Characterization of a *Mycobacterium tuberculosis* nanocompartment and its potential cargo proteins

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Running Title: Mtb nanocompartment and its cargo proteins

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Key Words: *Mycobacterium tuberculosis*, Nanocompartments, Protein-protein interaction, Electron microscopy, Protein self-assembly, oxidative stress.

Background: *Mycobacterium tuberculosis* has a probable nanocompartment (Mt-Enc).
Result: Mt-Enc self-assembles into a 60-subunit cage that encapsulates enzymes via their C-terminal tails, which remain active within Mt-Enc.
Conclusion: Cargo proteins are potentially involved in host oxidative stress response suggesting that enzyme encapsulation may be a mechanism to evade host immune assault.
Significance: Mt-Enc may be utilized as a novel therapeutic delivery mechanism.

Abstract

*Mycobacterium tuberculosis* (Mtb) has evolved various mechanisms by which the bacterium can maintain homeostasis under numerous environmental assaults generated by the host immune response. Mtb harbors enzymes involved in the oxidative stress response that aid in survival during the production of reactive oxygen species in activated macrophages. Previous studies have shown that a dye-decolorizing peroxidase (DyP) is encapsulated by a bacterial nanocompartment, encapsulin (Enc), whereby packaged DyP interacts with Enc via a unique C-terminal extension. Mtb also harbors an encapsulin homolog (CFP-29, Mt-Enc), within an operon with Mtb DyP (Mt-DyP), which contains a C-terminal extension. Together these observations suggest that Mt-DyP interacts with Mt-Enc. Furthermore it has been suggested that DyPs may function as either a heme-dependent peroxidase or a deferrochelatase. Like Mt-DyP, Mtb iron-storage ferritin protein, Mt-BfrB, and an Mtb protein involved in folate biosynthesis, 7,8-dihydroneopterin aldolase (Mt-FolB), have C-terminal tails that could also interact with Mt-Enc. For the first time, we show by co-purification and electron microscopy that mycobacteria via Mt-Enc can encapsulate Mt-DyP, Mt-BfrB, and Mt-FolB. Functional studies of free or encapsulated proteins demonstrate that they retain their enzymatic activity within the Mt-Enc nanocompartment. Mt-DyP, Mt-FolB and Mt-BfrB all have antioxidant properties, suggesting that if these proteins are encapsulated by Mt-Enc, then this nanocage may play a role in the Mtb oxidative stress response. This report provides initial structural and biochemical clues regarding the molecular mechanisms that utilize compartmentalization by which the mycobacterial cell may aid in detoxification of the local environment to ensure long-term survival.
Introduction

The etiologic agent of tuberculosis (TB), Mycobacterium tuberculosis (Mtba), infects approximately 11.1 million people per year, resulting in over 1.3 million deaths worldwide (1). With the emergence of Mtba strains resistant to the major anti-TB therapies, the discovery of novel drug targets is imperative to triumph over TB infection. Deciphering the complicated biology of Mtba, which has both active and latent forms, will aid in the discovery of new therapeutics to combat this highly successful pathogen.

In mammalian cells, exosomes are secreted vesicles proposed to be involved in mediating the adaptive immune response (2,3). Mycobacterial proteins have been discovered in exosomes of cells infected with Mtba. One mycobacterial protein identified within exosomes was a 29-kDa culture filtrate protein (CFP-29, Rv0798c) (4), reported to be secreted (5), although lacking a signal peptide. CFP-29 has 58% amino acid identity to Brevibacterium linens M18 linocin protein (also known as encapsulin, Bl-Enc) (5), implying that CFP-29 is an Mtba encapsulin homolog (Mt-Enc). Throughout the remainder of this manuscript, CFP-29 (Rv0798c) will be referred to as Mt-Enc. Additionally, the gene encoding Bl-Enc is within a two-gene operon containing a gene encoding for a dye-decolorizing peroxidase (Bl-DyP) with a ~25 amino acid C-terminal extension (Fig. 1A). The crystal structure of a close homolog of Bl-Enc, Thermotoga maritima encapsulin (Tm-Enc), has been determined, revealing a 60-subunit icosahedral nanocompartment (6). Electron microscopy (EM) shows that coexpression of Bl-DyP and Bl-Enc results in encapsulation of Bl-DyP within the Bl-Enc nanocage, where encapsulation of Bl-DyP is abolished upon truncation of its C-terminal extension (6). Finally, a search across bacterial genomes demonstrates that genes encoding for Enc are usually in two-gene operons with a gene encoding for either DyP or ferritin-like proteins, all with predominately hydrophobic C-terminal extensions (Fig. 1A) (6). These observations suggest that Enc proteins compartmentalize DyP or ferritin-like proteins via their C-terminal tails. Notably, both DyPs and ferritin proteins possess antioxidant properties (7,8).

Mt-Enc, the only homolog of Enc in Mtba, is part of a two-gene operon with the gene for a DyP-like peroxidase (Mt-DyP, Rv0799c), where Mt-DyP contains a similar C-terminal extension to Bl-DyP (Fig. 1A). DyP-type peroxidases are a novel family of fungal and bacterial heme-dependent peroxidases that can oxidize lignin and anthraquinone dyes in the presence of H$_2$O$_2$ (9,10). However, Escherichia coli DyP ortholog, Ec-YfeX, was proposed to possess deferrochelatase activity, where Ec-YfeX can catalyze the extraction of iron from heme without cleavage of the tetrapyrole ring (11); although in a parallel study Ec-YfeX was demonstrated to be a heme-dependent peroxidase with no detectable deferrochelatase activity (12). If Mt-DyP is a heme-dependent peroxidase, then Mt-DyP may protect Mtba against host oxidative assault by H$_2$O$_2$.

Notably, two other Mtba proteins have aliphatic C-terminal extensions beyond what is seen in other bacterial homologs and similar to Mt-DyP, suggesting that these proteins may also be cargo proteins for Mt-Enc. Mt-BfrB, one of the two iron-storage ferritin proteins within Mtba, has a C-terminal tail (Fig. 1A) and has displayed antioxidant properties (13,14). In addition, an enzyme involved in folate metabolism, 7,8-dihydroneopterin aldolase (Mt-FolB), also has an aliphatic C-terminal extension (Fig. 1B). In a previous study, Mt-FolB structure determination shows that this C-terminal extension is disordered, and Mt-FolB enzymatic activity is independent of the C-terminal tail. Furthermore, its substrate has also been implicated in Mtba resistance to oxidative stress (15-17).

Herein we describe the Mt-Enc encapsulation of three different Mtba enzymes, Mt-DyP, Mt-BfrB and Mt-FolB, all of which possess antioxidant properties. We show by EM that Mt-Enc can encapsulate Mtba cargo enzymes, and by biochemical analyses that the enzymes remain active within the Mt-Enc nanocompartment. We also carried out preliminary characterization of Mt-DyP and suggest that it is primarily a heme peroxidase. To our knowledge, this study provides the initial report into mycobacterial compartmentalization of enzymes, whereby protein encapsulation by Mt-Enc may function to combat oxidative stress within the human host.
Materials and Methods

Cloning
The Mtb genes encoding proteins Mt-BfrA (Rv1876), Mt-BfrB (Rv3841), Mt-DyP (Rv0799c), Mt-Enc (Rv0798c), and Mt-FolB (Rv3607c) were PCR-amplified from Mtb H37Rv genomic DNA using the KOD HotStart Polymerase Kit (Novagen) with 5′ and 3′ primers (MWG Operon) containing specific restriction sites, to either generate a C-terminal poly-histidine tag (His-tag) or a tagless protein. PCR products were ligated into pCR-BluntII-TOPO (Invitrogen), and then transformed into E. coli One Shot TOP10 cells (Invitrogen). Double digestions with specific restriction enzymes were performed from each vector of choice. Excised genes were ligated into the appropriate linearized pET vector and transformed into E. coli BL21-Gold (DE3) cells (Novagen). Each final construct was verified by DNA sequencing using T7 promoter and reverse primers (Laguna Scientific).

Overexpression and purification of Mtb proteins
Proteins were expressed from plasmids alone or in combination with one another, using E. coli BL21 Gold (DE3) cells. The following concentrations of antibiotics to the media were added where appropriate: kanamycin (30 µg/ml) and ampicillin (50 µg/ml). Cells harboring expression vector(s) were grown aerobically at 37 °C in LB media containing the appropriate antibiotic(s). Protein expression was induced at OD600nm ~0.8 by the addition of isopropyl-β-d-thiogalactopyranoside (IPTG, 1 mM). Cells were harvested after four hours of induction (apart from Mt-Enc and proteins in complex with Mt-Enc, which were induced overnight at 18 °C), by centrifugation at 5100 x g for 20 min. Additionally, where indicated in the results, cultures expressing Mt-Enc-DyP were supplemented with 0.7 mM δ-aminolevulinic acid (δ-ALA) at the time of induction.

Harvested cell pellets were resuspended in 20 mL of Buffer A (50 mM Tris-HCl, pH 7.4, 350 mM NaCl, 10 mM imidazole and 10% glycerol), followed by the addition of phenylmethylsulfonyl fluoride (PMSF) and hen egg-white lysozyme. Pellets containing Mt-Enc and proteins in complex with Mt-Enc were resuspended in Buffer A containing 0.2% lauryldimethylamine-oxide (LDAO) for solubilization. Cells were disrupted by sonication, clarified by centrifugation at 18000 x g for 30 min at 4 °C and syringe-filtered (1 µm pore size) for removal of cell debris. The clarified cell lysate was then loaded onto a 5 mL Ni2+-charged Histoprep column (GE Healthcare) pre-equilibrated with Buffer A. The protein(s) were eluted with a linear gradient of 10-500 mM imidazole (100 mL). Proteins that co-purify with Mt-Enc elute at a higher imidazole concentration (>250 mM imidazole). Eluted fractions were collected, analyzed by SDS-PAGE, and concentrated using Amicon concentrators with the appropriate molecular-weight cutoff (Millipore, Bedford, MA). Mt-DyP and Mt-BfrB were further purified on a Superdex 200 (S-200) HiLoad 16/60 gel filtration chromatography column (GE Healthcare) utilizing 50 mM Tris-HCl, pH 7.4, and 150 mM NaCl, and their respective molecular weights were calculated using molecular weight standards (Bio-Rad). Mt-BfrB was dialyzed into 20 mM Tris-HCl, pH 8.0, and 10 mM NaCl, and purified by ion exchange (5 mL, HiTrapTM Q HP, GE Healthcare). The protein was eluted with a linear gradient of 0.01 - 1.0 M NaCl (100 mL); the purified protein eluted between 400 and 600 mM NaCl. Protein concentration was determined by UV/vis spectroscopy and their respective molar extinction coefficients at 280 nm as predicted by the program Protein Calculator (Scripps), or determined by either modified Lowry (18) or Bradford (19) assay using bovine serum albumin as a standard.

Electron microscopy.
Samples were negatively stained as follows: ultra-thin carbon and silicon monoxide grids (Ted Pella catalog #01824 and #01829, respectively) were prepared by removing the formvar backing by dipping the grids in 100% chloroform for 10 seconds and glow discharging them for 30 seconds (Leica SCD500, Leica, Vienna). Samples were diluted 1:10 in buffer and 5µL of this was placed on a grid and immediately washed twice in ddH2O followed by a 1-min incubation in 1% uranyl acetate. Excess stain was then aspirated off and the grid was allowed to dry at room temperature. Following negative staining, the grids were imaged on a Zeiss Libra 120 PLUS EF-TEM (Carl Zeiss, Oberkochen, Germany).
**Generation and characterization of MtbΔmbtB strains deficient in Mt-DyP, MhuD, or both Mt-DyP and MhuD.**

MtbΔmbtB, a mycobactin-deficient mutant of Mtb, was used as the parental strain to construct the following double and triple mutants, MtbΔmbtBΔdyP, MtbΔmbtBΔmhuD and MtbΔmbtBΔmhuDΔdyP, via specialized transduction as previously described (20). To generate the MtbΔmbtBΔmhuD mutant, we constructed an allelic exchange substrate by cloning a 1.9 kb PCR product including the entire mhuD gene and flanking regions and then replacing 222 nucleotides of the mhuD gene (encoding amino acids 10-85 of the 105 amino acid (aa) MhuD protein) with an apramycin resistance cassette, using essentially the same strategy described previously for construction of MtbΔmbtBΔmmpL11 and MtbΔmbtBΔRv0203 (20). To generate the ΔdyP mutants, MtbΔmbtBΔdyP and MtbΔmbtBΔmhuDΔdyP, we first inserted an 849 nt in-frame, unmarked deletion of dyP (encoding aa 21-303 of the 335 aa DyP protein) flanked by a hygromycin resistance gene and sacB cassette (hyg-sacB) into the dyP region of the chromosome by specialized transduction. In a second step, we passaged hygromycin resistant clones that were sensitive to sucrose (due to expression of sacB) in the absence of hygromycin to allow for recombination to occur between homologous regions flanking the hyg-sacB cassette (eliminating the hyg-sacB cassette from the chromosome and leaving just the unmarked ΔdyP mutation), and then plated on 7H10 plates containing 2% sucrose. Sucrose resistant clones were confirmed to have lost hygromycin resistance by plating on 7H10 plates with and without hygromycin. All mutants were confirmed to have the correct genotype by PCR.

Heme utilization experiments were performed as previously described (20), except 7H9 growth medium was supplemented with 10% OADC and 0.01% Tyloxa pol (7H9-OADC-TLX). Log-phase bacteria were inoculated at an initial OD750 of 0.0005 into 30 mL 7H9-OADC-TLX containing no supplement, 0.2 µM heme, or 10 ng/mL mycobactin J and grown for 21 days until the growth of all strains had plateaued in the presence of mycobactin J. Growth of the double and triple mutants (MtbΔmbtBΔdyP, MtbΔmbtBΔmhuD, and MtbΔmbtBΔmhuDΔdyP) was compared with the growth of the MtbΔmbtB parental strain under identical conditions to determine whether the mutations impacted the ability of the bacteria to acquire iron from heme. For comparisons, all OD750 measurements were normalized to the OD750 measured for the MtbΔmbtB parental strain in the presence of mycobactin J at the same time point.

**Heme and protoporphyrin IX (PPIX) titration into Mt-DyP.**

Solutions were made and titration experiments were performed as previously described (21,22). Heme (23) and PPIX (24) solutions were freshly prepared prior to each experiment. Briefly, approximately 4 mg of heme was dissolved in 1mL of ice-cold 100 mM NaOH and vortexed periodically over a 20 min period. One mL of 1 M Tris (pH 7.4) was added to the solution, centrifuged for 10 min at 4 °C at 13000 rpm. The supernatant was then diluted with 50 mM Tris (pH 7.4) and 150 mM NaCl, centrifuged again at 13000 rpm for 10 min to remove undissolved heme. Final concentrations were determined using ε385 of 58.44 mM$^{-1}$ cm$^{-1}$. Crystals of PPIX were dissolved in 150 mM NaCl, vortexed periodically over 20 min and centrifuged. Supernatant was collected and diluted in 2.7 N HCl, where concentration was determined using an ε408 of 262 mM$^{-1}$ cm$^{-1}$. PPIX solutions were diluted in 50 mM Tris-HCl (pH 7.4) and 150 mM NaCl for experiments. Heme and PPIX solutions were protected from light and used within 12 h. Micromolar increments of either heme or PPIX were titrated into either 5 µM or 2.5 µM of purified apo-Mt-DyP, respectively, in 50 mM Tris-HCl (pH 7.4) and 150 mM NaCl.

**Kinetics of enzymatic activity within Mt-Enc.**

**Mt-DyP.** All enzymatic reactions were performed in 100 µL reactions. Peroxidase activity utilizing guaiacol as a substrate was monitored over 20 min as a change in absorbance at 470 nm, indicative of the production of tetraguaiacol. 2 µM holo-Mt-DyP (reconstituted with heme to obtain a 1:1 heme-to-protein molar ratio), apo-Mt-DyP, Mt-Enc, and Mt-Enc-DyP (grown in 0.7 mM δ-ALA, where the heme bound Mt-DyP concentration is estimated from Soret peak intensity utilizing UV/vis spectroscopy) were assayed in the presence of 10 mM H2O2 (Sigma-
Aldrich) and 10 mM guaiacol (Sigma-Aldrich) (25) in 100 mM sodium citrate at pH 4. Reactions were initiated upon the addition of H2O2, and were monitored over a 12 min period observing the change in absorbance at 470 nm by UV/vis spectrometry (DU-800 spectrophotometer, Beckman-Coulter) at 25°C. The rate of product formation was calculated using the extinction coefficient of 26600 M⁻¹ cm⁻¹ of tetraguaiacol at 470 nm (26).

Further analysis of the activity of Mt-DyP was carried out by utilization of 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS) as a substrate, as previously described (27). Briefly, 250 µM ABTS was added to a reaction mixture containing 1.25 µM Mt-DyP, Mt-Enc, or Mt-Enc-DyP (grown in 0.7 mM δ-ALA, where the heme bound Mt-DyP concentration is estimated from Soret peak intensity) in 50 mM HEPES at pH 5.5. Reaction was initiated upon the addition of 10 µM H2O2 and monitored for 10 min at 420 nm by UV/vis spectrometry (DU-800 spectrophotometer, Beckman-Coulter) at 25°C. The rate of product formation was calculated using the extinction coefficient of 36000 M⁻¹ cm⁻¹ of the ABTS radical cation at 420 nm (28).

To assess deferrocheletase activity, 5 µg of Mycobacterium smegmatis mc²155 cytosol and cell membrane was isolated as previously described (29,30) and incubated with exogenous, recombinant holo-Mt-DyP (5 µM) at 25°C. The change at 402 nm was monitored over 10 min by UV/vis spectrometry (DU-800 spectrophotometer, Beckman-Coulter).

Mt-BfrB. Ferrooxidase activity was measured following an amended protocol described by Khare, et al 2011, whereby ferrooxidase activity is monitored at 320 nm with some differences (31). Briefly, purified Mt-BfrB apo-Mt-BfrB, apo-Mt-BfrBA167-181, and Mt-Enc-BfrB proteins were dialyzed against 100 mM MES pH 6.5 and 100 mM KCl. Ferrous ammonium sulfate (FAS) was dissolved in degassed 0.1 M HCl for a 5 mM stock solution of FAS diluted in 100 mM MES pH 6.5 and 100 mM KCl. FAS was added to 1 µM of 24-subunit assembly of Mt-BfrB at 25°C to a final FAS concentration of 100 µM. Ferrous iron oxidation was monitored by observing the change in absorbance at 320 nm over 10 min, using a DU-800 spectrophotometer (Beckman-Coulter).

Mt-FolB. Mt-FolB aldolase activity was monitored by fluorimetry, as previously described (16). Briefly, Mt-FolB, Mt-Enc-FolB or Mt-Enc (1 µM) was assayed in the presence of 80 µM 7,8-dihydroneopterin (Sigma-Aldrich) in 50 mM Tris-HCl, pH 8.0, and 150 mM NaCl. The production of 6-hydroxymethyl-7,8-dihydroneopterin was determined by fluorimetry (Hitachi F-4500 Fluorescence Spectrometer), where the λex was 430 nm and the change in λem at 524 nm was monitored. Data was acquired over 35 min.

Results
Mt-Enc is a nanocompartment
Mt-Enc is the only Enc homolog in the Mtb proteome. Recombinant Mt-Enc was purified to near homogeneity, as shown by SDS-PAGE (Fig. 2A). EM analysis revealed that purified Mt-Enc forms a shell-like nanocompartment with a diameter of ~220 Å and shell thickness of ~25 Å, as determined using Image J (32) (Fig. 2B). The shell diameter and thickness of Mt-Enc is in good agreement with the previously solved structure of the 60-subunit Tm-Enc nanocompartment (6), suggesting that Mt-Enc also forms a 60-subunit assembly.

Preliminary Characterization of Mt-DyP
The gene that encodes for Mt-Enc is in a two-gene operon with Mt-DyP. Mt-DyP is a previously uncharacterized protein; therefore we first determined the function of Mt-DyP. Two DyP-like peroxidases from E. coli, Ec-YfeX and Ec-EfeB, were proposed to extract iron from heme while keeping the tetrapyrrole ring intact (11). This hypothesis was based on protoporphyrin IX (PPIX) accumulation upon overexpression of Ec-YfeX in E. coli (11). However, another study reported that Ec-YfeX is a typical heme-dependent dye-decolorizing peroxidase and does not possess deferrocheletase activity (12). Recently, Rhodococcus jostii DypB (Rj-DypB) found in an operon with Enc (Rj-Enc), was shown to be a heme-dependent dye-decolorizing peroxidase with lignin-degrading capabilities (27). Mt-DyP is 57% (over 343 amino acids) and 28% (over 152 amino acids) sequence identical to Rj-DypB (DyP Class B) and Ec-YfeX (DyP Class A), respectively.

Mt-DyP was purified to homogeneity by nickel-affinity chromatography (Fig. 3A). Purified Mt-DyP was colored pink suggesting heme-bound
Mt-DyP. Size exclusion chromatography was utilized to separate apo- and heme-bound species, where three distinct oligomeric states of Mt-DyP were separated and analyzed by UV/vis spectroscopy at 402 nm to determine heme bound fractions (Figs. 3C & 3D). Monomeric Mt-DyP was in its apo form, similar to other bacterial DyPs (DyP2 from Amycolatopsis sp. 75iv2 and E. coli apo-EfeB (33,34)), and a minor dimeric Mt-DyP species with a sub-stoichiometric quantity of heme was also observed. Tetrameric Mt-DyP had a near 1:1 molar stoichiometry of heme bound. Finally, Mt-DyP binds both heme and protoporphyrin IX (PPIX), Fig. 3B. Titration of apo-Mt-DyP with heme suggests a 1:1 molar stoichiometry, similar to the titration of Mt-DyP with protoporphyrin IX (PPIX), which also implies a 1:1 molar stoichiometry (data not shown).

To determine whether Mt-DyP possesses heme peroxidase activity, we tested the ability of tetrameric holo-Mt-DyP to form compound I, the heme-dependent peroxidase intermediate. The irreversible reaction of a heme-dependent peroxidase with H₂O₂ results in the formation of compound I, an oxidized form of heme, where Fe(III) is oxidized to Fe(IV)=O along with the formation of a porphyrin π-cation radical; compound I is then poised to oxidize its substrate. Upon addition of H₂O₂ to holo-Mt-DyP, a color change of brown to green occurs, which is indicative of compound I formation. Further, the UV/vis spectrum of holo-Mt-DyP after the addition of H₂O₂ at pH 7.4 displayed a decrease and blue-shifted, hypochromatic Soret peak from 402 nm to 398 nm, with the emergence of a slight shoulder at around 345 nm (Fig. 4A) and a broad hyperchromaticity between 576 and 648 nm (Fig. 4A, inset) similar to that observed for Rj-DypB (35) and horseradish peroxidase compound I intermediates (36) (Table 1). These results show that holo-Mt-DyP forms compound I in the presence of H₂O₂, indicating that Mt-DyP is a heme peroxidase.

Mt-DyP was tested for peroxidase activity using either guaiacol or 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) as substrates. First, peroxidase activities of holo- and apo-Mt-DyP were compared utilizing guaiacol as a substrate (Fig. 4B). Apo-Mt-DyP had no detectable peroxidase activity compared with holo-Mt-DyP, suggesting that Mt-DyP is a heme peroxidase that can utilize guaiacol as a substrate. The specific activity of Mt-DyP forming tetraguaiacol under the conditions tested was 0.041 ± 0.004 μmol min⁻¹ mg⁻¹, and the kcat is 1.46 ± 0.15 min⁻¹. Mt-DyP has a 50-fold lower kcat value than a DyP from a white rot fungus Phanerochaete chrysosporium (37); however it has been noted that Mtb enzymes may have slower rates of reactions compared to their homologs (38,39). Second, utilizing ABTS as the reducing substrate, holo-Mt-DyP produced an ABTS radical cation in the presence of H₂O₂ indicative of an active peroxidase, while apo-Mt-DyP displayed background activity (Fig. 4C). The specific activity of Mt-DyP producing ABTS radical cation under the conditions tested was 0.052 ± 0.005 μmol min⁻¹ mg⁻¹, and the kcat is 2.11 ± 0.25 min⁻¹. Mt-DyP has a 50-fold lower kcat value than Rj-DypB (40). These results suggest that Mt-DyP is a heme peroxidase.

Even though Mt-DyP has heme-dependent peroxidase activity, Mt-DyP deferrochelatase activity was also tested as we have shown that Mt-DyP can bind PPIX (Fig. 3B). Heme-bound Mt-DyP was incubated with either Mycobacterium smegmatis mc²155 cytosolic or membrane fractions, as previously described for Ec-YfeX (11). The reaction was followed by UV/vis spectroscopy and no significant change in the Soret region was observed under the conditions tested, indicating no PPIX product was formed as a result of iron extraction from heme (Fig. 5A). These results suggest that Mt-DyP does not possess deferrochelatase activity in vitro. However, deferrochelatase activity requires an electron source for iron extraction from heme, and the in vitro conditions tested may not contain the necessary components. Thus, we sought to investigate the function of Mt-DyP in vivo by observing the effects of Mt-DyP upon mycobacterial heme-iron acquisition.

Mycobacteria possess a heme uptake pathway (20,41) along with a cytosolic heme-degrading enzyme, MhuD, which has been shown to degrade heme by cleavage of the tetrapyrrole ring to release iron and mycobilins as products (42,43). To test for Mt-DyP deferrochelatase activity, we utilized a mycobactin-deficient Mtb strain, MtbΔmbtB, which has an interrupted mycobactin biosynthetic pathway to prevent...
siderophore-mediated iron acquisition (20,41) and cannot utilize Fe(III) in the absence of exogenous siderophore (mycobactin). Thus, MtbΔmbtB may only sequester iron via the heme uptake pathway. We assessed growth of MtbΔmbtB mutants lacking MhuD and/or Mt-DyP against MtbΔmbtB alone in limiting heme concentrations (Fig. 5B). MtbΔmbtB supplemented with 0.2 µM heme grows to ~60% of the growth achieved in the presence of exogenous siderophore (mycobactin). Thus, Mtb cannot utilize Fe(III) in the absence of exogenous siderophore-mediated iron acquisition (20,41) and significantly attenuated in the presence of 0.2 µM ΔmbtB in the presence of 0.2 µM heme. In contrast, the growth rate of MtbΔmbtBΔmhuD was significantly attenuated in the presence of 0.2 µM heme compared with MtbΔmbtB (20% vs. 60%), Fig. 5B. These results suggest that MhuD, but not Mt-DyP, is required for heme degradation when heme is the sole iron source, at least when MhuD is present. To determine whether Mt-DyP is required for iron sequestration in the absence of MhuD, we generated the triple mutant, MtbΔmbtBΔmhuDΔdyP. Growth of MtbΔmbtBΔmhuDΔdyP in the presence of 0.2 µM heme was significantly attenuated compared with MtbΔmbtB (18% vs. 60%), but no more so than MtbΔmbtBΔmhuD (18% vs. 60%). Since Mt-DyP is not required for iron sequestration from heme in either the presence or absence of MhuD. Taken together, these results indicate that under the conditions tested, Mt-DyP probably does not possess deferoxochelatase activity.

Co-purification of Mt-Enc and cargo proteins
Mt-DyP is found upstream of Mt-Enc and harbors a C-terminal extension, similar to that observed in other organisms that also contain Enc and DyP in a two-gene operon. Thus we propose that Mt-DyP interacts with Mt-Enc (6,27). The two-gene operon, whose gene products are Mt-Enc and Mt-DyP, was cloned into an expression vector so that Mt-DyP, has a diameter ranging from 70 – 90 Å as measured by ImageJ (32). The oligomeric state of encapsulated Mt-DyP is unknown. To test that the C-terminal extension of Mt-DyP is required to target Mt-DyP to be encapsulated by Mt-Enc, we deleted the last 24 C-terminal amino acids of Mt-DyP (Mt-DyPΔ312-335). Mt-Enc and Mt-DyPΔ312-335 were then coexpressed and upon purification, Mt-DyPΔ312-335 was observed in the flow-through and only Mt-Enc was eluted, as seen by SDS-PAGE (Fig. 6D). These results suggest that Mt-Enc encapsulates Mt-DyP via its C-terminal tail.

We previously carried out studies of Mt-BfrB (ferritin (44)) and Mt-FolB (second enzyme in folate biosynthesis (16)), and noted that both mycobacterial proteins have extra ~20 amino acid C-terminal extensions (Fig. 1) in comparison with most of their other bacterial homologs. Both Mt-BfrB (13) and Mt-FolB (16) are known to have antioxidant properties, along with DyPs (45,46). Due to the observation that ferritin-like proteins with C-terminal extensions are contained within operons with Enc, and are proposed to interact (6), we hypothesized that Mt-BfrB and Mt-FolB may also interact with Mt-Enc through their C-terminal extensions.

Mt-Enc encapsulates fully assembled Mt-BfrB, where Mt-BfrB forms a 24-subunit icosahedral shell (44), in contrast to Mt-Enc, which forms a 60-subunit icosahedral shell. We co-expressed Mt-Enc with a C-terminal His-tag together with full-length Mt-BfrB. Mt-Enc and Mt-BfrB co-eluted from an affinity column (Fig. 6B). Visualization of the Mt-Enc-BfrB complex by EM clearly shows a smaller spherical shell within the interior of a larger shell. The larger shell has a diameter of ~220 Å, similar to Mt-Enc alone, and the inner shell has a diameter of ~120 Å, similar to that of Mt-BfrB (Fig. 6B) (44), suggesting that Mt-Enc encapsulates fully assembled 24-subunit Mt-BfrB (Fig. 6B). As it is proposed that Mt-Enc encapsulates its target protein via a C-terminal extension, Mt-BfrB was truncated to remove 15 C-terminal residues (Mt-BfrBΔ167-181). First, we showed by analytical gel filtration analysis that Mt-BfrBΔ167-181 has a similar elution profile to Mt-BfrB, suggesting that it retains its 24-subunit assembly in the absence of its C-terminal extension (data not shown).
Second, upon coexpression of Mt-Enc and Mt-BfrB\textsubscript{A167-181} followed by affinity chromatography purification, Mt-BfrB\textsubscript{A167-181} was observed in the flow-through and does not coelute with Mt-Enc (Fig. 6E). The other Mtb ferritin homolog, Mt-BfrA, also self-assembles into a 24-subunit nanocage (38,39), however Mt-BfrA does not possess a C-terminal extension. When Mt-BfrA was co-expressed with Mt-Enc followed by affinity chromatography purification, it did not co-elute with Mt-Enc (Fig. 6F). These observations validate the importance of the C-terminal extension in potentiating Mt-BfrB binding to the interior of the Mt-Enc compartment.

Similarly, Mt-FolB contains an extended C-terminus composed of aliphatic amino acids (Fig. 1), thus it is possible that Mt-FolB might interact with Mt-Enc via its C-terminal tail. Coexpression of Mt-FolB and Mt-Enc resulted in co-purification of Mt-FolB with Mt-Enc EM visualization of the Mt-Enc-FolB complex, showed fully assembled Mt-Enc that contained a potential protein cargo (Fig. 6C). The diameter of the cargo is ~ 60 Å, similar to the diameter of either the tetrameric or octameric forms of Mt-FolB (16), suggesting that Mt-Enc also encapsulates Mt-FolB. Upon deletion of the C-terminal tail of Mt-FolB, where Mt-FolB\textsubscript{A118-133} retains its enzyme activity (16), coexpression with Mt-Enc followed by purification resulted in the elution of Mt-Enc alone (Fig. 6G). These results suggest that Mt-FolB can be encapsulated by Mt-Enc via its C-terminal extension. EM images show nearly all Mt-Enc nanocompartments are occupied by Mt-DyP (~80%), which were coexpressed from a single plasmid. Fewer Mt-Enc assemblies were occupied with Mt-BfrB or Mt-FolB (<30%); however Mt-Enc and Mt-BfrB or Mt-FolB were coexpressed from separate plasmids. Reduced encapsulation is not surprising as heterologous overexpression of mycobacterial proteins in E. coli is not representative of the coupled translation or tight regulation in Mtb.

**Analysis of enzymatic activity of cargo proteins within Mt-Enc**

We have demonstrated that Mt-DyP has heme-dependent peroxidase activity that may utilize guaiacol or ABTS as substrates. Previously, it has been shown that Mt-BfrB has ferroxidase activity (31), and Mt-FolB has aldolase activity even in its C-terminal truncated form (16). Furthermore, we have shown that coexpression of each of these proteins with Mt-Enc results in encapsulation of each cargo protein via its C-terminal extension (Fig. 6). Next, we examined whether Mt-DyP, Mt-BfrB and Mt-FolB retain their activities when encapsulated by Mt-Enc.

**Mt-DyP.** To ensure that C-terminal truncation of Mt-DyP did not result in loss of activity, we demonstrated that heme-bound Mt-DyP\textsubscript{A312-335} retained its peroxidase activity with both guaiacol and ABTS as substrates (data not shown). In an attempt to produce encapsulated holo-Mt-DyP, Mt-Enc and Mt-DyP were coexpressed in the presence of δ-ALA. After purification of the Mt-Enc-DyP complex, the heme concentration within the complex (and presumably bound to Mt-DyP) was quantified using UV/vis spectroscopy (to determine heme concentration) and SDS-PAGE gel-band intensity analysis (to determine Mt-DyP concentration) using ImageJ (32). Utilizing either guaiacol or ABTS as substrates in the presence of H\textsubscript{2}O\textsubscript{2}, we assessed peroxidase activity of holo-Mt-DyP within Mt-Enc. With guaiacol as a substrate, Mt-Enc-DyP shows minimal peroxidase activity compared to unencapsulated holo-Mt-DyP (Fig. 7A). In contrast, encapsulated Mt-DyP retains peroxidase activity with ABTS as a substrate similar to unencapsulated holo-Mt-DyP (Fig. 7B), although we observe a slightly decreased rate. Within Mt-Enc, we cannot monitor the oligomeric state of holo-Mt-DyP, and we have shown above that holo-Mt-DyP is active as a tetramer (Figs. 3C & 3D); thus the observed decrease in rate of activity of encapsulated Mt-DyP may be due to the presence of various heme-bound Mt-DyP oligomeric states. In light of these results, we have demonstrated that Mt-DyP retains heme peroxidase activity while encapsulated; however it appears to have a more limited substrate-set than unencapsulated holo-Mt-DyP.

**Mt-BfrB.** To determine the activity of encapsulated Mt-BfrB, ferroxidase activity of the Mt-Enc-BfrB complex was measured at 320 nm to monitor iron core formation (47). To determine if the Mt-Enc-BfrB complex maintained ferroxidase activity, we quantified the amount of encapsulated Mt-BfrB by densitometry measurements of SDS-PAGE gel bands using ImageJ (32). Upon
addition of ferrous iron to Mt-BfrB alone, we observe ferroxidase activity. The specific activity of Mt-BfrB oxidizing Fe(II) is 0.073 ± 0.006 μmol min⁻¹mg⁻¹, where the activity is similar to a previous study of Mt-BfrB (31). Truncation of the C-terminal tail of Mt-BfrB, Mt-BfrBΔ167-181, resulted in fully assembled ferritin (data not shown) with a slightly reduced oxidation rate of Fe(II) (data not shown), as also observed by Khare et al. (31). In contrast, Mt-Enc alone was not capable of oxidizing Fe(II) (Fig. 7C). Mt-BfrB encapsulated in Mt-Enc retained its ferroxidase activity with a similar rate to that of Mt-BfrBΔ167-181 (Fig. 7C). Reduction in the rate of Fe(II) oxidation by encapsulated Mt-BfrB similar to that of Mt-BfrBΔ167-181 suggests that Mt-BfrB’s C-terminal tail is probably involved in binding to the interior of Mt-Enc, rendering it unavailable to enhance ferroxidase activity to the level observed for unencapsulated full-length Mt-BfrB.

Mt-FolB. Aldolase activity of Mt-FolB within Mt-Enc was assayed by measuring the production of 6-hydroxymethyl-7,8-dihydropterin as a change in fluorescence emission at 524 nm. The concentration of encapsulated Mt-FolB was estimated by densitometry measurements from SDS-PAGE analysis using ImageJ (32). The level of aldolase activity achieved upon the addition of a 1.5 molar excess of encapsulated Mt-FolB was similar to that of unencapsulated Mt-FolB (Fig. 7D). The specific activity of Mt-FolB is 0.18 ± 0.02 μmol min⁻¹mg⁻¹, similar to our previous study of Mt-FolB (31). Mt-Enc alone did not exhibit aldolase activity. These results suggest that Mt-FolB retains aldolase activity while encapsulated.

Discussion

Encapsulins are a recently discovered family of conserved prokaryotic proteinaceous nanocompartments, first described by Sutter et al. (6). Enc cargo proteins have C-terminal tails required for their encapsulation, where Enc and cargo proteins are usually found in a two-gene operon, suggesting tight translational coupling. We have demonstrated that Mtb has an Enc nanocompartment similar in diameter to that of Tm-Enc (6), implying that it is also a 60-subunit icosahedral shell. Mt-Enc exists in a two-gene operon, where the upstream Mt-DyP harbors a C-terminal extension. Coexpression of Mt-DyP and Mt-Enc results in encapsulation of Mt-DyP by Mt-Enc, an interaction mediated by the C-terminal tail of Mt-DyP. Although it is possible that Mt-Enc and Mt-DyP are expressed as a polycistronic mRNA transcript, the transcriptional and translational regulation of these proteins in Mtb is unknown. Additionally, we have demonstrated that Mt-BfrB and Mt-FolB are also encapsulated by Mt-Enc, via an interaction mediated by the C-terminal extension of each protein cargo, where loss of this C-terminal tail abolishes the interaction with Mt-Enc and their subsequent encapsulation. These results show that Mt-Enc could potentially compartmentalize other mycobacterial enzymes that bear a C-terminal tail.

Compartmentalization is important for organization, increasing the local concentration of functionally related enzymes, and confining unstable reaction intermediates within the cell. Many bacteria produce protein-based microcompartments (MCPs) that are larger than encapsulin nanocages, but also encapsulate various cargo enzymes (48,49). In Salmonella enterica, MCPs are proposed to organize reactions as well as isolate “unstable” reaction intermediates that may form within the compartment (48), such as proteins involved in 1,2-propanediol utilization (Pdu) (50) and ethanolamine utilization (Eut) (48). Indeed, a recent comparative genomics study predicts that in some organisms MCPs may encapsulate bacterioferritin (51), similar to Mt-Enc. A common feature of MCP-compartmentalized enzymes is an additional short N-terminal sequence found to be necessary for targeting enzymes to the MCP interior (48,52), akin to the cargo protein C-terminal extensions required for their encapsulation in Enc (6). It could be surmised that engineering a C-terminal tail on an enzyme could potentiate encapsulation by Mt-Enc, and possibly deliver and/or release cargo enzymes under conditions where the compartment disassembles.

This study and previous ones (6,27) demonstrate that encapsulins from four different organisms self-assemble into 60-subunit nanocompartments with a diameter of ~220-240 Å, and can accommodate multiple copies of a single cargo protein. Distant sequence homologs of Tm-Enc, Pyrococcus furiosus PfV and the major caspids protein gp5 of the HK97 virus (18 and 9% sequence identity, respectively) are close
structural homologs of Tm-Enc (rmsds of 2.39 and 2.65 Å, respectively) (53). All three proteins form icosahedral cages; however Tm-Enc has \( T = 1 \) symmetry with 60 subunits/cage (6), PfV has \( T = 3 \) symmetry with 180 subunits/cage (53), and gp5 has \( T = 7 \) symmetry with 420 subunits/cage (54). Thus one can postulate that Enc homologs, such as Mt-Enc, may form higher symmetry icosahedral cages in vivo to allow for more than one cargo protein, or even an entire reaction pathway, to be encapsulated, as observed for MCPs (49).

Passage of substrates through encapsulin is currently unknown. Charged pores observed for Tm-Enc may mediate substrate specificity (6), regulating the flux of substrates in and out of encapsulin that are available to the protein cargo. This may also be the case with Mt-Enc and the differential activity of encapsulated Mt-DyP with various electron-donor substrates, where guaiacol is neutral and ABTS is negatively charged. Rhamanpour and Bugg reported peroxidase activity of Rj-DypB when encapsulated within Rj-Enc with lignin as a substrate (27), this was an unexpected result as the expected pore size of Enc (< 5 Å wide) is not large enough to accommodate the passage of lignin (27) suggesting that the Enc pore is dynamic. Indeed, utilizing dynamic light scattering, it was observed that native Rj-Enc has a diameter of 220 Å; however, after acidic disassembly followed by reassembly with Rj-DyP, the complex has a diameter of 310 Å (27). The flexibility of the Enc monomer may allow Enc to form compartments consisting of more than 60 subunits or to expand its pore size to modulate substrate entry (6,27).

Mycobacteria are resistant to assault by host oxidative metabolites (46), where the presence of iron-storage proteins and peroxidases aid in mycobacterial viability. A recent study demonstrated that an Mtb double deletion mutant of \( bfrA \) and \( bfrB \) exhibited vulnerability to oxidative stress (14), and the Mtb\( \Delta bfrB \) mutant has greater susceptibility than the Mtb\( \Delta bfrA \) mutant (13,14), highlighting the importance of Mt-BfrB in Mtb survival during oxidative assault. Mt-BfrB has antioxidant properties by storing iron, thereby preventing the generation of hydroxyl radicals upon oxygen exposure. Furthermore, in vivo it has been shown that Mt-BfrB is up-regulated under hypoxic conditions (55), assault by NO donors (56), and during adaptation to the stationary phase (57). We have demonstrated that Mt-DyP can function as a heme-dependent peroxidase; thus, Mt-DyP may aid in mycobacteria resistance to host oxidative assault (46). This could potentially hold true for Mt-FolB. Mt-FolB is a 7,8-dihydroneopterin aldolase that is involved in folate biosynthesis, converting folate precursor 7,8-dihydroneopterin to 6-hydroxymethyl-7,8-dihydropterin. Mt-FolB displays cooperative dependence on 7,8-dihydroneopterin binding from an apo-tetrameric form to active substrate-bound octameric form (16). Furthermore, its substrate possesses antioxidant properties (15,17). Thus, if Mtb encounters host oxidative stress, then 7,8-dihydroneopterin may function as an antioxidant maintaining a relatively low cellular concentration. However, when oxidative stress diminishes, the concentration of 7,8-dihydroneopterin would increase to the critical level to form active, octameric Mt-FolB and Mtb would continue with its normal folate biosynthetic pathway (16). Recently, an in vitro study showed that there were five Mt-FolB reaction products (58). Another potential benefit of encapsulation of Mt-FolB could be to a) prevent side products of the reaction forming and b) allow 7,8-dihydroneopterin to remain “free” within the cytosol to act as an antioxidant. Thus Mtb encapsulation of proteins may provide the bacterium multiple mechanisms for protecting itself from oxidative damage arising from host immune defenses.

Encapsulation of DyP and ferritin homologs has previously been observed in other organisms (6,27); however there is no evidence that Enc interacts with FolB in other organisms. A recent study suggests that a potential Haliangium ochraceum aldolase is compartmentalized by a bacterial BMC by its C-terminal extension (Fig. 1A) (59). Furthermore, Mtb in vivo microarray data suggests that Mt-Enc and Mt-FolB both are up-regulated under hypoxic conditions (60) and exposure to Cephalexin (61), further suggesting that they indeed may interact.

This study provides the first evidence of a mycobacterial nanocompartment that has the ability to encapsulate cargo proteins, where encapsulated cargo proteins retain their enzymatic activities. To date, there are no reports illustrating the formation of a proteinaceous nanocompartment in mycobacteria. It is unknown which cargo protein(s) Mt-Enc encapsulates in
vivo, how it is regulated and if it can accommodate multiple cargo proteins. As has been suggested for other bacterial compartments (62), the potential for carrying cargo and apparent protein stability make Mt-Enc an appealing vehicle to be exploited as a drug delivery system.

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Figure Legends

Figure 1: C-terminal extensions of potential Mt-Enc protein cargos. A. C-terminal extension residue sequences of Mt-DyP, Mt-BfrB, and Mt-FolB beyond what is seen in homologs that are not encapsulated as well as B. linens DyP (Bl-DyP, (6)), T. maritima ferritin-like protein (Tm-Flp, (6)), and a putative H. ochraceum aldolase (Ho-Ald, (59)) thought to be encapsulated by its C-terminal tail to BMCs. B. The top numbered grey line represents Mt-FolB residues and the brown and light orange boxes represent conserved 7,8-dihydroneopterin aldolase (DHNA) and FolB domains, respectively, from various organisms generated by the Conserved Domain Database (63).

Figure 2: Mt-Enc assembles into a nanoparticle. A. Elution fractions of Mt-EncHis purification. B. EM image of purified Mt-EncHis; inset shows a single particle with a double-sided arrow indicating ~220 Å Mt-Enc particle diameter and thickness of ~25 Å.

Figure 3: Preliminary characterization of Mt-DyP. A. Elution fractions from nickel-affinity purification of Mt-DyP. B. UV/vis spectra of heme- (solid line) or PPIX- (dashed line) bound Mt-DyP to a 1:1 molar ratio. C. Representation of three distinct elution peaks from a gel-filtration experiment performed on an S200 16/600 Superdex column, yielding an Mt-DyP tetramer (I, 116 kDa), dimer (II, 58 kDa), and a monomer (III, 29 kDa), where their respective molecular weights were calculated using molecular weight standards (Bio-Rad). D. A UV/vis spectra of elutions resulting from three elution peaks post-gel filtration from (C) demonstrating heme content at ~400 nm. The tetramer (solid line) has 1:1 heme to protein molar ratio; dimer (dashed line) has trace amounts of heme while the monomer is in its apo-form (dotted line).

Figure 4: Peroxidase activity of Mt-DyP. A. Absorbance spectrum of holo-Mt-DyP (~2.5 µM) in the absence (solid line) and presence (dashed line) of 100 µM H_2O_2, where in the presence of H_2O_2, the formation of Compound I is observed, indicative of a heme-dependent peroxidase. Inset: the visible region magnified 10-fold. B. Holo-Mt-DyP (2 µM) peroxidase activity utilizing guaiacol (10 mM) as a substrate in the presence of H_2O_2 (10 mM) at pH 4.0 was monitored by observing the production of tetraguaiacol at 470 nm. C. Holo-Mt-DyP (1.25 µM) peroxidase activity utilizing ABTS (250 µM) as a substrate in the presence of H_2O_2 (10 µM) at pH 5.5 was monitored by observing the change in absorbance at 420 nm.

Figure 5: Mt-DyP demonstrates no deferrochelatase activity. A. Top: M. smegmatis mc²155 cytosolic fraction (5 µg) and Bottom: M. smegmatis mc²155 TritonX-114-extracted membrane (5 µg) was incubated with holo-Mt-DyP (5 µg), respectively. Possible deferrochelatase activity was monitored over 20 minutes, where a decrease in Soret intensity (~410nm) would be observed over time. B. MtbΔmbtB, MtbΔmbtBΔdyP, MtbΔmbtBΔmuuD and MtbΔmbtBΔmuuDΔdyP were grown in 7H9-OADC containing 0.01% tyloxapol medium supplemented with either 0.2 µM heme or mycobactin J (Myc J) (10 ng/mL). Growth was monitored by measuring absorbance at 750 nm and the results shown are taken at 18-20 days, when all strains in the presence of Myc J plateau. The results shown are OD₇₅₀ measurements normalized to the growth of the MtbΔmbtB in the presence of Myc J at the same time point and are the mean ± SE of two independent experiments.

Figure 6: Characterization of Mt-Enc with cargo proteins. Mt-Enc and co-elution of putative interacting Mtb protein: A. Mt-DyP (~37 kDa); B. Mt-BfrB (~20 kDa); or C. Mt-FolB (~14 kDa). Left: SDS-PAGE of eluted fractions with a molecular weight marker (MW). Right: EM images with inset of a single magnified Mt-Enc particle with cargo protein with a double-sided arrow indicating 220 Å Mt-Enc particle diameter. Dashed arrow denotes the diameter of cargo protein. Panels D-G demonstrates that proteins lacking a C-terminal extension do not co-purify with Mt-Enc. Mt-Enc does not co-elute with D.
Mt-DyPΔ312-335, E. Mt-BfrBΔ167-181, F. Mt-BfrA, or G. Mt-FolBΔ118-133. “FT” represents flow-through and “E” indicates elution fractions analyzed by SDS-PAGE.

Figure 7: Analysis of enzymatic activity of cargo protein within Mt-Enc. A. Mt-DyP peroxidase activity utilizing guaiacol as a substrate in the presence of H₂O₂, was monitored by observing the production of tetraguaiacol at 470 nm. B. Mt-DyP peroxidase activity utilizing ABTS as a substract in the presence of H₂O₂, was monitored by observing the change in absorbance at 420 nm. C. Mt-BfrB ferroxidase activity was monitored by observing the oxidation of ferrous iron to ferric iron at 320 nm. The encapsulated BfrB was compared to the rate of the Mt-BfrBΔ167-181. D. Mt-FolB enzyme activity was monitored utilizing 7,8-dihydroneopterin as a substrate, by observing the change in emission at 524 nm.
Table 1: Absorption spectral comparisons of resting and compound I forms of heme-dependent peroxidases.

| Name   | Resting (nm) | Compound I (nm) | Ref    |
|--------|--------------|-----------------|--------|
| Mt-DyP | 402, 498, 634 | 398, 576, 613, 648 | This Study |
| Rj-DypB| 404, 503, 634 | 400, 580, 613, 648 | (35)   |
| HRP    | 403, 498, 640 | 400, 525 (sh), 577, 622 (sh), 651 | (36)   |
Mtb nanocompartment and its cargo proteins

Figure 1

|        | C-terminal extension |
|--------|----------------------|
| Mt-DyP | LPQATPLAAGSLIGSLKGSFPR 335 |
| Mt-BfrB | EEVDVAPAGPHAGGRL---- 181 |
| Mt-FolB | ---RR-GGARGVVP-AGGAV---- 133 |
| Bi-DyP | QTAEEAARCGGSLGKSRGQ 367 |
| Tm-Flp | ---EIEEETGGSSENTGDDLGIRKL 114 |
| Ho-Ald | ---RDDLVRIVREELVRLA---- 261 |

B

![Image of protein sequence and diagram]

DHNA

FolB
Figure 4

A

B

C

Figure 4

Mtb nanocompartment and its cargo proteins

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Figure 5

A

B

Normalized Growth (%)
Figure 6

A

Mt-DyP  
35  
25  
15

Mt-Enc  
50 nm

~ 70 - 90 A

B

Mt-Enc  
35  
25  
15

Mt-BfrB  
220 Å

C

Mt-Enc  
34  
26  
17

Mt-FolB  
220 Å

220 Å

D

Mt-DyP  
Δ312-335  
34  
26  
17

Mt-Enc

Mt-BfrB  
Δ167-181

E

Mt-Enc

Mt-BfrA

F

Mt-Enc

Mt-BfrA

G

Mt-Enc

Mt-FolB  
Δ118-133

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Figure 7

A. Mt-DyP + Guaiacol

B. Mt-DyP + ABTS

C. Mt-BfrB

D. Mt-FolB
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