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Unusual Diheme Conformation of the Heme-Degrading Protein from *Mycobacterium tuberculosis*

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Heme degradation plays a pivotal role in the availability of the essential nutrient, iron, in pathogenic bacteria. A previously unannotated protein from *Mycobacterium tuberculosis*, Rv3592, which shares homology to heme-degrading enzymes, has been identified. Biochemical analyses confirm that Rv3592, which we have termed MhuD (*m*ycobacterial *h*eme *u*tilization, *d*egrader), is able to bind and degrade heme. Interestingly, contrary to previously reported stoichiometry for the *Staphylococcus aureus* heme degraders, iron-regulated surface determinant (Isd)G and IsdI, MhuD has the ability to bind heme in a 1:2 protein-to-heme ratio, although the MhuD–diheme complex is inactive. Furthermore, the 1.75-Å crystal structure of the MhuD–diheme complex reveals two stacked hemes forming extensive contacts with residues in the active site. In particular, the solvent-exposed heme is axially liganded by His75 and is stacked planar upon the solvent-protected heme. The solvent-protected heme is coordinated by a chloride ion, which is, in turn, stabilized by Asn7. Structural comparison between MhuD–diheme and inactive IsdG and IsdI bound to only one highly distorted metalloporphyrin ring reveals that several residues located in α-helix 2 and the subsequent loop appear to be responsible for heme stoichiometric differences and suggest open and closed conformations for substrate entry and product exit.

Keywords: Mycobacterium tuberculosis; heme binding protein; heme degradation; iron metabolism; X-ray crystallography

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Introduction

Heme degradation is a metabolic role performed by diverse organisms, fulfilling various physiological functions. The identification of a mammalian microsomal heme oxygenase (HO) demonstrated the oxidative cleavage of heme to release biliverdin as its final product, along with free iron and carbon monoxide in equimolar amounts.[1] In eukaryotes, this reaction is coupled with the conversion of biliverdin to bilirubin by biliverdin reductase,[2] whereas prokaryotic HOs have been implicated in phycobilin and phytochrome biosyntheses.[3] Additionally, increasing lines of evidence indicate that HOs from several bacterial pathogens play a major role in iron availability;[3] host heme degraded by HOs in Gram-positive and Gram-negative pathogens (i.e., HmuO from *Corynebacterium diptheriae*[4] and HemO from *Neisseriae* sp.,[5] respectively) provides an alternate source of iron, an essential element for growth, survival, and pathogenicity.

Recently, a new family of heme degraders has been described in *Staphylococcus aureus* and *Bacillus anthracis*, as well as the non-pathogenic *Bradyrhizobium japonicum*.[6–8] While these proteins do not share sequence or structural homology to canonical HOs, they are able to degrade heme. Furthermore, complementation studies show that *S. aureus* iron-regulated surface determinant (Isd)I can restore growth in *Corynebacterium ulcerans* HO mutant (Δ*HmuO*) and demonstrate the ability of noncanonical heme degraders to function *in vivo* as HOs.[7]

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Abbreviations used: MhuD, mycobacterial heme utilization, degrader; *Mtb*, *Mycobacterium tuberculosis*; TB, tuberculosis; HO, heme oxygenase; Isd, iron-regulated surface determinant; ITC, isothermal titration calorimetry; PDB, Protein Data Bank.

The crystal structures of homodimeric IsdG and its homolog, IsdI, reveal overall topologies distinct from monomeric HOs albeit similar to monooxygenases involved in antibiotic synthesis in *Streptomyces* sp.[9] Additionally, the heme-bound structure of a catalytically inactive IsdG mutant revealed that the α-meso edge of heme, which is buried in the active site of HOs,[10–13] is exposed to solvent.[14] Together, these data imply that IsdG and IsdI undergo a different mechanism for heme degradation distinct from HOs.

Mycobacterium tuberculosis (*Mtb*), the causative agent of tuberculosis (TB), is a bacterial pathogen responsible for approximately 8 million new infections and 2 million deaths per year worldwide.[15] The ease of *Mtb* to enter latency and develop multidrug resistance becomes a deadly combination for AIDS patients, and there is an urgent need to discover new anti-TB drugs. Similar to other pathogenic bacteria, the acquisition of iron in *Mtb* is required for infectivity and pathogenicity; as such, its iron acquisition pathways are well characterized.[16,17] Because heme biosynthesis and degradation are intricately linked with iron cycling[18] and *Mtb* possesses a biosynthetic pathway for heme,[19] we hypothesized the presence of proteins that are able to catabolize heme. Therefore, proteins important for iron acquisition and/or heme degradation can potentially serve as new targets for anti-TB therapeutics. However, this effort has been hampered by the lack of identification and biochemical characterization of a heme-degrading protein in *Mtb*.

In this study, we searched the *Mtb* genome[20] to identify proteins that may function as heme degraders. We have identified, for the first time, a putative *Mtb* heme degrader, Rv3592, which shares sequence homology with *S. aureus* IsdG and IsdI. Additionally, we show that Rv3592, which we term MhuD (*m*ycobacterial *h*eme *u*tilization, *d*egrader), is able to bind and degrade heme. Significantly, MhuD can bind two molecules of heme per monomer, which is different from the monoheme IsdG and IsdI, although MhuD–diheme is inactive. Finally, we have also solved a 1.75-Å crystal structure of MhuD–diheme complex, which sheds light on the nature of heme binding with alternate conformations. The above results pave the foundation towards new TB therapeutics targeting heme-degrading proteins.

Results

MhuD is homologous to bacterial heme degraders

The existence and identity of a heme-degrading protein in *Mtb* has not been established prior to this study. Concurrent presence of heme biosynthetic and degradation pathways in many organisms has been suggested to be important in maintaining cellular homeostasis by controlling the availability of heme and/or iron.[18] Since *Mtb* possesses a biosynthetic pathway for heme,[19] we hypothesized the presence of enzymes that are able to catabolize heme. To prove this hypothesis, we conducted extensive genome mining on heme-degrading proteins in *Mtb*. A BLAST homology search of the entire *Mtb* genome[20] did not reveal the presence of a canonical HO gene. However, we identified a putative gene, *Rv3592*, which shares 46% and 43% sequence identity with *S. aureus* IsdG and IsdI, respectively, analogous heme degraders that are unrelated to HOs.[7] Homologous proteins are found across other pathogenic and non-pathogenic bacteria, including *B. anthracis* IsdG[8] and *B. japonicum* HmuQ.[6] Furthermore, *Mtb* MhuD is a conserved protein across all mycobacterial species, including *Mycobacterium avium* and *Mycobacterium leprae*. Multiple sequence alignments of these proteins show several key conserved residues that have been reported to be crucial for heme degrading activity in *S. aureus* IsdG (Asn7, Trp67, and His77, Fig. 1). In particular, mutational analyses of these residues have been shown to maintain heme binding albeit abolishing heme degradation.[9] MhuD also contains these invariant residues corresponding to Asn7, Trp66, and His75, suggesting that it could function as a heme-degrading protein.

MhuD binds and degrades heme

As a first report for mycobacteria, we found that MhuD both binds and degrades heme. To test our hypothesis that apo-MhuD is a novel protein involved in *Mtb* heme degradation, we investigated its ability to bind heme. Hemin was incrementally titrated into 5 μM purified apo-MhuD and the spectral range between 300 and 700 nm was measured using a dual-beam spectrophotometer. The resulting difference spectra are generated by subtracting the free heme spectra from the heme-titrated MhuD spectra. MhuD exhibits the hallmarks of a heme-binding protein with the appearance of a Soret peak at 410 nm, as well as a broad peak around 575 nm corresponding to the Q band region (Fig. 2a). Plotting the absorbance difference at 410 nm against heme concentrations reveals that heme binding is saturable (Fig. 2a, inset).

To gain further insight into MhuD heme binding, we used isothermal titration calorimetry (ITC) to investigate the heme:MhuD stoichiometry and to obtain binding constants. The ITC experiments in which apo-MhuD (in the cell) was titrated with hemin (in the syringe) generated a binding isotherm consistent with saturation of heme binding (Fig. 2b). Using the Origin software (MicroCal), we tried one-site ($\chi^2 = 7.0 \times 10^4$) and two-site ($\chi^2 = 3.35 \times 10^4$) binding models and found that the best-fit parameters were obtained with the sequential binding site model ($\chi^2 = 1.29 \times 10^4$), whereby two heme binding sites per monomer were specified to be fit in a sequential manner. The calculated heme association constants (K_a) correspond to two heme sites of $1.2 \pm 0.3 \times 10^5$ and $2.0 \pm 0.3 \times 10^5$ M^{-1},