Identification Of Genes Encoding Acyl-coa Reductases And Aldehyde Reductases In Mycobacterial Genome By Characterization Of The Reductases Expressed In E. Coli

Harminder Singh
University of Central Florida

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IDENTIFICATION OF GENES ENCODING ACYL-COA REDUCTASES AND ALDEHYDE REDUCTASES IN MYCOBACTERIAL GENOME BY CHARACTERIZATION OF THE REDUCTASES EXPRESSED IN E.COLI.

by

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A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in the Department of Molecular Biology and Microbiology in the Burnett School of Biomedical Sciences at the University of Central Florida Orlando, Florida

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ABSTRACT

*Mycobacterium tuberculosis* has been long known to produce wax esters. However, the enzymes involved in their biosynthesis have not been identified. Here we report the identification of Rv3391 and Rv1543 as genes that encode fatty acyl-CoA reductases and Rv1544 as one that encodes an aldehyde reductase. When expressed in *E.coli*, the products of Rv3391 and Rv1543 catalyzed reduction of fatty acyl-CoA to fatty alcohol with the corresponding aldehyde as an intermediate with an optimal pH of 7.0. Both enzymes showed a strong preference for NADPH over NADH as a reductant. Apparent Km for NADPH was 38 µM for Rv3391 product and 202 µM for Rv1543 product. Both enzymes reduced saturated fatty acyl-CoA such as palmitoyl-CoA and stearyl-CoA but showed a preference for oleoyl-CoA. Apparent Km for oleoyl-CoA was 13 µM for Rv3391 product and 7 µM for Rv1543 product.

The Rv1544 product catalyzed fatty aldehyde reduction to fatty alcohol but not acyl-CoA reduction. The optimal pH for aldehyde reduction was 8.0. This aldehyde reductase showed a strong preference for NADPH with an apparent Km of 83 µM. All three reductases were inhibited by SH directed reagents.
I dedicate my thesis to my parents.
ACKNOWLEDGMENTS

This work was supported by grants AI46582 and AI35272 from the National Institutes of Health. We thank Dr. Stefan H.E. Kaufmann for the gift of the pSD31 expression plasmid. I would like to thank my friends not only from my lab but also from Dr. Self lab for their extended help. I would also like to thank my friend Dr. Anurag Pande for his availability and help.
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<tr>
<th>Acronym</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>IPTG</td>
<td>Isopropyl β-D-1-thiogalactopyranoside</td>
</tr>
<tr>
<td>NADH</td>
<td>Nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NADPH</td>
<td>Nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>NEM</td>
<td>N-Ethylmaleimide</td>
</tr>
<tr>
<td>PAGE</td>
<td>Polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PCMB</td>
<td>p-chloromercuribenzoate</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
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</table>
CHAPTER ONE: INTRODUCTION AND LITERATURE REVIEW

Tuberculosis is a leading cause of preventable deaths (2, 10). The emergence of multi-drug resistant and the more recent reports of nearly untreatable extremely drug resistant strains pose great threat to public health (11). The ability of the pathogens to survive in asymptomatic people in a drug-resistant latent state for decades makes cure and eradication of tuberculosis extremely difficult. One third of the world population is reported to have latent pathogen. Understanding of the biochemical steps critically needed for the latent pathogen could help identify targets for novel drugs that can eliminate latent pathogen.

There is strong evidence that the pathogen uses stored lipids as the energy source for persistence in the host (12, 21, 24). Triacylglycerols and wax esters are two forms of long terms storage lipids used by living organisms (8, 20, 27, 30, 31). The biochemical processes involved in the storage and utilization of triacylglycerols in M.tuberculosis has been studied (8, 28). However, the biosynthesis of wax esters by M.tuberculosis has not been studied although such lipids have long been known to be present in this pathogen (31). Here we report the identification of genes that encode the enzymes that catalyze the synthesis of fatty alcohols for wax ester biosynthesis in M.tuberculosis and report some of the properties of these reductases.
**CHAPTER TWO: METHODOLOGY**

**Cloning and Expression of M.tuberculosis reductase genes in E.coli.**
Rv1543, Rv1544 and Rv3391 were amplified from *M.tuberculosis* genomic DNA by PCR amplification using *Pfu* Turbo Hotstart DNA polymerase (Stratagene) and primers shown in Table 1. Amplified DNA were cloned into pET200 directional-Topo expression vector (Invitrogen) and sequence was confirmed. BL21star (DE3) host cells were transformed with each expression construct and grown overnight at 37°C and diluted by 1:50 with fresh LB medium. This culture was induced with 1mM IPTG at OD<sub>600</sub> 0.5-0.8 followed by incubation at room temperature for 16 hours. Cell lysates were obtained by sonicating the induced cells in 0.1M phosphate buffer.

**Cloning and Expression of M.tuberculosis reductase gene in M.smegmatis.**
Rv1543 was amplified from *M.tuberculosis* genomic DNA by PCR using *Pfu* Turbo Hotstart DNA polymerase (Stratagene). Amplified DNA was cloned into pCR –Blunt II-topo vector and transformed in *E.coli* Top 10 (Invitrogen). Sequence integrity was confirmed and the inset was subcloned into the mycobacterial expression vector pSD31 (25). After selection of the proper orientation the expression construct was released by digestion with SfoI, electroporated into *M.smegmatis* mc<sup>2</sup>155. Single colony was grown in 7H9 complete medium (7H9 medium (BD) supplemented with 10% ADS, 0.05% Tween-80 and 0.2% glycerol) overnight with 50µg/ml hygromycin and diluted by 1:50 in expression medium (7H9 medium (BD) without ADS but supplemented with 0.05% Tween-80, 0.2% glucose and 0.2% glycerol). This culture was induced with 1 %
acetamide at OD<sub>600</sub> 0.6 followed by incubation at 30°C. Cell lysates were obtained by sonicating the induced cells in 0.1M phosphate buffer (25).

**Assay for Acyl-CoA reductase.**

Acyl-CoA reductase activity was routinely measured by measuring conversion of [1-<sup>14</sup>C]Oleoyl-CoA to [1-<sup>14</sup>C]Oleyl alcohol and [1-<sup>14</sup>C] Oleyl aldehyde. The reaction mixture containing 15 µM [1-<sup>14</sup>C]Oleoyl-CoA (55Ci/mole;American Radiolabeled Chemicals), 100 µM NADPH (Sigma Aldrich) in a final volume of 250 µl of 0.1 M phosphate buffer, pH 7 was incubated at room temperature. Reaction was stopped after 45 min with 50µl of 6M HCl. Lipids were extracted with chloroform-methanol (2:1[v/v]). Recovered lipid products were applied to silica gel plates and developed with hexane:diethylether:formic acid (40:10:1v/v). Regions on silica gel matching with nonradioactive co-chromatographed octadecanal and octadecanol were scraped off and assayed for <sup>14</sup>C in Beckman LS3801 scintillation counter (13). The kinetic parameters were calculated using nonlinear regression analysis with the Michaelis-Menten equation. (GraphPad Prism version 4; GraphPad Software).

**Preparation of [1-14C]Oleyl aldehyde.**

[1-<sup>14</sup>C]Oleic acid (55Ci/mole;American Radiolabeled Chemicals) in anhydrous tetrahydrofuran was reduced with LiAlH<sub>4</sub> to oleyl alcohol (55Ci/mole) and this oleyl alcohol in CH<sub>2</sub>Cl<sub>2</sub> was oxidized to oleyl aldehyde with pyridinium chlorochromate (1). TLC on silica gel with hexane:diethyl ether:formic acid (40:10:1) as developing solvent was used to purify labeled oleyl aldehyde and oleyl alcohol (18).
Assay for Aldehyde reductase.
Aldehyde reductase activity was assayed by measuring conversion of $[1^{-14}C]$oleyl aldehyde to oleyl alcohol. Reaction mixture containing 7µM $[1^{-14}C]$oleyl aldehyde (55Ci/mole) and 100µM NADPH in a final volume of 250µl of 0.1 M phosphate buffer pH8, was incubated at room temperature. Reaction was stopped after 30min with 50µl of 6M HCl. Lipids, extracted with chloroform-methanol (2:1[v/v]) were applied to silica G plates and developed with hexane:diethylether:formic acid (40:10:1v/v) as the solvent system. Regions of silica gel matching with co-chromatographed unlabeled oleyl alcohol was scrapped and assayed for $^{14}C$ in Beckman LS3801 scintillation counter (18).

Identification of the aldehyde intermediate and oleyl alcohol.
The $^{14}C$-labelled material that matched with co-chromatographed authentic oleyl aldehyde, recovered from TLC was treated with NaBH$_4$ in methanol at room temperature for one hour and the recovered reduction product was subjected to TLC with authentic oleyl alcohol as standard and developed with the same solvent system. The $^{14}C$-labeled material that comigrated with oleyl alcohol was treated overnight with 2:1 v/v acetic anhydride and pyridine at room temperature. The reaction product was subjected to TLC with oleyl acetate as the authentic standard.
CHAPTER THREE: FINDINGS

Expression of Rv3391 and Rv1543 in E.coli.
Even though expression of these gene products was high at 37°C it was found that enzymatic activity in cell lysates were higher when expression was done at room temperature. Therefore all expression experiments were carried at room temperature. Induction of the two reductase genes in pET 200 vector at room temperature overnight, followed by SDS-PAGE analysis of cell lysates, showed accumulation of proteins of the size expected from the size of the ORFs expressed: 74 KDa for Rv3391, 40 KDa for Rv1543. (Fig. 1).

Product Identification.
Cell free extracts expressing Rv3391 and Rv1543 were assayed for acyl-CoA reductase activity. It was found that both proteins produced alcohol and aldehyde which comigrated with oleyl alcohol and oleyl aldehyde, respectively. The $R_f$ values for oleyl alcohol was 0.26 whereas oleyl aldehyde was 0.71. The alcohol product when treated with acetic anhydride gave a product that comigrated with authentic oleyl acetate with a $R_f$ of 0.6 whereas oleyl aldehyde was reduced at room temperature by NaBH₄ to a product that comigrated with authentic oleyl alcohol with a $R_f$ of 0.15.

Effect of pH on the two acyl-CoA reductases.
Both proteins with acyl-CoA reductase activity showed optimum activity at pH 7 (Fig. 2, 3). Both alcohol and aldehyde formation showed optimum at pH 7. Alcohol formation by both enzymes increased rectilinearly with increasing time of incubation. Formation of the aldehyde intermediate increased during the early time periods and then decreased as it
was being converted to alcohol (data not shown). With both reductases, product formation taken as the sum of aldehyde and alcohol increased rectilinearly with increasing protein concentration up to 320 µg/ml.

**Cofactor requirement.**
Both reductases exhibited strong preference for NADPH; NADH was ineffective (data not shown). Both reductases displayed typical Michaelis-Menten kinetics (Fig.4, 5). Rv3391 protein showed higher affinity towards NADPH with apparent Km of 38 µM than Rv1543 protein whose apparent K_m was found to be 202 µM. V_max for Rv1543 was 25.3 µmolemin⁻¹ and Rv3391 showed a lower V_max at 4.5 µmolemin⁻¹.

**Substrate Specificity.**
When assayed for substrate preference it was found that both the proteins showed highest specificity towards oleoyl-CoA. Ranking of acyl-CoA reduction by both the proteins was in the following order oleoyl-CoA>stearoyl-CoA>palmitoyl-CoA. Both proteins showed typical substrate saturation with oleoyl-CoA. Rv1543 protein displayed higher affinity for oleoyl-CoA with K_m of 7µM than Rv3391 protein with an apparent K_m of 13.44µM (Fig.6, 7). V_max for Rv1543 protein was found to be 20.5 µmolemin⁻¹ whereas V_max for Rv3391 was found to be 6.8 µmolemin⁻¹.

**Effect of Inhibitors.**
Both acyl-CoA reductases were found to be sensitive to thiol directed reagents (4). N-Ethylmaleimide reduced the activity of Rv3391 protein resulting in drastic decrease in the formation of alcohol and aldehyde, whereas with Rv1543 protein only alcohol formation was drastically reduced whereas aldehyde formation was not affected. p-Chloromercuribenzoate severely inhibited both alcohol and aldehyde production catalyzed
by the 74 KDa reductase (Fig. 8). On the other hand \( p \)-Chloromercuribenzoate drastically inhibited alcohol production catalyzed by the 40 KDa reductase but aldehyde production was not affected. (Fig. 9).

**Reductase encoded by Rv1544.**

When Rv1544, located next to the gene encoding the 40 KDa acyl-CoA reductase in the genome (7), was expressed in *E.coli* a 31 KDa protein was formed (Fig.1). This protein showed no oleoyl-CoA reductase activity. However, it showed oleyl aldehyde reductase activity. (Figure. 10).

**Effect of pH, Cofactor requirement and Effect of Inhibitors on the catalytic activity of Rv1544 product.**

The 31 KDa aldehyde reductase showed optimal activity at pH 8 (Fig. 11). Oleyl aldehyde reduction increased rectilinearly with increasing protein concentration and time of incubation. NADPH was strongly preferred as the reductant; NADH was ineffective. Typical Michealis-Menten saturation kinetics was observed with increasing concentrations of NADPH with an apparent \( K_m \) 83 \( \mu \)M and \( V_{max} \) 13 \( \mu \)mole/min (Fig. 12). Oleyl aldehyde saturation displayed apparent \( K_m \) 93 \( \mu \)M and \( V_{max} \) 136 \( \mu \)mole/min (data not shown). \( p \)-Chloromercuribenzoate severely inhibited the aldehyde reductase activity (Fig.13). A combination of this aldehyde reductase with the 40 KDa acyl-CoA reductase gave very efficient conversion of oleyl-CoA to oleyl alcohol. In view of the finding that in other biological systems, aldehyde generating reductase and aldehyde reductase activity were found to be catalyzed by separate enzymes and both reductase activities were catalyzed by a larger reductase (13), we further tested whether Rv1543 product could indeed reduce acyl-CoA and aldehyde. When Rv1543 was expressed in *M.smegmatis* the protein was found to be located in the membrane fractions as predicted.
from its sequence. The membrane fraction from the transformed *M.smegmatis* catalyzed reduction of [1-\(^{14}\)C]Oleoyl-CoA to aldehyde and alcohol. The R\(_f\) values for oleyl aldehyde and oleyl alcohol were 0.67 and 0.21 respectively. The membrane fraction from untransformed *M.smegmatis* showed no aldehyde reductase activity. Therefore we concluded that Rv1543 product catalyzed both reductase steps. Confirming this conclusion the membrane fraction containing the Rv1543 product catalyzed reduction of oleyl aldehyde to oleyl alcohol with a R\(_f\) of 0.24.
**Table 1: Primers used for amplification of reductases genes from *M.tuberculosis* genome**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer pairs(5'-3')&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rv3391</td>
<td>F: CACCATGCGGTACGTCGTTACCAGGC</td>
</tr>
<tr>
<td></td>
<td>R: CTACCAATGCACCCGGGCAACCAG</td>
</tr>
<tr>
<td>Rv1543</td>
<td>F: CACCATGAATCTTTGGTGACTTAACG</td>
</tr>
<tr>
<td></td>
<td>R: TTTTCACCAATGGATCCCTCGGGT</td>
</tr>
<tr>
<td>Rv1544</td>
<td>F: CACCATGACCCCTCAGGAACCCGAAC</td>
</tr>
<tr>
<td></td>
<td>R: TCAGCGGCTGGCCCGC</td>
</tr>
<tr>
<td>Rv1543 in <em>M.smegmatis</em></td>
<td>F: GGGGCGCCATGAATCTTTGGTGACTTA</td>
</tr>
<tr>
<td></td>
<td>R: GGGGCGCCTCAACATGGATCCCT</td>
</tr>
</tbody>
</table>

<sup>a</sup>F, Forward; R, Reverse
Figure 1: Expression of M.tuberculosis Rv3391, Rv1543 and Rv1544 in E.coli

SDS PAGE of cell lysate was done followed by coomassie staining. Lane 1: Markers, Lane 2: BL 21 untransformed, Lane 3: Rv3391, Lane 4: Rv1543, Lane 5: Rv1544.
Figure 2: Effect of pH on acyl-CoA reductase activity of mycobacterial Rv3391 protein

Acyl-CoA reductase activity was measured with 15µM [1-14C]Oleoyl-CoA, 100µM NADPH, 70µg protein in a final volume of 250µl of 0.1 M phosphate buffer for 45min.
Figure 3: Effect of pH on acyl-CoA reductase activity of mycobacterial Rv1543 protein

Acyl-CoA reductase activity was measured with 15µM [1-14C]Oleoyl-CoA, 100µM NADPH, 70µg protein in a final volume of 250µl of 0.1 M phosphate buffer for 30min.
Figure 4: Effect of NADPH concentration on the rate of reduction of Oleoyl-CoA by Rv3391 protein.

Assay was conducted with 70µg protein from cell free lysate of E.coli expressing Rv3391 with 15µM [1-14C]Oleoyl-CoA in a final volume of 250µl of 0.1 M phosphate buffer for 45min.
Figure 5: Effect of NADPH concentration on the rate of reduction of Oleoyl-CoA for Rv1543 protein

Assay was conducted with 70µg protein from cell free lysate of *E.coli* expressing Rv1543 with 15µM [1-14C]Oleoyl-CoA in a final volume of 250µl of 0.1 M phosphate buffer for 30min.
Figure 6: Substrate saturation curve for Rv3391 protein

Assay was done with 70µg cell free lysate of E.coli expressing Rv3391 protein with 15µM [1-14C]Oleoyl-CoA, 100µM NADPH in a final volume of 0.1 M phosphate buffer incubated for 45 min.
Figure 7: Substrate saturation curve for Rv1543 protein

Assay was done with 70µg cell free lysate of *E. coli* expressing Rv1543 protein with 15µM [1-14C]Oleoyl-CoA, 100µM NADPH in a final volume of 0.1 M phosphate buffer incubated for 30 min.
Figure 8: Effect of thiol-directed reagents on acyl-CoA reductase activity on Rv3391 protein

Assay was done with 70µg cell free lysate of E.coli expressing Rv3391 protein with 15µM [1-14C]Oleoyl-CoA, 100µM NADPH in a final volume of 0.1 M phosphate buffer incubated for 45 min.
Figure 9: Effect of thiol-directed reagents on Acyl-CoA reductase activity on Rv1543 protein

Assay was done with 70µg cell free lysate of *E.coli* expressing Rv1543 protein with 15µM [1-^14^C]Oleoyl-CoA, 100µM NADPH in a final volume of 0.1 M phosphate buffer incubated for 30 min.
Figure 10: Identification of product expressed by M. tuberculosis Rv1544 in E.coli

TLC showing alcohol production from aldehyde by Rv1544 protein. Lane 1: BL21 untransformed, Lane 2: Rv1544
Aldehyde reductase activity was carried out with 7µM [1-14C]oleoyl-CoA, 100µM NADPH, 70µg protein in a final volume of 250µl of 0.1 M phosphate buffer for 30min respectively.

Figure 11: pH curve of reductase encoded by Rv1544
Aldehyde reductase activity was carried out with 7µM [1-14C]oleoyl-CoA, 100µM NADPH, 70µg protein in a final volume of 250µl of 0.1 M phosphate buffer for 30min respectively.
Figure 13: Effect of inhibitors on reductase encoded by Rv1544

Aldehyde reductase activity was carried out with 7µM [1-14C]oleoyl-CoA, 100µM NADPH, 70µg protein in a final volume of 250µl of 0.1 M phosphate buffer for 30min respectively
CHAPTER FOUR: CONCLUSION

Wax esters are known to be one form of energy storage in both the plant and animal kingdom (26). Wax esters display diverse functions apart from acting as rich energy source (16, 26). Wax esters protect plants from desiccation by coating their surfaces (29). Evaporation of moisture from tear film is retarded in the eye by the secretion of wax from the meibomian gland (3, 22). Biosynthesis of wax esters involves three different enzymatic steps: acyl-CoA is reduced to fatty aldehyde that undergoes reduction to fatty alcohol followed by esterification by a fatty alcohol: acyl-CoA transacylation (30, 32). These reactions, first demonstrated in cell free extracts of *Euglena gracilis* (15), have been demonstrated in numerous organisms in both plant and animal kingdoms. (6, 9, 14, 15, 17 18, 19).

Fatty acyl-CoA can be reduced to alcohol without any significant release of fatty aldehyde intermediate as seen in *E.gracilis*, jojoba seeds and sebaceous glands (15, 18, 23). In such cases a single protein catalyzes both acyl-CoA reduction to aldehyde and reduction of the aldehyde to alcohol (15, 18). In other cases two separate proteins generate fatty alcohol from fatty acyl-CoA; the first one generates the aldehyde and the second catalyzes aldehyde reduction (13). In *M.tuberculosis* genome Rv3391 that would encode a 74 KDa protein has been annotated as acyl-CoA reductase (5, 7). We found by homology that Rv1543 might also encode a smaller 40 kDa acyl-CoA reductase with an adjacent ORF that could encode a 31 KDA reductase, annotated as keto reductase. Expression of these three genes in *E.coli* and examination of their enzymatic
activities show that Rv3391 and Rv1543 encode acyl-CoA reductase whereas Rv1544 is an aldehyde reductase (7).

Both acyl-CoA reductases generate both aldehyde and alcohol. The large reductase appear to have two putative NADPH binding sites, in Rv1543 only one NADPH binding site is obvious. Here we demonstrate that it catalyzes reduction of fatty acyl-CoA and fatty aldehyde. Since the two small reductases together equal the size of the larger one, in analogy to the situation found in plants (13), we suspected that Rv1543 product would generate aldehyde and and Rv1544 product would reduce the aldehyde to alcohol. It was surprising that Rv1543 product can catalyze conversion of acyl-CoA to both aldehyde and alcohol. Expression of Rv1543 in M.smegmatis and examination of oleoyl-CoA reduction products generated by subcellular fractions showed that Rv1543 product was located in the membranes as predicted by its sequence. The membrane-located Rv1543 product generated both aldehyde and alcohol confirming the results obtained with the product generated in E.coli. Interestingly, thiol-directed reagents inhibited acyl-CoA reduction to alcohol by Rv1543 product whereas aldehyde formation catalyzed by the same enzyme was not inhibited. One possible explanation is that acyl-CoA reduction involves a critical thiol group and subsequent reduction of the enzyme bound aldehyde to alcohol manifests severe inhibition. However, reduction of the free aldehyde intermediate that comes off the enzyme is less sensitive to inhibition by thiol modification. In fact, the enzyme was found to catalyze reduction of exogenous aldehyde and this reduction was found to be much less sensitive to inhibition by PCMB (data not shown). The cell free preparations of M.tuberculosis H37RV catalyze conversion of [1-$^{14}$C] oleoyl-CoA to fatty alcohol and wax ester demonstrating the presence of the enzymatic
activities required for wax ester synthesis (unpublished). The present results identify mycobacterial genes involved in the reduction of acyl-CoA to alcohol but the genes encoding the esterification process remains to be elucidated. Whether wax ester storage is involved in latency is unknown.
LIST OF REFERENCES


