Rv0132c of *Mycobacterium tuberculosis* Encodes a Coenzyme F$_{420}$-Dependent Hydroxymycolic Acid Dehydrogenase

Endang Purwantini$^{1,2}$, Biswarup Mukhopadhyay$^{1,2,3,4,*}$

$^1$ Department of Biochemistry, Virginia Tech, Blacksburg, Virginia, United States of America, $^2$ Virginia Bioinformatics Institute, Virginia Tech, Blacksburg, Virginia, United States of America, $^3$ Departments of Biological Sciences, Virginia Tech, Blacksburg, Virginia, United States of America, $^4$ Virginia Tech Carilion School of Medicine, Virginia Tech, Blacksburg, Virginia, United States of America

Abstract

The ability of *Mycobacterium tuberculosis* to manipulate and evade human immune system is in part due to its extraordinarily complex cell wall. One of the key components of this cell wall is a family of lipids called mycolic acids. Oxidation of mycolic acids generating methoxy- and ketomycolic acids enhances the pathogenic attributes of *M. tuberculosis*. Thus, the respective enzymes are of interest in the research on mycobacteria. The generation of methoxy- and ketomycolic acids proceeds through intermediary formation of hydroxymycolic acids. While the methyl transferase that generates methoxymycolic acids from hydroxymycolic acids is known, hydroxymycolic acids dehydrogenase that oxidizes hydroxymycolic acids to ketomycolic acids has been elusive. We found that hydroxymycolic acid dehydrogenase is encoded by the rv0132c gene and the enzyme utilizes F$_{420}$ a deazaflavin coenzyme, as electron carrier, and accordingly we called it F$_{420}$-dependent hydroxymycolic acid dehydrogenase. This is the first report on the involvement of F$_{420}$ in the synthesis of a mycobacterial cell envelope. Also, F$_{420}$-dependent hydroxymycolic acid dehydrogenase was inhibited by PA-824, and therefore, it is a previously unknown target for this new tuberculosis drug.

Introduction

The cell wall of *Mycobacterium tuberculosis* (Mt$b$), the causative agent of tuberculosis [1,2], has an extraordinarily complex and very hydrophobic structure. Consequently it offers an exceptionally low permeability and makes the Mt$b$ cells poorly accessible to drugs and less vulnerable to attack by the host immune system [3]. For this reason, cell wall synthesis enzymes of Mt$b$ have been targeted for TB drug development [4]. Mycolic acids (MAs) are some of the key lipid components of the mycobacterial cell wall. These “high-molecular weight beta-hydroxy fatty acids with a long alpha-alkyl side chain” [5] (Fig. S1) are constituents of mycolyl-arabinogalactan-peptidoglycan complex and trehalose mono-/di-mycolates (TMM and TDM) [6–8]. By helping to build a strong cell wall and being immunogenic [7,9,10], these complexes contribute to the development of TB [3,10–15]. Mt$b$ generates three structural types of MAs which are called $\varepsilon$-, methoxy- and keto-mycolic acids ($\varepsilon$-, M- and K-MAs) and under *in vitro* growth conditions it does not contain epoxymycolic acids ($E$-MAs) that are found in *Mycobacterium smegmatis* [16]; the respective chemical structures are shown in the Supporting Material (Fig. S1). The keto- and methoxy-derivatives enhance the pathogenic nature of Mt$b$ [17,18], and the bacterium uses these compounds to modulate the host immune response [9,19–21]. A recent report shows that K-MAs allow Mt$b$ to form pellicle structures, which in turn make this pathogen drug-resistant [22]. Thus, the enzymes that introduce keto- and methoxy-groups in mycolic acids are of research interest [3,17,23–26]. These oxygenated lipids are generated through common immediate precursors, hydroxymycolic acids (H-MAs) (Fig. 1) [3,24,27]. Whereas it is known that in Mt$b$ the conversion of H-MAs to M-MAs is catalyzed by an adenosylmethylene-thionine-dependent methyltransferase (Mma$3$ or Cma$B$) encoded by the ORF Rv0643c [7,24,26] (Fig. 1), the enzyme that oxidizes H-MAs to K-MAs remains unknown. We call this unknown enzyme hydroxymycolic acid dehydrogenase (HMAD). In this report we describe the gene that encodes HMAD in Mt$b$ and demonstrate that the enzyme utilizes coenzyme F$_{420}$, a deazaflavin derivative, as electron carrier (Fig. 1). Thus, we named the enzyme HMAD for F$_{420}$-dependent Hydroxyl Mycolic Acid Dehydrogenase. Also, we show that HMAD is inhibited by PA-824, a nitroimidazopyran and a new TB drug that is currently on clinical trial [28].

Results and Discussion

Identification of Rv0132c as Coenzyme F$_{420}$-dependent Hydroxymycolic Acids Dehydrogenase (fHMAD) in *M. tuberculosis*

This work began with an analysis of the available data, and the resulting hypothesis was tested via genetic analysis of an Mt$b$ gene.
in *Mycobacterium smegmatis*. The rationale for the selection of *M. smegmatis* as the experimental host has been elaborated below.

Selection of *Mycobacterium smegmatis* as a facile screening host in a search for the HMAD encoding gene of *Mycobacterium tuberculosis*. As mentioned above, *Mtb* produces α-, K- and M-MAs, and it does not contain epoxymycolic acids (E-MAs) under *in vitro* growth conditions [16]. In this regard *Mycobacterium bovis* strain BCG (BCG) is similar to *Mtb* except some of the strains of the former do not produce M-MAs as the cmaB or mma3 gene of the organism is non-functional [16,25,26]. *M. smegmatis* produces α-, a9- and E-MAs but is devoid of K- and M-MAs [16,29]. The investigation described in this report concerns only the longer aliphatic chains (the R groups) of the MAs (Fig. S1 and Fig. 1).

Fig. 1 shows the proposed pathways for synthesis of H-, K-, M- and E-MAs in wild-type and recombinant *Mtb*, BCG and *M. smegmatis* [17,23,24,27]; a common precursor for the aliphatic chains of various MAs serves as the starting point in this scheme. The deletion of the hma gene (also called mma4 and cmaA) in *Mtb* abolishes the production of K- and M-MAs and causes the production of E-MAs and an intermediate that is similar to α-MAs of *M. smegmatis* [17,23,27]. Heterologous expression of the *Mtb* or BCG hma gene (orf rv0642 or mb0661, respectively) in *M. smegmatis* allows the synthesis of H-MAs and reduces the production of α- and E-MAs in the recombinant strain [23,24,27]. Therefore, in *M. tuberculosis* the hma gene encodes the enzyme that generates H-MAs as precursors for both keto and methoxy forms, and this process competes well with the E-MA formation. The accumulation of H-MAs in a *M. smegmatis* strain carrying heterologous hma shows that the organism lacks both Mma3 (or CmaB) and HMAD and therefore cannot transform this intermediate into M-MAs and K-MAs [23,24,27]. Accordingly, a recombinant *M. smegmatis* strain carrying *Mtb* hma could be used to screen candidate *Mtb* genes for HMAD activity via complementation. This is advantageous, as unlike *Mtb*, *M. smegmatis* is not pathogenic and it grows much faster than *Mtb* or BCG [30].

Identification of rv0132c as a candidate gene encoding HMAD. We searched for this gene in the *Mtb* H37Rv genome [31] by using the following criteria. It must be present in both *Mtb* and BCG while absent in *M. smegmatis*. It should encode a dehydrogenase capable of catalyzing a two-electron transfer process, as the conversion of H-MAs to K-MAs in the recombinant strain [23,24,27]. Accordingly, a recombinant *M. smegmatis* strain carrying *Mtb* hma could be used to screen candidate *Mtb* genes for HMAD activity via complementation. This is advantageous, as unlike *Mtb*, *M. smegmatis* is not pathogenic and it grows much faster than *Mtb* or BCG [30].

**Figure 1. Proposed pathways for the synthesis of hydroxy-, keto-, methoxy- and epoxymycolic acids in mycobacteria** [7,24]. A common intermediate for various R groups is used as the starting point. Where MmaA2 and CmaA2 are involved in the formation of cis cyclopropane group, CmaA2 and an yet to identified enzyme (indicated by?) catalyze trans-cyclopropanation [48,67]. The details of the individual R groups are shown in Fig. S1. * indicates that it is not known whether the cyclopropanation step follows or precedes oxygenation. All protons (except for the isolated groups) that have been target for NMR data analysis have been shown in red. The OH group shown in italics and underlined in the box at the left corner of the figure was converted to a methoxy group during saponification of mycolic acids; the process generated mycolic acids methyl esters (MAMEs).

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homolog of coenzyme F420-dependent glucose-6-phosphate (G6P) dehydrogenase (Fgd1 or Fgd1) that catalyzes two-electron oxidation of G6P [glucose-6-phosphate + F420 \rightarrow 6-phosphogluconolactone + reduced F420 (F420H2)] [33,34]. Coenzyme F420 is a deazaflavin derivative that is found in all mycobacteria [35,36]. At the ground state it functions similar to nicotinamide coenzymes or NAD(P), mediating hydride transfer reactions [37]. Fgd2 does not oxidize G6P and its substrate remains unknown [32,38]. M. smegmatis expresses Fgd1 and it lacks Fgd2, whereas both Mtb and BCG carry Fgd1 and Fgd2 ([31,32]; NCBI Accession Number: NC_0006596). Both Fgd1 and Fgd2 are also homologs of F420-dependent methylenetetrahydromethanopterin reductase (M) that are found in methanogenic archaea [34]. To obtain some clues to the nature of the substrate that Fgd2 or Rv0132c acts on, we analyzed the primary structure of this protein based on X-ray crystallographic structures of three well characterized Mer homologs: Fgd1 of Mtb (PDB ID: 3B4Y) [32], F420-dependent methylenetetrahydromethanopterin reductase from Methanopyrus kandleri (MkMer; PDB ID: 1EZW) [39], and an F420-dependent secondary alcohol dehydrogenase (Adf; PDB ID: 1RHC) from Methanoculleus thermophilicus [40]. M. kandleri and M. thermophilicus are methanogenic archaea. In Fgd1, His190, Ser73, Val74 and Glu109 help to bind F420 and these residues, except Ser, are functionally conserved in MkMer and Adf [39,40] as well as in Rv0132c (Fig. 2). Ser73 of Fgd 1 interacts with F420 via the oxygen atom of the backbone carbonyl [32], and in Rv0132c and MkMer this residue has been substituted with Gly and in Adf the equivalent position is occupied by a Cys (Fig. 2). Ser, Cys and Gly are highly compatible in terms of their hydrophobicities and sizes [41–43]. Hydrophobic compatibility indices for Ser-Cys and Ser-Gly pairs in a scale 1–20 (1 and 20 being least and fully compatible, respectively) are 17.7 and 16.8, respectively [41]. The isoelectric points of Ser, Cys and Gly are 5.7, 6.0 and 5.1, respectively [41].

In our next analysis we tried to understand whether Rv0132c has the potential to transform hydrophobic substrates such as mycopic acids. Adf and MkMer interact with hydrophobic substrates whereas Fgd1 accommodates charged glucose-6-phosphate. In Adf the Val195 and Leu227, which are hydrophobic, not only interact with the hydroxymethyl unit of F420 but also help to position the hydrocarbon chain of the substrate [40]. Similarly, Ala197 and Tyr229 in MkMer interact with both the F420 and the hydrophobic perin ring of tetrahydromethanopterin [40]. Rv0132c shows partial conservation of these characteristics, as homologous residues in this protein are Ala and Glu, respectively (Fig. 2). In contrast, the equivalent positions in Fgd1 are occupied by Ser and Glu [32], which are less hydrophobic and polar, respectively. In Fgd1, Lys232, which has a charged side chain, helps to bind a citrate ion, which is a competitive inhibitor of the enzyme [32]. In Rv0132c, Adf and MkMer, this residue has been replaced with Phe, Trp and Cys, respectively (Fig. 2). Additionally, Fgd1 utilizes a positively charged pocket formed by Lys190, Lys253, and Arg201 to hold the phosphate group of glucose-6-phosphate [32] and these residues are not conserved in Rv0132c (Fig. 2). Hence, Rv0132c has the potential of interacting with a hydrophobic substrate.

We had observed that two tandem Arg residues in the NH2-terminus (amino acid residues 1–27, MTGISRRTFGLAAGF-GAIGAGGGLGCCC; bold and underlined, characteristic residues) form a signature for translocation into the periplasmic space via a Tat-dependent protein export pathway which exists in the mycobacteria [45] and the features shown underlined (see above) represent a putative prokaryotic membrane lipoprotein lipid attachment site (PS00013) where Cys27 could carry lipophilic substrates [46]. Indeed, as our work was complete, Rv0132c was found to be exported to the cell envelope of Mtb [38]. Thus, it is reasonable to assume that Rv0132c could interact with the hydrocarbon chains of the mycopic acids (R group, Fig. 1). The relevance of the demonstrated cellular location of Rv0132c to our findings has been discussed below.

**Experimental elucidation of the function of Rv0132c.** We have tested whether Rv0132c represents an F420-dependent hydroxymycolic acids dehydrogenase (HMAD) by introducing this gene and *hma* into *M. smegmatis*. As expected, the plasmid pEP-hma, which was constructed based on the *E. coli*-mycobacterium shuttle vector pSMT3 [47] and carried *Mtb hma* gene (r0642c)
under the control of its native promoter element, produced H-MAs in *M. smegmatis* mc²¹⁵⁵ or wild-type (wt) strain (Fig. 3A, lane hma). The expression of both *hma* and *rv0132c* genes from pEP-rv0132c/hma led to the production of K-MAs (Fig. 3A, lane rv0132c/hma); *rv0132c* alone (pEP-rv0132c) did not provide either H-MAs or K-MAs (Fig. 3A, lane rv0132c). *M. smegmatis*, as such (host control; Fig. 3A, lane None) or while carrying pSMT3 (vector control; Fig. 3A, lane pSMT3), also did not produce either H-MAs or K-MAs; they contained α-, α’-, and E-MAs.

The initial identification of the individual mycolic acid bands on the TLC plates was performed via comparison with previously reported patterns [10,23,24]. Then we carried out mass spectrometric and NMR spectroscopic analysis with materials recovered from the relevant TLC bands. For the H-MAs and K-MAs bands, MALDI-TOF mass spectrometry yielded spectra that were characteristics of respective mycolic acids with 77–82 carbon atoms (Fig. 4) [23]. The mass for every characteristic H-MA ion (Fig. 4A) was 2 units higher than that for a K-MA (Fig. 4B) and this is consistent with the respective structures shown in Fig. 1. ¹H NMR data provided more detailed characterization of relevant mycolic acid species and we discuss the findings below with a focus on the H-atoms marked in red in Fig. 1. This analysis is based on previously reported NMR data on mycolic acids [23,27,29,48,49]. The resonances at 2.7 ppm observed with the E-MA preparation obtained from *M. smegmatis* mc²¹⁵⁵ cells (Fig. 5A) were characteristics of the methine protons associated with a trans-cyclopropane group [23]. In the spectrum obtained with the H-MAs preparation from *M. smegmatis* (pEP-hma) strain (Fig. 5B) the resonances for the above-mentioned epoxy group were not seen and instead it exhibited a resonance at 3.5 ppm representing the methine proton on the carbon that carried the characteristic hydroxyl group of H-MA. Similarly, the ¹H resonances of the methylene and methine groups that flank the carbonyl group in K-MAs were found at 0.40, 0.48, and 0.58 ppm for cis-epoxide (Fig. 5C); 0.50 ppm for the K-MA preparation from *M. smegmatis* (pEP-rv0132c/hma) strain (Fig. 5C). The spectra for the E-MA, H-MA, and K-MA preparations exhibited the following common resonances and this observation is consistent with previous reports [23,27,29,48,49] (Fig. 5A–C): 1.29 ppm – broad, isolated methylene proton; 0.85 ppm - triplet, terminal methyl groups; 3.71 ppm – singlet, methyl ester; 2.50 ppm - multiplet, methine at position C-2 with respect to the terminal carboxyl group (see within the box at the left corner of Fig. 1). None of the above spectra showed the resonances of the protons that are associated with the cyclopropane groups of mycolic acids produced by *Mtbc*; these resonances appear at −0.40, 0.50, and 0.58 ppm for cis-cyclopropanation and 0.01–0.16 ppm for trans-cyclopropanation [49]. Major mycolic acids produced by *M. smegmatis* lack cyclopropanation under normal growth conditions [23,50]. This modification occurs during growth at 25°C [51] and the growth temperature in our study was 37°C.

The above-described analysis showed that the heterologous expression of *Mtb* hma in *M. smegmatis* caused the suppression of the synthesis of E-MAs and the production of H-MAs, and Rv0132c protein converted the H-MAs to K-MAs. Thus, in *Mtb* Rv0132c encoded a hydroxymycolic acid dehydrogenase (HMAD).

The next step was to determine if HMAD was coenzyme F₄₂₀-dependent. The *fbiC* is a key gene for the production of F₄₂₀ chromophore in mycobacteria [52] and mycobacterial strains lacking a functional *fbiC* gene are devoid of this coenzyme [52,53]. We found that a *M. smegmatis* *AfbiC* strain [53] generated H-MAs but not K-MAs when complemented with pEP-rv0132c/hma (Fig. 3B, right most lane or the lane rv0132c/hma for *AfbiC*). Complementation with pEP-hma also provided H-MAs in *M. smegmatis* *AfbiC* (Fig. 3B, lane hma) and pEP-rv0132c did not provide either K-MAs or H-MAs (Fig. 3B, lanes rv0132c), the left most lane (lane rv0132c/hma for wild-type (wt) strain) served as a positive control, where production of K-MAs was observed. Hence, HMAD required F₄₂₀ for activity and we call it hereafter hHMAD.

In this context we address two sets of contradicting reports in the literature that concern the biosynthesis of H-MAs and K-MAs in *M. smegmatis* strains carrying clones for the *Mtb* *hma* gene. In one case the *hma* gene caused the synthesis of both H-MAs and K-MAs [23,24] and in the latter only H-MAs were found in the recombinant [10]. Our result is consistent with the latter [10], as the conversion of a hydroxyl group to a keto group would be catalyzed by an electron transfer enzyme or dehydrogenase such as hHMAD, and not by a methylase/hydrase activity such as in Hma.

![Figure 3. Thin layer chromatography (TLC) profiles of methyl esters of mycolic acids extracted from various Mycobacterium smegmatis strains grown in the absence and presence of PA-824.](image)

Wild type (wt) and *AfbiC* strains of *M. smegmatis* carrying the indicated plasmids were analyzed (lane label, name of plasmid): None, no plasmid; pSMT3, pSMT3 (vector control); hma, pEP-hma; rv0132c, pEP-rv0132c; rv0132c/hma, pEP-rv0132c/hma. (+) and (−) cultivation of *M. smegmatis* (pEP-rv0132c/hma) with and without PA-824 (100 microgram per ml), respectively. Mycolic acid types: α, α’, epoxy (E), hydroxy (H), and keto (K) [Fig. S1 shows the respective chemical structures.]. Panel A: rv0132c causing the conversion of H-MAs to K-MAs in wild-type *M. smegmatis*; Panel B: Requirement of *fbiC* for the production of K-MAs in *M. smegmatis* (pEP-rv0132c/hma) [Note: The left most lane is for wt strain, used as control]. Panel C: Inhibition of the production of K-MAs in *M. smegmatis* (pEP-rv0132c/hma) by PA-824.

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Fig. 1 shows two mycobacterial MA oxygenation pathways, one of which leads to H-MAs, K-MAs and M-MAs, and the other is for the production of E-MAs. It has been shown that when the former operates, the latter is suppressed [17,23,24,27]. We observed a more stringent form of this regulation in our studies. The data in Fig. 3A show that when \textit{M. smegmatis} was made capable of producing K-MAs, it did not produce E-MAs; a comparison of rv0132c/hma lane with any other lane in Fig. 3A leads to this conclusion. This effect was not due to the Rv0132c protein or the DNA elements cloned into pEP-rv0132c/hma, as their presence did not suppress E-MA production when the host lacked \textit{fbiC} (Fig. 3B, lanes hma, rv0132c and rv0132c/hma). Hence it could be hypothesized that K-MAs either inhibit one or more E-MA synthesis enzymes and/or suppresses the expression of E-MA production.

Figure 4. MALDI-TOF mass spectra of methyl esters of hydroxymycolic acids (A) and ketomycolic acids (B) recovered from engineered \textit{Mycobacterium smegmatis} strains. Hydroxymycolic acids were obtained from the lane hma and ketomycolic acids were from lane rv0132c/hma (Fig. 3A). Only a part of each spectrum is shown and the annotations for the ion masses are based on reference [23]: labels H & K, ions from hydroxy- and ketomycolic acids preparations; numbers 77–82: total number of carbon atoms in free acids. The unlabeled peaks belong to unidentified species that were present in both preparations.

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of respective genes. Other possibilities are the interference with the translocation of the precursor of E-MA s to the modification site such as periplasm or a flux-based competition between the two pathways. We also observed that the cellular level of H-MA s increased when K-MA s were produced (Fig. 3A, lane pEP-rv0132c/hma). It is possible that K-MA s enhanced the activities of one or more enzymes that generate H-MA s from α-mycolic acids (Fig. 1) and/or increased the expressions of their genes, and the prevailing fHMAD activity was not at par with the rate of H-MA production. The other explanation is that in M. smegmatis (pEP-rv0132c/hma) the cellular level of Hma activity was much higher than that of fHMAD. In this context we note that the overproduction of M-MA s through over-expression of Mma3 or CmaB suppresses K-MA s in Mtb [18,22]. A more detailed study is needed to elucidate the mechanisms underlying these competitions between mycolic acids oxygenation pathways.

**Inhibition of fHMAD by PA-824**

PA-824, a new TB drug, inhibits the formation of K-MA s and causes an accumulation of H-MA s in Mtb [54]. We tested whether this effect is specifically due to the inhibition of fHMAD. As shown in Fig. 3C, in the presence of PA-824, M. smegmatis (pEP-rv0132c/hma) accumulated a high level of H-MA s and contained a reduced level of K-MA s (Fig. 3C). Hence, PA-824 inhibited the heterologously expressed fHMAD. To establish further that fHAMD was inhibited by PA-824, we determined the relative levels of K-MA s in M. smegmatis (pEP-rv0132c/hma) cultivated in the presence of this drug at various concentrations. The results showed that the inhibition began at a PA-824 concentration between 10–25 microgram per ml culture and increased further as the drug concentration was raised (Fig. S2). Such a dose-dependent increase in the inhibition of K-MA synthesis by PA-824 has been reported also for M. tuberculosis [54]. However, the K-MA synthesis process in M. smegmatis (pEP-rv0132c/hma) was much less sensitive to PA-824 than that observed in wild-type M. tuberculosis; in M. tuberculosis this inhibition begins at a PA-824 concentration between 30-60 nanogram per ml culture [54]. It is possible that the higher minimum inhibitory concentration of PA-824 observed with M. smegmatis (pEP-rv0132c/hma) was due to the presence of a higher level of Rv0132c protein in this recombinant strain; rv0132c was expressed from a multi-copy plasmid [47] and was driven by both the native promoter as well as the strong and constitutive mycobacterial hsp60 promoter [47,55]. Another explanation is that compared to M. tuberculosis, M. smegmatis takes up PA-824 poorly and as a result for achieving an inhibitory concentration of the drug inside the cell, it had to be supplied in the culture medium at a higher concentration; wild-type M. smegmatis is naturally resistant to PA-824 [56]. Nevertheless, the results presented in Figs. 3C and S2 show that the phenomenon of inhibition of K-MA synthesis by PA-824 that was observed in wild-type M. tuberculosis could be reproduced in a M. smegmatis strain carrying cloned hma and rv0132c genes from the former.

PA-824 kills Mtb under both aerobic and anaerobic conditions [54,57,58]. The anaerobic killing occurs through the reduction of PA-824 by an F_{120H_2}-dependent nitroreductase called Ddn, which is followed by the production of toxic NO [57]. Since F_{120H_2} is produced by Fgd1, Mtb and BCG strains lacking Fgd1 activity are resistant to PA-824 [54]. The aerobic killing of Mtb by PA-824 has been thought to occur due to the elimination of K-MA s via unknown mechanisms [54,58]. Our data has now linked this concept to a gene, rv0132c. Curiously, F_{120H_2} is an integral part of both mycobacterial systems, Ddn and fHAMD, that interact with PA-824.

As mentioned above, fHMAD found to be exported to the cell envelope of Mtb [38]. Also, it is thought that complete mycolic acids are transported to the plasma membrane as trehalose monomycolates or TMM [39]. In combination these observations suggest that in Mtb at least one additional modification, formation

Figure 5. Proton NMR spectra of methyl esters of mycolic acids recovered from engineered *Mycobacterium smegmatis* strains. The sources of hydroxymycolic acids or H-MA s (A) and Ketomycolic acids or K-MA s (B) were same as that indicated in the legend of Fig. 4. Epoxymycolic acids or E-MA s (C) were from lane “None” in Fig. 3. In each case the inset shows expansion of the relevant regions.

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of keto group, of otherwise complete mycolic acids occur within the cell envelop.

Conclusion

The hydroxymycolic acid dehydrogenase of \textit{Mbb} was shown to be an F_{420}\textsuperscript{+}-dependent enzyme encoded by the ORF Rv0132c and it is inhibited by PA-824, a new TB drug.

Our data suggest that there is only one \textit{bona fide} Fgd in the mycobacteria. Citing the lack of glucose-6-phosphate dehydrogenase activity in Rv0132c, it has been recently suggested that this protein should no longer be called Fgd2, and Fgd1 should be called simply Fgd [38]. Our data supports this proposal and provides a functional name for Rv0132c, \textit{F}_{420}\textsuperscript{+}-dependent hydroxymycolic acid dehydrogenase (HMAD). Coenzyme \textit{F}_{420} is universally present and essential in the strictly anaerobic methanogenic archaea [37]. In the bacterial domain, a similarly wide distribution of this deazaflavin derivative is seen in the Actinobacteria phylum, which includes the mycobacteria [35,36]. Every mycobacteria examined thus far contains \textit{F}_{420} [35,36]. As mentioned above, in the hydride transfer function \textit{F}_{420} mimics nicotinamides [37]. Perhaps in the mycobacteria \textit{F}_{420} participates in a set of hydride transfer reactions that cannot be accomplished at all or efficiently by the nicotinamides due to thermodynamic reasons, such as a need to operate at a lower redox potential. Such a specialized role has now been seen in the neutralization of nitrosative stress [53] (via a chemical reaction with Fgd-derived \textit{F}_{420}\textit{H}2 and in the introduction of a key functionality to the complex mycobacterial cell envelope (the HMAD reaction as demonstrated here). Both of these actions bring resilience to the mycobacteria against environmental stresses such as those imposed by the human immune system. It is noteworthy, that the current report presents the first example for the involvement of \textit{F}_{420} in the biosynthesis of mycobacterial cell wall. The nitroreductase (Dnh) that helps to activate PA-824 with \textit{F}_{420}\textit{H}2 and the \textit{F}_{420}\textsuperscript{+}-dependent enzymes that allow the mycobacteria to decolorize triphenylmethane dyes or to degrade aflatoxins [57,60–62] could also fulfill yet to be described key and normal physiologically relevant cellular functions in these organisms.

Materials and Methods

Oligonucleotides, Plasmid, DNA, Bacteria and Growth Conditions

Oligonucleotides, plasmids and bacteria used in this study have been described in the Supporting Material (Table S1). \textit{M. tuberculosis} H37Rv chromosomal DNA was obtained from the National Institutes of Health’s TB Vaccine Testing and Research Materials Contract (TBVTRMC) at the Colorado State University. \textit{E. coli} was grown in Luria-Bertani broth or solid media. \textit{Mycobacterium smegmatis} strains were grown in Middlebrook 7H9 broth or on agar solidified medium with 0.2% glycerol as the carbon and energy source [63]. For liquid cultures \textit{Tween 80} at the concentration of 0.05% was also included. When required, \textit{M. smegmatis} strains bearing antibiotic resistance genes were selected on or grown with kanamycin and hygromycin at the concentration of 20 and 150 microgram/ml, respectively, and for similar work with \textit{E. coli} strains ampicillin, kanamycin, and hygromycin concentrations were 100, 20, and 150 microgram/ml, respectively. To study the effect of PA-824 on the mycolic acids content of \textit{M. smegmatis} (pEP-rv0132c/hma), a freshly inoculated culture was grown overnight to an optical density of 0.3 at 600 nm (as measured by use of a DU800 UV/Vis Spectrophotometer, Beckman Coulter, Brea, CA). It was then supplemented with PA-824 to a desired final concentration from a stock solution (80 mg/ml) in DMSO and grown for additional 36 h. The control culture received DMSO at a concentration of 1.25 ml/liter. PA-824 was a gift from Global Alliance for TB Drug Development (New York, NY) through the Global Health program of the RTI International (Research Triangle Park, NC).

Molecular Biology Techniques

\textit{M. smegmatis} chromosomal DNA was isolated as described previously [63]. Transformation of \textit{M. smegmatis} with plasmids was performed via electroporation [64] at 2.5 KV using an Electro-oporator 2510 (Eppendorf North America, Hauppauge, NY) and a cuvette with a 0.2 cm electrode-gap. For PCR amplification, Phusion polymerase with the GC buffer (Finnzymes Inc., Woburn, MA) was used. Plasmid purification and DNA recovery from agarose gels were done using Qiaprep and Qiaquick columns (Qiagen Inc., Valencia, CA), respectively. Manipulations of DNA were performed using standard methods [65].

Construction of Protein Expression Plasmids and Bacterial Strains

The protein expression plasmids were based on pSMT3, a mycobacteria–\textit{Escherichia coli} shuttle vector that allows selection for hygromycin resistance and gene expression under the control of the strong and constitutive \textit{hsp60} promoter [47,55]. To generate the plasmids pEP-hma and pEP-rv0132c for the expression of \textit{hma} (rv0642c) and rv0132c of \textit{M. tuberculosis}, respectively, the corresponding coding sequences along with the respective upstream regions bearing the promoters and ribosome-binding sites (253 bp for \textit{hma} and 316 bp for \textit{rv0132c}) and a bit of the downstream sequences (4 bp for \textit{hma} and 20 bp for \textit{rv0132c}) were PCR-amplified from \textit{M. tuberculosis} H37Rv chromosomal DNA and cloned into pSMT3; the cloning sites were EcoRV and ClaI for \textit{hma} and BamHI and EcoRV for \textit{rv0132c}. The primers used for this work have been described in the Supporting Material (Table S1). The cloned genes in pEP-hma and pEP-rv0132c were expressed in \textit{M. smegmatis} from their native promoters and perhaps also from the plasmid resident mycobacterial \textit{hsp60} promoter. The plasmid pEP-rv0132c/hma that allowed simultaneous expression of \textit{hma} and \textit{rv0132c} was constructed by cloning the \textit{hma} coding sequence along with the respective upstream and downstream sequences as mentioned above at the EcoRV and ClaI sites (or at the 3’ end of the \textit{rv0132c} segment) of pEP-rv0132c. The construction of \textit{M. smegmatis} \textit{AfbiC::aph} strain has been described previously [53].

Preparation and Analysis of Mycolic Acid Methyl Esters

Mycolic acid methyl esters (MAMEs) were prepared as described previously [66]. Briefly, pelleted mycobacterial cells were saponified via incubation in 15% tetrabutyl ammonium hydroxide at 110°C overnight, followed by the addition of water, diazomethane, and dichloromethane and shaking at room temperature. From this mixture the MAMEs were recovered in the dichloromethane fraction and washed sequentially with equal volumes of water, 0.1 N HCl and water. The dichloromethane solution of MAMEs was dried under a stream of nitrogen, dissolved in a toluene-acetonitrile mixture (2:1), and then precipitated at room temperature with an addition of acetonitrile (final toluene:acetonitrile, 2:3). The pellet of MAMEs was dissolved in dichloromethane. Analysis of MAMEs was carried out by thin layer chromatography (TLC) on an aluminum-backed silica gel plate (10 x 10 cm, Merck 5735-silica gel 60F254) by multiple developments using a solvent comprised of petroleum
ether and diethyl ether (9:1). Mycolic acid spots were revealed by charring at 110 °C for 15 min after spraying with 5% ethanolic molybdophosphoric acid [66].

Mass Spectrometric and NMR Analysis of Mycolic Acids This work concerned the methyl esters of hydroxy- and keto-mycolic acids. After performing multi-lane TLC separation for a sample, a terminal lane was cut off and processed for color development as described above. Then using a relevant band in this lane as a guide, the desired mycolic acid spots (silica layer) were scrapped off from the rest of the lanes. From the recovered silica particles, mycolic acids were extracted with dichloromethane and analyzed via MALDI-TOF mass spectrometry at School of Chemical Sciences Mass Spectrometry Laboratory at the University of Illinois at Urbana-Champaign. The Bruker peptide calibration mixture II (Angiotensin II, Angiotensin I, Substance P, Bombesin, ACTH clip 1–17, ACTH clip 18–39, Somatostatin 28, Bradykinin Fragment 1–7, and Renin Substrate Tetradecapeptide porcine Covered mass range: ~700 Da – 3200 Da) was used for calibration and the matrix was 2,5-dihydroxybenzoic. A Bruker UltrafleXtreme mass spectrometer (Fahrenheitstr. 4,D-28359 Bremen, Germany) equipped with a smart beam II laser was used in the positive mode to acquire MALDI-TOF mass spectra. Samples were analyzed in the Reflectron mode.

A Bruker Avance III 600 MHz available at the NMR Laboratory, Department of Chemistry, Virginia Tech, was used to obtain 1H NMR spectra of the purified mycolic acid methyl esters preparations described above. The solvent was CDCl3 (100% D) and the reported chemical shifts were relative to the methyl resonances of tetramethylsilane (0 ppm).

Supporting Information
Figure S1 Structures of mycolic acids in and Mycobacterium tuberculosis complex and Mycobacterium smegmatis. The detailed structures of R groups in various mycolic acids are shown [4]. The reference cited here is listed in File S1.

(TIF)

Figure S2 Dose-dependent inhibition of K-MA production in M. smegmatis (pEP-rv0132c/lhma) by PA-824. Wild-type M. smegmatis was used as control; the data in Fig. 3 show that neither the expression constructs pEP-lhma and pEP-rv0132c nor the vector pSMT3 allow the production of K-MA in M. smegmatis. The other details of the study have been presented in the MATERIALS AND METHODS. Mycolic acid types: z, z′, epoxy (E), hydroxy (H), and keto (K) [Fig. 1S shows the respective chemical structures.].

(TIF)

Table S1 Oligonucleotides, plasmids, and strains. The references cited in this table are listed in File S1.

(DOC)

File S1 References for Table S1 and Figure S1.

(DOC)

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Author Contributions
Conceived and designed the experiments: EP BM. Performed the experiments: EP. Analyzed the data: EP BM. Wrote the paper: EP BM.
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