl-CoA (3-OCS-CoA).

Scheme 1. *Mtb* Pathway for Cholesterol Activation Upstream of Side Chain β-Oxidation



Unlike classic fatty acyl β-oxidation, *Mtb* utilizes a structurally and evolutionary distinct class of acyl-CoA dehydrogenases (ACAD) to generate α,β-unsaturated steroid enoyl CoAs.[5,9] These ACADs from *Mtb* are assembled from two adjacent gene products and form an obligate $\alpha_2\beta_2$ heterotetrameric architecture.[5,6,8,9] ChsE4-ChsE5 is the only ACAD protein regulated by KstR1 that can oxidize 3-OCS-CoA, the first acyl-CoA metabolite in the side chain β-oxidation cycle.[6]

We discovered that the 3-OCS-CoA α,β-dehydrogenation reaction catalyzed by ChsE4-ChsE5 is stereospecific.[6] The α,β-dehydrogenation of 1:1 (25*R*,25*S*)-3-OCS-CoA catalyzed by ChsE4-ChsE5 proceeds to only 50% completion (Figure 1a,

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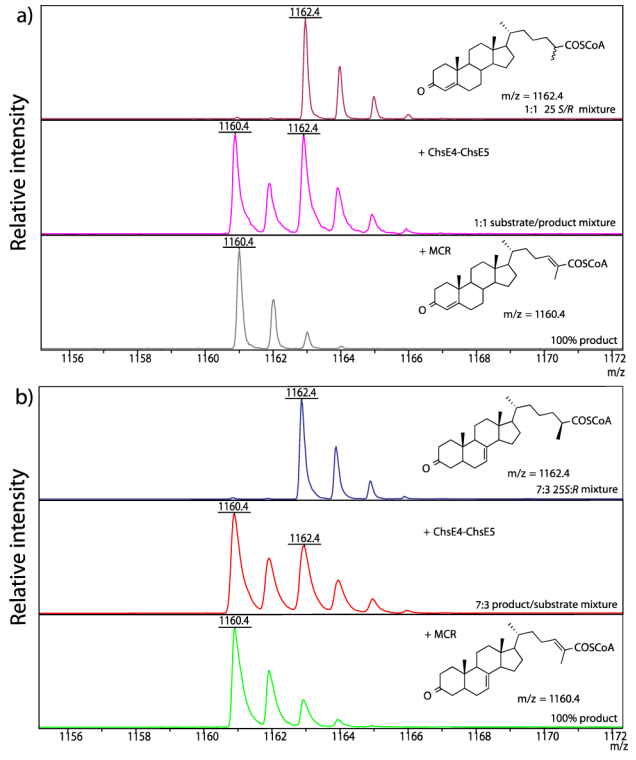

Figure 1. ChsE4-ChsE5 and MCR product analysis by matrix-assisted laser desorption ionization time-of-flight mass spectrometry illustrating that ChsE4-ChsE5 is stereospecific for the 25*S* steroyl-CoA diastereomer: (a) 1:1 (25*R*:25*S*)-3-OCS-CoA substrate (top), product of ChsE4-ChsE5-catalyzed dehydrogenation (middle), product after addition of MCR to the ChsE4-ChsE5 reaction mixture (bottom) and (b) 7:3 (25*S*:25*R*)-Δ^7-dafachronyl-CoA substrate (top), product of ChsE4-ChsE5-catalyzed dehydrogenation (middle), and product after addition of MCR to the ChsE4-ChsE5 reaction mixture (bottom). In each case, reaction mixtures were monitored until no further changes in product distribution occurred.

middle).[6] An increasing incubation time or enzyme concentration does not lead to further reaction; the substrate:product ratio remains 1:1.[6] The inability of ChsE4-ChsE5 to utilize both diastereomers raises the puzzling question of how the 25*R*-specific CYP142 can compensate for knockout of the 25*S*-specific CYP125 in cholesterol metabolism. Moreover, the stereochemistry of the ChsE4-ChsE5 substrate cannot be predicted by analogy given the structural divergence of the ChsE4-ChsE5 ACAD from classical homotetrameric ACADs. We reasoned that an α-methyl racemase/epimerase might function in this pathway to interconvert the 25*R* and 25*S* thioesters and thereby allow utilization of both stereoisomers in cholesterol metabolism.

In the *Mtb* genome, there are three genes that might encode the requisite α-methyl racemase/epimerase: *mcr*, *far*, and *Rv3727*. The MCR protein (Rv1143) is known to bind several α-methyl acyl CoA thioesters, including 3,7,12-trihydroxycoprostanoyl-CoA (THCA-CoA), and to catalyze the interconversion of (2*R*,2*S*)-methylmyristoyl-CoA and (2*R*,2*S*)-ibuprofenoyl-CoA.[17,18] The crystal structure of MCR liganded to ibuprofenoyl CoA reveals a relatively large substrate-binding site, which raised the possibility that cholesterol metabolites may be physiologically relevant substrates for this mycobacterial enzyme.[19,20] Moreover, the ability of MCR to bind THCA-CoA, a steroyl-CoA thioester intermediate in the cholic acid biosynthesis pathway, encouraged us to explore its potential function in the cholesterol degradation pathway. We tested the epimerase activity of MCR with a 1:1 (25*R*:25*S*)-3-OCS-CoA mixture using a matrix-assisted laser desorption ionization time-of-flight-based assay coupled to ChsE4-ChsE5 for detection of activity. In the absence of MCR, as previously described, ChsE4-ChsE5 can dehydrogenate only one of the two 3-OCS-CoA diastereomers (Figure 1a, middle). Upon addition of MCR, both diastereomers are consumed by ChsE4-ChsE5 (Figure 1a, bottom). We conclude that in the coupled assay, MCR catalyzes the interconversion of (25*R*)-3-OCS-CoA and (25*S*)-3-OCS-CoA, resulting in the dehydrogenation reaction catalyzed by ChsE4-ChsE5 proceeding to completion (Figure 1a, bottom).

ChsE4-ChsE5 is clearly stereospecific for a single diastereomer of 3-OCS-CoA, an α-methyl-branched acyl CoA substrate. However, the stereochemistry of the active diastereomer was not known. Mitochondrial and peroxisomal ACAD enzymes stereospecifically catalyze dehydrogenation of (*S*)-α-methyl acyl-CoA diastereomers.[21] Therefore, we obtained a commercially and synthetically prepared Δ^7-dafachronic acid sample that is predominantly the biologically active 25*S* diastereomer.[22] From the acid, a 7:3 mixture of (25*S*:25*R*)-Δ^7-dafachronyl-CoA was prepared using *mt*FadD19. Consistent with our earlier work, ChsE4-ChsE5 dehydrogenates a single diastereomer, which is the major isomer (25*S*)-Δ^7-dafachronyl-CoA (Figure 1b, middle). Upon addition of MCR to epimerize the remaining (25*R*)-Δ^7-dafachronyl-CoA, dehydrogenation proceeded to completion (Figure 1b, bottom).

Upon demonstrating the epimerization activity of MCR with (25*R*,25*S*)-3-OCS-CoA and the 25*S* stereospecificity of ChsE4-ChsE5, we determined the steady state kinetic rate constants for MCR. The MCR enzyme kinetic assays were coupled with ChsE4-ChsE5 to monitor reaction progress. The assay reaction mixtures were first incubated with ChsE4-ChsE5 and (25*R*,25*S*)-3-OCS-CoA (1:1), in the absence of MCR to consume all of the (25*S*)-3-OCS-CoA. The epimerization of the remaining (25*R*)-3-OCS-CoA was initiated by adding MCR and the reaction followed by monitoring the appearance of the dehydrogenation product. The (25*R*)-3-OCS-CoA steady state kinetic parameters are as follows: k_{cat} = 3.7 ± 0.2 s^{-1} and K_m = 6.5 ± 1.4 μM at pH 8.5 and 25 °C. Compared with the steady state kinetic parameters of MCR with a nonphysiologic substrate, (2*R*)-ibuprofenoyl-CoA (at pH 8.0 and 37 °C, k_{cat} = 228 ± 9 s^{-1} and K_m = 71 ± 9 μM), obtained from a continuous circular dichroism-based assay,[18] the K_m for (25*R*)-3-OCS-CoA is 10-fold lower and the specificity approximately the same. Likewise, the MCR epimerase preferentially binds bulky hydrophobic steroid substrates like (*R*,*S*)-THCA-CoA, which is bound 20 times tighter than acetyl-CoA, a small aliphatic moiety.[20]

Cholesterol ester is an abundant form of cholesterol in low-density lipoprotein (LDL)[23] and in the lipid droplets formed in foamy macrophages, the presumed natural source of cholesterol for *Mtb*.[24,25] Ortiz de Montellano and co-workers recently reported that Cyp142 preferentially oxidizes cholesterol ester as opposed to cholest-4-en-3-one.[26] On the basis of the crystal structures of MCR[17,20] and ChsE4-ChsE5,[6] we suggest that the cholesterol ester-derived acyl-CoA metabolites produced via Cyp142 oxidation are accepted as substrates by both MCR and ChsE4-ChsE5, as well. Thus, *Mtb* could effectively catabolize cholesterol ester directly, thereby bypassing a requirement for conversion of cholesterol to cholest-4-en-3-one by 3β-

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hydroxysteroid dehydrogenase (*hsd*)[27] before initiating side chain β-oxidation to generate energy. The existence of such a bypass is consistent with the absence of a phenotype for the *hsd* knockout in *in vivo* models of infection.[28] Some of the more than 150 genes in the *Mtb* genome regulated by cholesterol that have no assigned biochemical function[29] may contribute to this bypass.

In summary, ChsE4-ChsE5 specifically catalyzes the dehydrogenation of (25*S*)-3-oxo-cholest-4-en-26-oyl-CoA, and the activity of the MCR epimerase allows flux of the 25*R* steroyl CoA metabolite into the cholesterol side chain degradation pathway. These results explain the compensatory effect of *cyp142* expression in the H37Rv *cyp125* knockout and suggest that cholesterol ester be added to the panoply of carbon sources utilized by *Mtb in vivo*.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.biochem.5b00911.

Detailed Materials and Methods (PDF)

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Notes

The authors declare no competing financial interest.

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