An Unconventional Hexacoordinated Flavohemoglobin from *Mycobacterium tuberculosis*<sup>★</sup>‡

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**Background:** Flavohemoglobins are involved in diverse redox reactions and stress response(s).

**Results:** *Mycobacterium tuberculosis* carries a hexacoordinated flavohemoglobin (MtbFHb) that exhibits α-lactate metabolizing and antioxidant activities.

**Conclusion:** MtbFHb is an unconventional flavohemoglobin that oxidizes α-lactate in a FAD-dependent manner.

**Significance:** This study unravels unique features of a new class of flavohemoglobin and sheds light on its function in the biology and pathogenesis of *M. tuberculosis*.

Being an obligate aerobe, *Mycobacterium tuberculosis* faces a number of energetic challenges when it encounters hypoxia and environmental stress during intracellular infection. Consequently, it has evolved innovative strategies to cope with these unfavorable conditions. Here, we report a novel flavohemoglobin (MtbFHb) from *M. tuberculosis* that exhibits unique features within its heme and reductase domains distinct from conventional FHbs, including the absence of the characteristic hydrogen bonding interactions within the proximal heme pocket and mutations in the FAD and NADH binding regions of the reductase domain. In contrast to conventional FHbs, it has a hexacoordinate low-spin heme with a proximal histidine ligand lacking imidazolate character and a distal heme pocket with a relatively low electrostatic potential. Additionally, MtbFHb carries a new FAD binding site in its reductase domain similar to that of α-lactate dehydrogenase (α-LDH). When overexpressed in *Escherichia coli* or *Mycobacterium smegmatis*, MtbFHb remained associated with the cell membrane and exhibited α-lactate:phenazine methosulfate reductase activity and oxidized α-lactate into pyruvate by converting the heme iron from Fe<sup>3+</sup> to Fe<sup>2+</sup> in a FAD-dependent manner, indicating electron transfer from α-lactate to the heme via FAD cofactor. Under oxidative stress, MtbFHb-expressing cells exhibited growth advantage with reduced levels of lipid peroxidation. Given the fact that α-lactate is a byproduct of lipid peroxidation and that *M. tuberculosis* lacks the gene encoding α-LDH, we propose that the novel α-lactate metabolizing activity of MtbFHb uniquely equips *M. tuberculosis* to balance the stress level by protecting the cell membrane from oxidative damage via cycling between the Fe<sup>3+</sup>/Fe<sup>2+</sup> redox states.

*Mycobacterium tuberculosis*, the causative agent of tuberculosis, is an obligate aerobe that requires oxygen for its growth, but it paradoxically has remarkable ability to survive under hypoxia (1–3). The adaptability of tubercle bacillus within the intracellular environment, where a high level of toxic reactive species and scarcity of oxygen limits its survival, depends primarily on its specialized multi-tier defense system and metabolic flexibility to balance the oxidative/reductive reactions for energy generation (4–6). Flavohemoglobins (FHbs),<sup>4</sup> carrying an oxygen binding globin domain and a FAD binding reductase domain, constitute an integral part of virulence and stress response in several pathogenic microbes due to their ability to maintain the cell redox homeostasis at the aerobic/anaerobic interface and scavenge nitric oxide (NO) with high efficiency (7–9). In *M. tuberculosis*, a similar function is carried out by a single-domain truncated hemoglobin, HbN, that displays a potent nitric oxide dioxygenase activity and detoxifies NO 15-fold faster than myoglobin (10, 11), very similar to FHbs. Therefore, it has been anticipated that *M. tuberculosis* may not need a FHb-like protein. Recently, we have shown that the Rv0385 gene of *M. tuberculosis* may encode a novel FHb-like protein (MtbFHb) having a very low nitric oxide dioxygenase activity (12). Its amino acid sequence identity with conventional FHbs, however, appeared to be less than 25% as compared with 40–47%, found among various bacterial, yeast, and fungal FHbs (12). Mining of microbial genome data indicated that the occurrence of this unusual FHb may be restricted to few microbes, mainly belonging to actinomycetes. Interestingly, homologs of MtbFHb were detected in the majority of mycobacteria, including virulent and avirulent species. Bioinformatic analysis of the mycobacterial genome indicated its coexistence with a conventional FHb in some species (12), suggesting their distinct function(s) in cellular metabolism.

FHbs represent an interesting example of a multidomain protein where two domains with different functional properties

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<sup>4</sup> The abbreviations used are: FHb, flavohemoglobin; PMS, phenazine methosulfate; 6CLS, hexacoordinated low spin; LDH, lactate dehydrogenase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Mtb-FHb, *M. tuberculosis* flavohemoglobin.
Flavohemoglobin of M. tuberculosis

interact together and perform an entirely new function(s). Physiological functions of FHbs have been the subject of intense debate over the last decade, and several putative functions have been proposed (8, 9, 13). Experimental evidence, however, strongly supports its key role in protection from the deleterious effects of NO by detoxifying it to nitrate via NO-dioxygenation reaction (14, 15). FHbs have also been found to modulate oxidative stress response in several cases by conferring sensitivity or resistance against the stress (16, 17). One striking common feature among FHbs is their close association with the cellular membrane (9, 18) and a flexible distal pocket that can accommodate large polar molecules including phospholipids, suggesting the possibility of a different, as yet undefined, function for this protein. Diverse function of FHb in cellular metabolism is also supported by the fact that more than one FHb is present in some bacteria, yeast, and fungi (12, 19) that may carry out different cellular functions due to their distinct redox capabilities.

The presence of a two-domain FHb like protein along with two single domain-truncated hemoglobins, HbN and Hbo, in M. tuberculosis is particularly intriguing considering the fact that HbN has been proposed as a key protein involved in NO scavenging and nitrosative stress protection (10, 11) that is usually carried out by conventional FHbs (8, 14, 15). Heme and reductase domains of conventional FHbs display significant conservation within their cofactor binding regions. In comparison, MtbFHb exhibits several critical differences within the functionally conserved regions of its heme and reductase domains (12). The most notable difference within the globin domain is the lack of conserved hydrogen bonding interactions between F8H-H23E-G5Y within the proximal site, indicating the absence of peroxidase-like imidazolate character of the proximal histidine present in conventional FHbs (20). The cofactor (FAD and NAD) binding sites of the reductase domain in MtbFHb are also distinct from other FHbs, indicating significant differences in the interactions between the heme and the reductase domains as well as their associated redox reactions. Thus, MtbFHb and its homologs may constitute a distinct class within the FHb family and may have a novel function(s).

Structural and functional properties of MtbFHb or any of its homologs are unknown at present. In this communication we report the characteristics of this new class of FHb present in M. tuberculosis. We show that the heme and reductase domains of MtbFHb are distinct from those of conventional FHbs. We also show that MtbFHb plays a crucial role in electron transfer and oxidative stress management. These unique properties of MtbFHb may be vital for M. tuberculosis to sustain its cellular metabolism under highly stressed and oxygen-deficient intracellular environment. This study, therefore, adds new information on the bewildering world of microbial hemoglobins as well as provides information for a better understanding of the role of this novel FHb in the biology of stress response in M. tuberculosis

EXPERIMENTAL PROCEDURES

Bacterial Strains, Plasmids, and Culture Conditions— Escherichia coli BL21DE3 was used for the cloning and expression of recombinant proteins. Mycobacterium smegmatis mc² 155 and M. tuberculosis H37Rv having FHb encoding gene identical to Rv0385 were used for the expression of gene in mycobacterial hosts. Cultures of E. coli were grown in Luria Bertani (LB) or Terrific broth (containing 24 g of yeast extract, 12 g of Bacto-tryptone, 12.3 g of K₂HPO₄, 2.3 g of KH₂PO₄) at 37 °C at 180 rpm unless mentioned otherwise. M. smegmatis and M. tuberculosis H37Rv strains were grown in Middlebrook 7H10 agar or 7H9 liquid broth (Difco) supplemented with ADC (10% bovine serum albumin fraction V, dextrose, and sodium chloride), 0.2% glycerol, and 0.05% Tween 80 at 37 °C, 200 rpm. Kanamycin (50 μg/ml) and hygromycin (100 μg/ml) were supplemented in the growth medium whenever required. Details of bacterial strains and plasmids are provided in supplemental Table S1.

Cloning, Expression, and Purification of MtbFHb and Its Globin and Reductase Domains— MtbFHb and its heme (MtbFHb₉₁) and reductase (MtbFHb₁₃₉) domains were retrieved from the genomic DNA of M. tuberculosis H37Rv and expressed in E. coli using standard PCR techniques (see supplemental Table S2 for the list of primers). Authenticity of PCR-amplified genes was checked by nucleotide sequencing. Recombinant genes were cloned into the expression plasmid, pET9b, or pET15b at NdeI-BamHI sites and expressed in E. coli BL21DE3 cultured in Terrific broth supplemented with δ-aminolevulinic acid (500 μM) and FeCl₃ (20 μM) at 37 °C and 180 rpm. MtbFHb was expressed in M. smegmatis and M. tuberculosis H37Ra in an E. coli–mycobacteria expression vector, p19Kpro, at BamHI-HindIII sites. Recombinant MtbFHb when expressed in E. coli appeared strongly associated with the cell membranes and was retrieved after treating the cell pellet with 1% Triton X-100. The reductase domains of MtbFHb were purified after treating the cell pellet with 1% 2-mercaptoethanol. Purification of recombinant proteins was carried out on a nickel nitriilotriacetic acid column (Qiagen) following the manufacturer’s instructions. It resulted in nearly 70% pure preparation of protein exhibiting a distinct reddish pink color. These fractions were further purified using an ion-exchange column (DEAE-Sepharose CL4B; GE Healthcare) equilibrated with 10 mM Tris (pH 8.0) and eluted using a 0.1 M NaCl gradient. The protein and hemoglobin profile was monitored at 280 and 414 nm. Heme content of the cell was determined as described previously (21).

Optical Absorption and Resonance Raman Spectroscopy— Absorption spectra of whole cells or purified protein preparation were recorded using a Cary 100 spectrophotometer. The resonance Raman measurements were carried out with previously described instrumentation (20). Briefly, the output at 413.1 nm from a krypton ion laser (Spectra Physics) was used as the excitation source unless otherwise indicated. The laser line was focused into the sample cuvette, rotating at ~6000 rpm to avoid photo damage of the sample. The scattered light was collected at right angles to the incident beam and focused on the entrance slit (100 μm) of a polychromator (Spex, Metuchen, NJ), where it was dispersed by a 1200 grooves/mm grating and subsequently detected by a liquid nitrogen cooled charge-coupled device (CCD) (Princeton Instruments, Trenton, NJ). A halographic notch filter (Kaiser, Ann Arbor, MI) was used to eliminate the Rayleigh scattering. The Raman spectra were calibrated with indene (Sigma) for the 200–1700-cm⁻¹ spectral region or acetone/
potassium ferrocyanide for the 1700–2300-cm⁻¹ spectral region. Optical absorption spectra were acquired before and after the Raman measurements to ensure the integrity of the samples. The Raman data were processed by GRAMS software (Galactic Industries Corp.).

Site-directed Mutagenesis of FAD Binding Motif of MtbFHb and Cofactor Binding Properties of MtbFHb Mutant—The reductase domain of MtbFHb carries two structural motifs, GX₆GS and AX₆AXN, that are similar to FAD binding sites (GX₆GS and AX₆AXN) of d-lactate dehydrogenases (d-LDHs) (22). To ascertain the site of FAD binding in MtbFHb, the conserved GS residues were converted to alanine following standard overlap PCR techniques using PCR primer pairs specific to MtbFHb. The sequence of oligonucleotides used for this purpose is listed in supplemental Table S2. Incorporation of the mutation within the gene was confirmed by nucleotide sequencing. Hereafter, the MtbFHb carrying this mutation is designated as MtbFHbFAD.

Spectrofluorimetric Analysis of FAD Associated with MtbFHb—The potential association of FAD with MtbFHb, MtbFHbRD, and MtbFHbFAD was checked by published procedure (23). Briefly, the flavin cofactor associated with the protein was extracted after boiling for 5 min and centrifuging at 14,000 rpm for 20 min. The supernatant was checked for the presence of flavin by a fluorometer (Cary Eclipse). The excitation and emission spectra were obtained with emission monitored at 525 nm and excitation set at 450 nm, respectively. The FAD content was calculated by using free FAD, with an extinction coefficient of 11.300 M⁻¹ cm⁻¹, as a standard (24).

Cellular Localization of MtbFHb—The cellular localization of MtbFHb was probed by Western blotting using polyclonal antiserum raised against purified MtbFHb. The cytoplasmic and the membrane fractions of E. coli and M. smegmatis were separated after cell lysis and ultracentrifugation as described earlier (25) and resolved on 12.5% SDS-PAGE. The localization of MtbFHb was analyzed after transferring the gel onto a nitrocellulose membrane (0.45 μm) in a mini trans-blot apparatus (Bio-Rad) and probing it with primary (anti MtbFHb) and secondary (horseradish peroxidase-conjugated anti-rabbit IgG) antibodies using the fluorescence kit (Millipore).

Quantification of Lipid Peroxide and Characterization of Lipids Associated with MtbFHb—The lipid peroxides were determined through colorimetric assay by a FOX II reagent kit (G-Bioscience) following the manufacturer’s instructions. The lipid fraction from purified MtbFHb was extracted following the method of Bligh and Dyer (26). Briefly, 3.75 ml of 1:2 (v/v) chloroform:methanol was added to 1 ml of 0.5 mg/ml protein and mixed thoroughly, and then 1.25 ml chloroform was added and mixed followed by the addition of 1.25 ml of water and centrifugation at 14,000 rpm for 5 min. The bottom phase was recovered, evaporated to dryness under nitrogen stream, and further resuspended in 200 μl of chloroform. Separation of the lipid mixture was performed by two-dimensional TLC on silica gel G (Merck) by spotting lipid solution in one corner of the plate and developing in the first dimension with a mixture of chloroform/methanol/water (65:25:5, v/v/v) and in the second dimension with a mixture of chloroform/methanol/acetic acid/water (80:12:15:4, v/v/v/v). Detection of lipid was done by staining with molybdenum phosphate blue reagent. Further characterization of the lipid component was done by fatty acid methyl ester analysis through gas chromatography (GC)-MS using a fragmentation profile of fatty acid methyl esters already contained in the spectral library data.

D-Lactate Oxidation by MtbFHb and Assay for D-Lactate: Phenazine Reductase Activity—Oxidation of d-lactate by full-length MtbFHb and its globin domain was checked spectrophotometrically by recording the spectral changes in the protein in the presence of d-lactate as described elsewhere (27). The spectral changes exhibiting conversion of ferric MtbFHb and MtbFHbRD into the ferrous state in the presence of d-lactate were checked spectrophotometrically by Cary 100 after recording the spectra from 350 to 600 nm. LDH activity was determined with phenazine methosulfate (PMS) and MTT (Calbiochem) as described previously (28). Briefly, assays were carried out in a 1.5-ml reaction containing 0.05 M Tris-Cl buffer, 120 μg/ml PMS, 60 μg/ml MTT, 4 mM L- or d-lactate, and 200 nm concentrations of protein. The reaction was incubated for 10 min at 37 °C, heated at 65 °C for 5 min, chilled on ice, and then filtered by 0.2-μm syringe filter. Product analysis was carried out on a spectrophotometer (Shimadzu) using a Phenomenex 87H 300 × 6.5 mm column (C18) at 25 °C. Samples were eluted with isocratic elution buffer containing 99% 50 mM NaH₂PO₄ and 1% acetonitrile with a flow rate of 1 ml/min. A UV detector at 210 nm was used for product detection using commercially available L-lactate, d-lactate, and pyruvate as standard.

Transcriptional Fusion and Determination of Promoter Activity—The transcriptional fusion of MtbFHb was made in an E. coli-M. tuberculosis shuttle vector, pSC301 (29), by replacing the superoxide dismutase gene promoter, upstream of GFP, with upstream sequences of MtbFHb-encoding gene. The transcriptional fusion was made by PCR amplification of a sequence encompassing −240/+26 relative to the translational start codon of the Rp0385 gene using complementary oligonucleotide primers (supplemental Table S2) essentially as described earlier (29). The recombinant plasmid pSCFHp thus created was transformed into M. tuberculosis H37Ra using standard protocol. The recombinant strains carrying the control plasmid, pSC301, and pSCFHp were exposed to 5 mM H₂O₂ and 5 mM H₂O₂, and cells were taken out at an A₆₀₀ nm of 0.6 (early exponential phase), 1.2 (late exponential phase), and 2.0 (early stationary phase). The relative florescence units were measured using a 490-nm excitation filter and a 520-nm emission filter in a spectrofluorometer (PerkinElmer Life Sciences). All measurements were carried out in triplicate. For in vivo analysis of promoter activity, cells of M. tuberculosis H37Ra, carrying pSC301 and pSCHmpP, were infected into THP-1 macrophages and analyzed through a flow cytometer following the earlier described procedure (29).

Peroxidase and NADH Oxidase Activity Assays—Peroxidase assay was performed in a solution containing 3 mM 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid (ABTS) and 2.5 mM H₂O₂ in 10 mM phosphate buffer at pH 7.0, and formation of ABTS cation radical was followed at 415 nm. NADH oxidase activity of purified protein was determined by measuring the rate of NADH consumption (decrease in absorbance at 340 nm).
The assay was performed in 50 mM phosphate buffer (pH 7.0) containing 0.5 mM NADH and 1 mM EDTA as described elsewhere (30).

RESULTS

Unusual Structural Features of Heme and Reductase Domains of MtbFHb—MtbFHb, encoded by the Rv0385 gene of M. tuberculosis, is a 43.5-kDa monomeric protein having a distinct globin and reductase domain (12). Sequence comparison of its heme binding domain with that of other FHbs (Fig. 1A) indicated that it carries necessary structural features for adopting a three-over-three globin-fold and signature sequences of typical microbial globins, e.g. B10Tyr, CD1Phe, E7Gln, F8His, but it displays several crucial differences within the functionally conserved regions of its cofactor binding site. The most notable difference is the lack of hydrogen-bonding interactions between F8H-H23E-G5Y (Fig. 1, A and B) at the proximal site that is conserved among FHb family (7, 8). Additionally, H12Y that interacts directly with G5Y (Fig. 1B) and modulates the geometry of proximal site is substituted by an alanine. The data suggest that the proximal histidine of MtbFHb lacks the peroxidase-like imidazole character present in a typical FHb (7, 20).

The reductase domain of MtbFHb is also highly modified. It is devoid of the critical residues important for FAD and NADH binding. For example, the RXYS motif, the hallmark for FAD binding, and the GXGXXP motif, required for NADH binding...
(31), are mutated in MtbFHb. The lack of a conserved lysine residue at F7 within the globin domain and the glutamine residue at 394 position of the reductase domain, required for the electron transfer from reductase to globin domain in conventional FHbs (9), suggests that the interactions between the globin and reductase domains in MtbFHb may be different. Although the conserved FAD binding site is mutated in MtbFHb, two sequence motifs, A7XAXN and G7XGS, which are similar to the FAD binding motifs (A7XAXN and G7XGS) of the respiratory Δ-LDHs (22, 32), overlap with the mutated NADH binding site (Fig. 1A). It is interesting to note that these motifs are present in the majority of the MtbFHb homologs of virulent mycobacteria, whereas in many non-pathogenic mycobacteria (supplemental Fig. S1) and certain members of actinomycetes, these FAD binding motifs are not fully conserved. MtbFHb also displays multiple lipid binding motifs, HW, RW, and RKY (33) along with one high affinity lipid binding site (RKY) within the proximal heme site that is present in the majority of the mycobacterial FHbs (supplemental Fig. S1). These unusual features suggest that the structural and functional properties of the cofactor binding domains of MtbFHb are different from conventional FHbs.

Cloning and Expression of MtbFHb and Its Heme (MtbFHb\textsubscript{HD}) and Reductase (MtbFHb\textsubscript{RD}) Domains—The Rv0385 gene of \textit{M. tuberculosis}, encoding MtbFHb, was cloned and expressed in \textit{E. coli} with and without a His tag. In both cases the protein appeared to be associated with the cell membranes and was recovered after the treatment with chaotropic agents, indicating that MtbFHb is a peripheral membrane-associated protein. The appearance of reddish brown tinge in the protein indicates that it is bound with heme. In contrast, the expression of MtbFHb\textsubscript{HD} in \textit{E. coli} led to a cytoplasmic protein, suggesting that the integration of reductase domain with the globin module in MtbFHb confers structural/conformational changes required for its membrane association. The appearance of an intense red tinge in the cell expressing MtbFHb\textsubscript{HD} and the spectral measurements (discussed below) indicate that the protein is able to fold and bind heme efficiently. When the MtbFHb\textsubscript{RD} was expressed in \textit{E. coli} and purified from the cell lysate, it appeared colorless, indicating that FAD may not be associated with the protein.

\textit{MtbFHb} Carries Hexacoordinated Heme Domain; Spectral Characteristics of MtbFHb and MtbFHb\textsubscript{HD}—Initial studies on MtbFHb indicated that the protein predominantly exists in the ferric state, exhibiting Soret and visible bands at 414 and 536/570 nm (Fig. 2A), respectively, suggesting a hexacoordinated low spin (6CLS) heme with an intrinsic amino acid residue or exogenous ligand bound to its distal site. The absorption spectrum of the ferrous species shows Soret and visible bands at 428 and 533/559 nm, respectively, substantiating the 6CLS configuration of heme, consistent with the presence of a sixth ligand. Exposure of the ferrous protein to CO caused the absorption bands to shift to 423 and 540/567 nm, respectively, typical for a CO-bound heme (with histidine as the proximal ligand), indicating that the distal ligand is displaced by the CO.

The resonance Raman spectrum of the ferric species of MtbFHb\textsubscript{HD} exhibits \(\nu\textsubscript{2}, \nu\textsubscript{3},\) and \(\nu\textsubscript{4}\) at 1577, 1505, and 1375 cm\(^{-1}\), respectively (Fig. 2B), whereas the ferrous species exhibits \(\nu\textsubscript{2}, \nu\textsubscript{3},\) and \(\nu\textsubscript{4}\) at 1585, 1495, and 1361 cm\(^{-1}\), respectively. These modes are characteristic for a 6CLS heme, which is in good agreement with the absorption spectral data. The CO derivative of MtbFHb\textsubscript{HD} shows \(\nu\textsubscript{2}, \nu\textsubscript{3},\) and \(\nu\textsubscript{4}\) at 1580, 1495, and 1372 cm\(^{-1}\), respectively, typical for a 6-coordinate CO-bound heme (with histidine as the proximal ligand). The Fe-CO stretching mode \(\nu\textsubscript{Fe-CO}\) and Fe-C-O bending mode \(\nu\textsubscript{Fe-C-O}\) were identified at 524 and 586 cm\(^{-1}\), respectively, whereas the C-O stretching mode \(\nu\textsubscript{C-O}\) was found at 1924 cm\(^{-1}\) (Fig. 2C).

The location of the \(\nu\textsubscript{Fe-CO}/\nu\textsubscript{C-O}\) data point in the inverse correlation line shown in Fig. 3C indicates that MtbFHb has a distal heme pocket with a medium positive polar electrostatic potential, which is distinct from that of conventional FHbs, such as Hmp from \textit{E. coli} (34). As shown in Fig. 3C, Hmp exists in two conformations, Hmp\textsubscript{C} and Hmp\textsubscript{O}, which exhibit relatively high and low electrostatic potentials, respectively (20).
The presence of a single conformation instead of the dual conformations found in typical FHbs (20, 34) suggests that Mtb-FHb has a restricted conformational freedom.

Unlike the full-length protein, MtbFHb HD appeared mainly in the cytoplasmic fraction. In addition, the spectral data indicate that the ferrous species has a five-coordinate high spin configuration (Fig. 3, A and B), whereas the ferric species has a mixed hexacoordinated high spin and low spin configuration (indicating a water-bound ferric heme). These spectral features are comparable with typical globins (35), indicating that the structure of the heme domain is regulated by its cognate reductase domain in MtbFHb and that the binding of the sixth ligand in the active site is stabilized by interdomain interactions.

Furthermore, the Fe-His stretching mode ($\nu_{Fe-His}$) of the ferrous MtbFHb HD is identified at 220 cm$^{-1}$ (Fig. 3D), ~24 cm$^{-1}$ lower than that of Hmp (34, 35), consistent with the interruption of the proximal H-bonding interactions shown in Fig. 1B. It is noteworthy that the $\nu_{Fe-His}$ modes of ferrous heme proteins are only active in the pentacoordinated ferrous state and not in the hexacoordinated configuration; hence, this mode is not observable in the full-length MtbFHb. Like the full-length protein, CO-bound MtbFHb HD also exhibited a single conformation instead of a dual conformation (Fig. 3C), indicating lack of conformational flexibility in the globin domain. These spectral features further substantiate unusual characteristics of MtbFHb.

**Cofactor Binding and Functional Properties of MtbFHb RD**—Because the conventional NADH and FAD binding sites of FHbs are mutated in MtbFHb, we attempted to check whether these cofactors are associated with MtbFHb. The NADH oxidase activity of MtbFHb appeared extremely low in comparison to the HMP of E. coli (supplemental Fig. S2). During gel filtration analysis of MtbFHb, a yellowish ring appeared on the column, indicating the possibility of FAD association with the protein. The presence of FAD in MtbFHb and its isolated reductase domain (MtbFHb RD) was further investigated by extracting the cofactor from the protein after heat denaturation. The fluorescence spectra of the extract validated the association of FAD with the MtbFHb ($\approx 0.85–0.95$ mol of FAD/mol of protein) but not with MtbFHb RD (Fig. 4). To interrogate the FAD binding site within the reductase domain of MtbFHb, a mutant protein (MtbFHb FAD) in which the GS residues conserved in the FAD binding site of d-LDH were mutated to alanine, was constructed and examined with fluorescence measurements. The data revealed the lack of FAD in the mutant (data not shown), confirming that the wild type MtbFHb contains a FAD binding site that is similar to that of d-LDH.
Membrane Vesicles of MtbFHb-expressing Cells Display D-Lactate:phenazine Methosulfate Reductase Activity—To examine the functional role of the unusual FAD-binding motif that resembles that of D-LDH, we examined if MtbFHb is able to oxidize D-lactate by testing the D-lactate: PMS reductase activity of the soluble and membrane fractions of MtbFHb-expressing cells. The data show that the membrane fraction of MtbFHb-expressing cells displayed significant D-lactate-dependent PMS reductase activity (2.5 ± 0.38 μmol/min/mg), which was significantly higher than that of the membrane fraction of the control cells (0.048 ± 0.007 μmol/min/mg). No such activity was detected in the soluble fraction of the MtbFHb-expressing cells or control cells. When the membrane fraction of MtbFHb-FAD was tested, no increase in the D-lactate-dependent PMS reductase activity was observed, suggesting that FAD is required for the reduction of PMS. When D-lactate was replaced by L-lactate, D-malate, pyruvate, or mycolate, no PMS reductase activity was observed, suggesting D-lactate acts as a specific electron donor for MtbFHb.

Electron Transfer from D-Lactate to Heme Domain of MtbFHb Is Mediated by FAD during D-Lactate Oxidation—D-LDHs are known to transfer electrons from D-lactate directly or indirectly (via cytochrome c) to the quinone pool in the respiratory chain (27, 36). Because E. coli and mycobacteria do not have cytochrome c (37, 38), we examine if the heme domain of MtbFHb is able to act as an electron acceptor during D-lactate oxidation. Our data show that the addition of D-lactate to the ferric MtbFHb (with Soret and visible bands at 414 and 536/570 nm) resulted in a species with Soret and visible bands at 426 and 529/559 nm (Fig. 5A), indicating the reduction of the heme. In the control experiment with MtbFHbRD, no heme reduction was observed (supplemental Fig. S3). HPLC analysis of the product resulting from the reaction of MtbFHb with D-lactate revealed a peak corresponding to pyruvate (Fig. 5B), indicating the reduction of the heme is associated with the conversion of D-lactate to pyruvate. Under the experimental conditions, the specific D-lactate oxidation activity was ~28.3 μmol/min/mg, which was comparable with that of the respiratory D-LDH of Megasphaera elsdenii (20.3 μmol/min/mg) (39). No activity was observed when MtbFHb was replaced by MtbFHbRD, MtbFHbRD, or Hmp from E. coli. In addition, no activity was observed when the ferric MtbFHb was replaced by its ferrous or CO-bound derivative (Fig. 5B), suggesting that the redox state of the protein modulates its lactate metabolizing activity. Considering the fact that MtbFHb is in close proximity to the respiratory membrane in M. tuberculosis, we hypothesize that the heme domain of MtbFHb accepts an electron from D-lactate via FAD and subsequently passes it to the respiratory apparatus embedded in the membrane.

MtbFHb Remains Associated with Lipids and Protect Its Host under Oxidative Stress—When MtbFHb was overexpressed in E. coli or M. smegmatis, the protein remained localized to the cell membrane (Fig. 6B). Because MtbFHb comprises an unusual hexacoordinated heme, which has been reported for lipid-bound Vitreoscilla hemoglobin (40), we examined if the purified protein is associated with any lipid moiety. TLC analysis of MtbFHb extract validated the association of lipids with the protein (Fig. 6C). Removal of lipids from this protein could not be achieved by hydroxyapatite column; it was only feasible with organic solvent extraction. GC profile and fatty acid methyl ester analysis revealed that the lipids contained a heterogeneous mixture of sterol and fatty acids having cyclopentane heptanoic acid, hexadecanoic acid, palmitic acid, and C16 unsaturated and cyclopropanated chains (Fig. 6A). It is likely that the lipid binds to the heme active site of the protein, leading to the unusual 6CLS configuration of the heme iron. This scenario is consistent with the observation that when the reductase domain is deleted from MtbFHb, the protein (MtbFHbH9260) lost its membrane association property (data not shown); in addition, it displayed a typical five-coordinate high spin configuration instead of the unusual 6CLS configuration (Fig. 3A). Lipid binding to MtbFHb, thus, may play a regulatory role in keeping the heme domain in a 6CLS state to prevent oxygen binding and/or to tune the redox potential to keep the protein in the active ferric state for metabolizing D-lactate. The presence of sterol derivatives (androst-4-ene-3,17-dione) in the lipid extract suggests that MtbFHb is able to bind complex lipids, which may be combined with complex molecules such as sterol esters and waxes, which make the important components of the M. tuberculosis cell wall (41, 42).

Unlike conventional FHbs and HbN of M. tuberculosis, MtbFHb expression in E. coli or M. smegmatis did not provide any protection against nitrosative stress imposed by acidified nitrite (Fig. 7, A and B) This observation is consistent with the low nitric oxide dioxygenase activity of MtbFHb reported previously (12). However, surprisingly, it improved the survival of E. coli and M. smegmatis under sublethal concentrations of H2O2 (Fig. 7, A and B). We found that the protective effect of MtbFHb against oxidative stress is not a result of its peroxidase activity, as the protein did not exhibit peroxidase activity.
Additional studies showed that the levels of lipid peroxidation in the wild type E. coli and M. smegmatis were nearly 25–30% higher than the isogenic cells expressing MtbFHb (see supplemental Table S3), suggesting that the protective effect of MtbFHb is connected to its ability in minimizing lipid peroxidation.

Transcriptional Activities of Rv0385 (MtbFHb) Gene Is Upregulated in Vitro under Oxidative Stress and in Vivo during Macrophage Infection—To gain more insight into the physiological function of MtbFHb in its native host, we created a transcriptional fusion of GFP with the upstream region of the Rv0385 gene and studied its expression profile under different physiological conditions in vitro and within the infected macrophage.
TABLE 1
Flow cytometric analysis of transcriptional activities of Rv0385 (MtbfHb) in M. tuberculosis H37Ra during macrophage infection

| Time post-infection | Mean fluorescence intensity of in vivo grown cells* |
|---------------------|---------------------------------------------------|
| h                   |                                                   |
| 4                   | 8.8 ± 0.30                                        |
| 12                  | 24.8 ± 0.13                                       |
| 24                  | 21.2 ± 0.30                                       |
| 48                  | 19.1 ± 0.11                                       |

*THP-1 macrophages were infected with M. tuberculosis H37Ra carrying the transcriptional fusion of Rv0385 in triplicate sets at a multiplicity of infection of 1:20 as mentioned under "Experimental Procedures." The cells were incubated at 37 °C in 95% air-CO2, for 4 h, after which they were extensively washed with saline phosphate buffer (pH 7.2) to remove non-ingested bacteria. The infected monolayer of macrophages was then further infected for 12, 24, and 48 h, and mean fluorescence intensity of in vivo grown cells was analyzed through flow cytometry.

phagocytes. M. tuberculosis H37Ra was transformed with the plasmid, pSCFHbβ, carrying fusion of GFP with Rv0385 promoter. It was used to test the transcriptional activities of MtbfHb in vitro and in vivo. The promoter activity of the Rv0385 gene increased during the late exponential and stationary phase in the aerobically grown culture of M. tuberculosis H37Ra. In the presence of H2O2, the promoter activity was further elevated by 1.5-fold (Fig. 7C). When M. tuberculosis H37Ra, carrying the transcriptional fusion of Rv0385, was infected into THP-1 macrophages, a 2–3-fold increase in the promoter activity was observed at 24 and 48 h after the infection (Table 1).

Absence of D-Lactate Metabolizing Gene in M. tuberculosis; Implications of D-Lactate Utilization by MtbfHb under Oxidative Stress—M. tuberculosis cell wall is highly enriched in complex lipids and is constantly exposed to host-induced oxidative stress that may result in oxidative damage to the membrane via lipid peroxidation, thereby producing large amounts of malondialdehyde and methylglyoxal. It is well known that D-lactate is an abundant byproduct of methylglyoxal and, if accumulated, can generate hydroxyl ions by interacting with cellular iron (43), thereby inducing additional lipid peroxidation as illustrated in Fig. 8. Upon macrophage infection, M. tuberculosis may accumulate D-lactate in the cell due to enhanced lipid peroxidation that can elevate the oxidative stress in vivo and reduce its intracellular survival. The presence of MtbfHb in M. tuberculosis, thus, may be advantageous.

To understand the functional significance of the D-lactate metabolizing activity of MtbfHb, we surveyed D-lactate metabolizing pathways in mycobacteria. We found that M. tuberculosis lacks the D-LDH gene required for D-lactate utilization (44); in addition, D-LDH activity has not been detected in any mycobacterial species. These observations substantiate the physiological importance of MtbfHb in D-LDH utilization in M. tuberculosis.

**DISCUSSION**

This study shows that MtbfHb is a peripheral membrane-associated protein that catalyzes the oxidation of D-lactate into pyruvate in a FAD-dependent manner, similar to the respiratory (NAD-independent) D-LDHs (32). Because M. tuberculosis lacks a D-lactate-metabolizing gene, the accumulation of D-lactate under oxidative stress as a byproduct of lipid peroxidation may be highly toxic to the cell due to increased OH· generation. The ability of MtbfHb to associate with the cell membrane and oxidize D-lactate into pyruvate, thus, may be advantageous in protecting the respiratory membranes under oxidative stress and maintaining the flow of electrons to the electron transport chain for the energy generation within the oxygen and nutrient-deficient intracellular environment.

Our spectroscopic data show that the heme iron in MtbfHb exists in a 6CLS configuration; in addition, the proximal histidine ligand of the heme lacks the characteristic imidazole character required for the nitric oxide dioxygenase activity in conventional FHbs (9, 20). These results ruled out the involvement of MtbfHb in NO-detoxification and nitrosative stress protection. This conclusion is consistent with the observations that the FAD/NAD binding sites and NADH oxidase activity found in conventional FHb are absent in MtbfHb. Interestingly, although the conventional FAD binding site is not present in MtbfHb, the protein has a new FAD binding site similar to that of the respiratory D-LDHs (22), which binds FAD with 1:1 stoichiometry.

Our studies show that the dissociation of the heme domain from the reductase domain in MtbfHb induces significant conformational changes to both domains. The isolated heme domain exists as a cytoplasmic protein, with the heme iron in a globin-like five-coordinate high spin configuration, in contrast to the membrane-associated 6CLS wild type protein. It is likely that the interdomain interactions in MtbfHb are required for the stabilization of the sixth ligand in the active site and for the exposure of the surface residues critical for its membrane aso-
MtbFHb remains associated with the membrane and bound with lipids when overexpressed in E. coli or M. smegmatis. The hydrophobicity profile of MtbFHb is significantly high. MtbFHb carries high affinity lipid binding motifs, with several basic residues that may interact with electronegative phospholipids head groups. The prominent lipid species associated with MtbFHb are fatty acids (unsaturated and cyclopropanated) and sterol (androst-4-ene-3,17-dione), demonstrating its ability in interacting with wide range of lipids. This scenario is supported by the observation that the transcriptional activity of the MtbFHb encoding gene (Rv0385) is significantly increased in the presence of lung surfactant (45) that is rich in complex lipids (such as phospholipids, cholesterol, and free fatty acids). Although the relevance of lipid binding in MtbFHb is not obvious at present, the relatively lower level of lipid peroxidation in MtbFHb-expressing cells under oxidative stress as compared with the control supports its direct or indirect role in protecting the integrity of cell membranes under stress conditions.

The lipid content of the M. tuberculosis cell envelope is unusually high. The lipids may be prone to damage under oxidative burst during macrophage infection. The proximity of MtbFHb with the cell membrane, therefore, may be advantageous. This hypothesis is supported by the observation that the E. coli and M. smegmatis cells expressing MtbFHb display distinct growth advantage over control cells when exposed to \( \text{H}_2\text{O}_2 \). Under elevated levels of reactive oxygen, unsaturated fatty acids that constitute a major component of the mycobacterial cell wall (46) may decompose into toxic methylglyoxal, generating \( \text{D}-\text{lactate} \) as a byproduct. Recent studies demonstrated that methylglyoxal levels are significantly increased during mycobacterial infection of macrophages (47), which may lead to the accumulation of \( \text{D}-\text{lactate} \) that enhances stress level by interacting with \( \text{Fe}^{3+} \) iron to produce OH- radicals. The association of MtbFHb with the cell membrane and its \( \text{D}-\text{lactate} \)-metabolizing ability may allow it to utilize accumulated \( \text{D}-\text{lactate} \) as an alternate energy source within the nutrient and oxygen-deficient intracellular environment. This possibility is supported by the fact that the membrane fractions of MtbFHb-expressing cells were able to donate electrons to artificial electron acceptors, PMS, ferricyanide, and dichloroindophenol in the presence of \( \text{D}-\text{lactate} \). When incubated with \( \text{L} \)- and \( \text{D} \)-lactate, MtbFHb converted \( \text{D}-\text{lactate} \) into pyruvate but did not react with \( \text{L}-\text{lactate} \), indicating that this activity is specific for the \( \text{D}-\text{lactate} \). FAD is essential for the reaction, as MtbFHb \( \text{FAD} \) (which does not bind FAD) did not exhibit any activity. The interactions between the heme domain and reductase domain, required for maintaining the active conformation and membrane-association of the protein, are also critical for the reaction, as each individual domain exhibited no activity. Some flavin-containing dehydrogenases have been found to use methemoglobin or cytochrome c as an electron acceptor (28, 48). MtbFHb represents a unique two-domain flavin-containing protein in which the heme domain acts as an electron mediator that picks up an electron from \( \text{D}-\text{lactate} \) via FAD bound to the reductase domain and then passes it onto the respiratory appa-ratus. The electron transfer function of MtbFHb is supported by the observation that the addition of \( \text{D}-\text{lactate} \) to MtbFHb, but not the heme domain alone, changes the heme iron from the \( \text{Fe}^{3+} \) to \( \text{Fe}^{2+} \) state. Respiratory FAD-dependent \( \text{D}-\text{LDH} \) is known to be a peripheral membrane protein that participates in electron transfer (32); such a role for MtbFHb may be envisioned due to its close proximity with the respiratory membrane. This is supported by the observation that the transcriptional activity of Rv0385 increases significantly in the presence of bicyclic nitroimidazoles like PA-824 that mainly affect genes involved in cell wall biogenesis and respiration (49). Phylogenetic analysis has also indicated that MtbFHb may form a separate cluster from conventional FHbs (12) and may be more related to electron transferring proteins, whereas conventional FHbs are closer to dioxygenases (12).

Our studies show that cells expressing MtbFHb show enhanced protection against oxidative stress. The conservation of a high affinity lipid binding motif within the heme pocket of MtbFHb and its homologs suggest that lipid binding may be a common property of these FHbs and may be related to the protection of membrane integrity under oxidative stress. Under our experimental conditions, MtbFHb did not display any alkyl hydroperoxide reductase activity, indicating that the protection effect of MtbFHb is not a result of its peroxidase activity. MtbFHb was found associated with complex lipids, including cyclopropanated and unsaturated fatty acids. It is likely that a change in the redox balance of the cell due to the expression of MtbFHb promotes lipid modification and cyclopropanated fatty acids accumulation, thereby conferring a stabilizing/rigidifying effect on the membrane as reported in other cases (50, 51).

Although the precise mechanism of MtbFHb function in electron transfer and oxidative stress response requires further exploration, our experimental studies suggest its critical role in cellular metabolism and pathogenicity of M. tuberculosis. Transcriptional activity of Rv0385 is induced during oxidative stress and stationary phase of growth when the availability of oxygen and nutrients is low. Similar physiological conditions exist when M. tuberculosis resides within the macrophage during its pathogenic phase. This assumption is supported by the observation that the promoter activity of Rv0385 increases after 24 and 48 h of M. tuberculosis infection of macrophages. It is also supported by recent proteomic and microarray data on M. tuberculosis showing significant induction of Rv0385 in M. tuberculosis within the infected dendritic cells in response to oxidative stress (52).

In summary, MtbFHb has a hexacoordinated cytochrome-like heme in the heme domain and a FAD-containing reductase domain with \( \text{D}-\text{lactate} \) oxidation activity. It represents the first example of a two-domain protein that is able to oxidize \( \text{D}-\text{lactate} \) to pyruvate and at the same time sequester an electron and pass it onto the electron transfer chain in the respiratory apparatus as proposed in the model presented in Fig. 8. It is conceivable that by cycling between the \( \text{Fe}^{3+}/\text{Fe}^{2+} \) redox states, MtbFHb is able to balance the stress level induced by the oxidative burst exerted by macrophage under infection conditions.
Truncated hemoglobin, HbN, from *M. tuberculosis* is also localized on the cell membrane of *M. tuberculosis*. Its ability in detoxifying NO, thereby relieving stress on cellular respiration, lies in its NO dioxygenase activity that requires one redox equivalent, which can be obtained from cellular ferrous iron by converting it from Fe²⁺ to the Fe³⁺ states. The potential ability of MtbFHb in recycling Fe³⁺ to Fe²⁺ and the co-localization of MtbFHb and HbN at the site where respiratory apparatus locates hence can be advantageous. Unidirectional conversion of α-lactate into pyruvate by MtbFHb may be a unique survival strategy developed by *M. tuberculosis* to cope with hazardous level of reactive oxygen/nitrogen species, thereby sustaining its energy generation within the hypoxic and nutrient-deficient intracellular environment.

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