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A single mutation in the *Mycobacterium tuberculosis* heme-degrading protein, MhuD, results in different products

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Abstract

Mycobacterium tuberculosis heme-degrading protein MhuD degrades heme to mycobilin isomers and iron, while its closest homologs from *Staphylococcus aureus,* IsdG and IsdI, degrade heme to staphylobilin isomers, formaldehyde and iron. Superposition of the structures of the heme bound complexes reveal that the heme molecule in the MhuD active site is rotated ~90° about the tetrapyrrole plane with the respect to IsdG and IsdI active site heme molecules. Therefore, the variation in IsdG/IsdI and MhuD chromophore products may be attributed to the different heme orientations. In MhuD, two arginines, Arg22 and Arg26, stabilize the heme propionates, and may account for heme orientation. Herein, we demonstrate that the MhuD-R26S variant alters the resulting chromophore product from mycobilin to biliverdin IXα (α-BV), whereas the R22S variant does not. Surprisingly, unlike canonical heme oxygenase (HO) that also degrades heme to α-BV, the MhuD-R26S variant produces the C1-product formaldehyde rather than carbon monoxide as observed for HO. The MhuD-R26S variant is an important tool to further probe the mechanism of action of MhuD, and to also study the fate of the MhuD product in mycobacterium.

Keywords

Heme degradation; *Mycobacterium tuberculosis*; mycobilin; biliverdin; iron

In living organisms, heme (iron-protoporphyrin IX) degradation contribute to a variety of crucial functions including maintaining iron homeostasis[1], cell signaling[2], and antioxidant defense[3]. The most well-studied heme degradation enzyme, human heme oxygenase 1 (hHO-1, UniProtKB P09601), catalyzes the regiospecific breakdown of heme to biliverdin IXα (α-BV) (Figure 1A), the C1-product carbon monoxide (CO) and ferrous iron[4–6]. HO-

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ASSOCIATED CONTENT

Supporting Information

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MhuD P9WKH3

IsdG Q7A649

IsdI Q7A827

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like enzymes have also been found in prokaryotes and share high structural homology with hHO-1 and predominately produce the same heme degradation products[7–11]. Other heme-degrading enzymes have been reported with no sequence homology to HOs[12, 13]. These non-canonical heme-degrading enzymes produce chromophores distinct from α-BV[14–16]. *Staphylococcus aureus* heme-degrading proteins, IsdG and IsdI (UniprotKB Q7A649 and Q7A827, respectively), break down heme and produce staphylobilin isomers (Figure 1A), formaldehyde and free iron[14, 17]. In addition, IsdG-type heme degrading enzymes are structurally distinct from HOs[12]. HO enzymes are composed exclusively of α-helices[18], whereas the structures of IsdG-type enzymes are composed of a dimeric eight-stranded β-barrel decorated with α-helices[12]. The *Mycobacterium tuberculosis* heme degrading enzyme, MhuD (UniProtKB P9WKH3), belongs to the IsdG-type protein family[13, 19]. Despite high sequence and structural homology to IsdG-type proteins, MhuD degrades heme to two mycobilin isomers (Figure 1A) and iron but no free C1-product[15]. Mycobilin isomers differ from staphylobilins as heme ring cleavage occurs at the β- or δ-meso carbons for staphylobilins and at the α-meso carbon for mycobilins, and there is no loss of the vicinal C–O moiety in mycobilins. Both products, however, are oxygenated at their β- or δ-meso carbons[14, 15], whereas the HO product, biliverdin, has no additional meso carbon carbonyl group.

Superposition of the heme bound complex structures of MhuD (PDB code: 4NL5) with *S. aureus* IsdG and IsdI (PDB codes: 2ZDO and 2ZDP, respectively) reveal that the heme molecule in the MhuD active site is rotated ~90° about the tetrapyrrole plane in comparison to those of IsdG and IsdI[19,20]. This difference in heme orientation possibly accounts for the variation in the resulting heme degradation chromophore products of IsdG/IsdI and MhuD, staphylobilin and mycobilin (Figure 1A), respectively. Within the HO protein family, the *Pseudomonas aeruginosa* HO (*pa*-HO) heme molecule is rotated ~100° from canonical HOs[21]. As a consequence, *pa*-HO heme degradation produces a mixture of β- and δ-biliverdin products instead of α-BV[21]. Furthermore, mutating residues within the canonical HO heme binding pocket results in alteration of the α-regioselectivity of biliverdin[22]. Thus we examined the MhuD active site to identity residues that may impact heme orientation in the active site of MhuD and regioselectivity of heme degradation (Figure 1B). Two residues, Arg22 and Arg26, which form electrostatic interactions with the heme propionates, are likely to be important in maintaining correct heme orientation. This study examines the impact that MhuD Arg22Ser and Arg26Ser mutations have upon heme binding, heme degradation activity and product formation.

To determine the effect of the MhuD R22S and R26S mutations on heme binding, we measured the heme off rates ($k_{\text{-heme}}$) for wild-type (WT) MhuD-heme and its variants. The heme off rate was measured using the apo-H64Y/V68F-myoglobin assay, whereby the apomyoglobin mutant acts as a high-affinity heme scavenger[23, 24]. The $k_{\text{-heme}}$ values for MhuD-WT and the R26S variant are similar with ~$k_{\text{-heme}} = 1.50 \times 10^{-3}$ min^{-1}. However, MhuD-R22S exhibits an increased $k_{\text{-heme}}$ value compared to MhuD-WT by ~2-fold, suggesting that R22S mutation has a weaker affinity for heme (Table S1 & Figure S1).

Single-turnover heme degradation reactions by MhuD-WT and the R22S and R26S variants were carried out using ascorbate as an electron donor in the presence of catalase to prevent

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non-enzymatic hydrogen peroxide heme degradation. The mutations of either R22 or R26 to serine did not prevent the degradation of heme as compared to MhuD-WT (Figure S2). A combination of high performance liquid chromatography (HPLC, Figure 2A & Table S2), UV/vis spectroscopy (Figure S2A) and mass spectrometry analyses (MS, Figure S3B) confirmed that the products of the MhuD-R22S variant were the characteristic mycobilin isomers as described for MhuD-WT[15]. However, the predominant tetrapyrrole product for the MhuD-R26S variant has similar characteristics to that of the canonical HO product, biliverdin (Figure 2A, Supplemental Figure S3 & Table S2). Notably, minor quantities of both mycobilin-a and mycobilin-b were produced by the MhuD-R26S variant (Table S2 & Figure 2A), suggesting multiple heme conformations and/or heme-iron electronic states within the MhuD-R26S variant. The MS/MS fragmentation patterns of the MhuD-R26S predominant product confirmed that it was α-BV (Figure 2B). This result suggests that Arg26 plays a role in MhuD product formation and perhaps the oxygenation of the β- and δ-meso carbons of the mycobilin isomers (Figure 1A).

Since the MhuD-R26S variant break down of heme results in α-BV, the R26S variant should produce a C1-product (ie CO) similar to hHO-1. CO production was detected using the H64L variant of myoglobin (Mb) that has a high CO affinity[15]. Figure 3A shows the Soret region difference spectra of ferrous H64L-Mb induced by CO generated by heme degradation. As previously reported, hHO-1 exhibits a significant difference spectrum with a near-stoichiometric formation of CO.[15] However, both MhuD-WT and the MhuD-R26S produce negligible amounts of CO.

Other C1-products that could be produced are formaldehyde, which was observed for *S. aureus* IsdG-type enzymes[17], and the oxidized product of formaldehyde, formic acid. To test for formic acid, formic acid dehydrogenase was utilized to convert formic acid to NADH, which was then quantified by HPLC[25]. Heme degradation catalyzed by either MhuD-WT or the R26S variant produced negligible amounts of NADH (Figure S4), suggesting that formic acid is not the C1-product for MhuD-R26S. Lastly, we tested for the production of formaldehyde by an acetylacetone method that converts formaldehyde to 3,5-diacetyl-1,4-dehydrolutidine[26], quantified by HPLC. Quantification of 3,5-diacetyl-1,4-dihydrolutidine shows that *S. aureus* IsdI produces formaldehyde while MhuD WT does not (Figure 3B). However, the MhuD-R26S variant does generate formaldehyde in near equimolar amounts similar to the IsdI heme degradation reaction[17] (Figure 3B), suggesting that the C1-product generated by MhuD-R26S is predominately formaldehyde.

MhuD Arg22 and Arg26 form electrostatic interactions with the heme propionates and the original hypothesis was that these interactions may impact heme orientation within the MhuD active site, resulting in the observed tetrapyrrole product variation between *S. aureus* IsdG/IsdI and MhuD heme degradation. This study shows that mutation of a single MhuD arginine, Arg26, to serine, results in heme degradation to a different tetrapyrrole product, α-BV, while mutation of Arg22 to serine, has no observable effect. The products of MhuD-WT and MhuD-R26S heme degradation, mycobilins and α-BV (respectively), are both cleaved at the α-meso carbon, suggesting that Arg26 does play a role in MhuD heme degradation but does not govern regiospecific tetrapyrrole ring cleavage. One hypothesis is that the heme orientation in the active site of the MhuD-R26S variant is similar to that of WT MhuD, and

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instead the R26S mutation alters the electrostatic environment within the MhuD-R26S active site. However, regiospecific heme cleavage is not always dependent on the heme orientation within the active site of heme degradation enzymes. In the case of hHO-1, where R183 also interacts with one of the heme propionates, the R183E variant results in altered α-regioselective cleavage with the predominant formation of δ-biliverdin[22]. Further analysis revealed that the heme molecule within the hHO-1 R183E variant retains a similar orientation to that of WT hHO-1[27] and the difference in degradation products was attributed to an altered electrostatic environment and hydrogen-bonding network within the mutant hHO-1 R183E active site[27]. In support of this, it was also demonstrated that the positive to negative charge reversal of the hHO-1-R183E variant decreased the rate of reduction of heme substrate, and thus suggests that the R183E mutation changed the heme-iron electronic environment from that of WT hHO-1[27].

Unlike canonical HO heme degradation that also produces α-BV, the MhuD-R26S variant does not generate CO. Instead, MhuD-R26S produces the C1-product, formaldehyde, similar to *S. aureus* IsdG and IsdI[17]. Thus, one may speculate that the MhuD-R26S variant predominately degrades heme by a different mechanism to that of HO and MhuD. HO degrades heme by three successive monooygenation reactions[6], whereas MhuD degrades heme by a monoxygenation step followed by a dioxygenation step(Figure S5)[28]. During the second HO monooxygenation reaction, the spontaneous conversion of *meso*-hydroxyheme to the key intermediate, verdoheme, results in the release of the α-*meso* carbon as CO (Figure S5)[6]. The lack of CO generation by MhuD-R26S suggests that this variant does not proceed through the formation of α-verdoheme, as has previously been determined for non-canonical HOs, MhuD and *S. aureus* IsdG and IsdI [28–30]. The production of formaldehyde by MhuD-R26S suggests that formylbilin is generated, followed by conversion into α-BV and formaldehyde, similar to the proposed IsdG/I intermediate albeit without the meso-oxo group[29]. A possible pathway for MhuD-R26S to produce formylbilin, could be via an intermediate step described for the HO degradation of 5-phenylheme to α-BV and benzoic acid rather than the C1-product CO[31]. It was proposed that after formation of the ferric hydroperoxo species, an additional electrophilic oxidation step occurs to give an epoxide, or after the addition of water, a diol intermediate, which would be followed by an additional oxidation step to yield the desired products (Figure S6)[31].

In summary, this is the first time that a single mutation within a heme-degrading protein has resulted in the formation of different products. If the only role of R26 is to properly orient heme within the active site, then the R26S might be expected to give similar WT products but with cleavage at a different meso carbon position. This clearly is not the case, which indicates that the R26S variant operates by a substantially different mechanism from WT MhuD and canonical HOs. Additional biophysical characterization is required to determine the heme orientation and/or heme-iron electronic environment of the MhuD-R26S variant compared to MhuD-WT, in order to fully understand the consequence of this single mutation. This study further underscores the delicate balance in the local electrostatic environment and, possibly, active site solvent structure in controlling the mechanism of heme degradation and products formed.

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Supplementary Material

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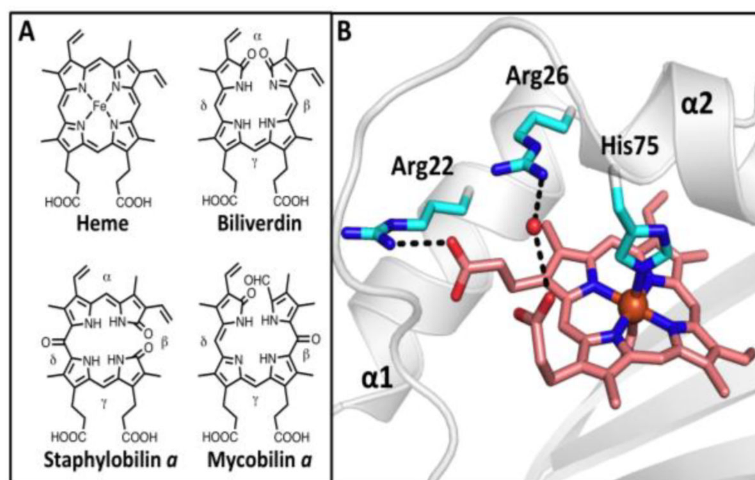

Figure 1. Structures of heme degradation products and MhuD-monoheme.

(A) The structure of heme and the heme chromophore degradation products. **(B)** The structure of Mtb MhuD in complex with cyano-derivatized monoheme is depicted (cartoon, white - PDB code: 4NL5). His75 coordinates the heme iron, and Arg22 and Arg26 residues form electrostatic interactions with the heme propionates are depicted in stick (cyan).

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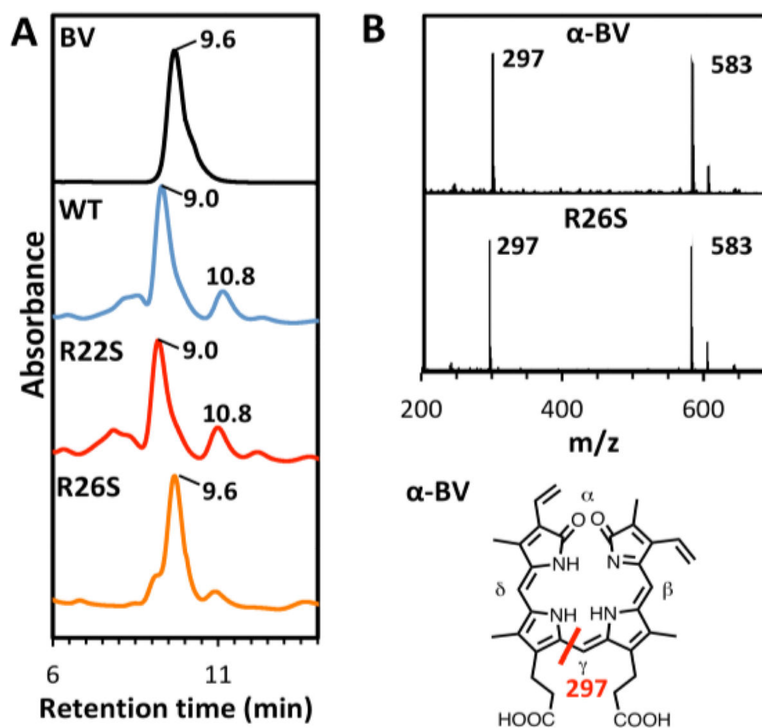

Figure 2. Tetrapyrrole product identification for MhuD and its variants

(A) HPLC chromatograms from top to bottom panels - Biliverdin standard (BV, black), MhuD-WT (WT, blue), MhuD-R22S (R22S, red) and MhuD-R26S (R26S, orange). BV elutes at 9.6 min, WT mycobilin a and mycobilin b elute at 9.0 and 10.8 min, respectively. (**B**) The top panel shows the MS/MS fragmentation of α-BV standard and the R26S BV product. The bottom panel is the expected fragmentation daughter ion for α-BV of 297 m/z.

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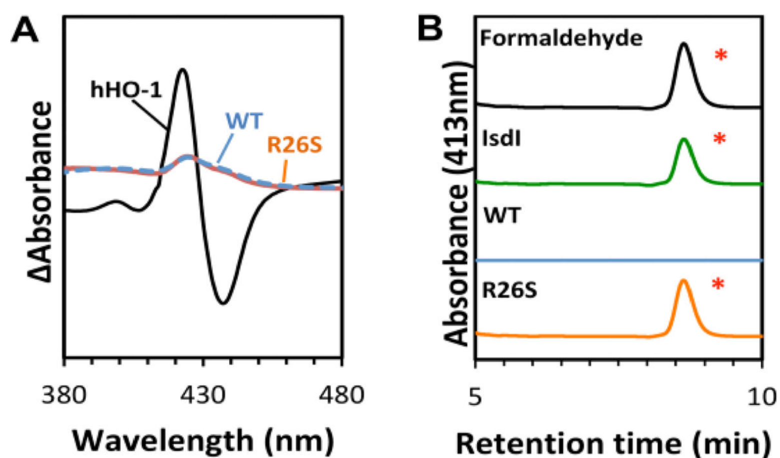

Figure 3. Quantitative analysis of MhuD-R26S C1-product

(A) CO quantification during heme degradation. Spectral changes of ferrous H64L Mb were calculated from the spectra taken before and after heme degradation of 5 μM heme complexes of hHO-1 (black line), MhuD-WT (WT, blue line) and MhuD-R26S (R26S, orange line). (B) HPLC detection of formaldehyde as 3,5-diacetyl-1,4-dihydrolutidine produced by formaldehyde standard (black line, 50 μM), IsdI (green line), MhuD-WT (WT, blue line) and MhuD-R26S (R26S, orange line).