Supplementary Information

For

The affinity of MhuD for heme is consistent with a heme degrading function *in vivo.*

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Supplemental Experimental

All materials were purchased from Fisher Scientific and used without further purification unless otherwise noted. All water was obtained from a Synergy water purification system (Millipore).

Cloning, Expression, and Purification. The cloning and expression of MhuD (*Rv3592*) with a C-terminal His_6 tag into pET-22b (Amp^r, Novagen) has been previously described.[1] The MhuD gene was recloned into a pET30a vector to encode a protein with an enterokinase cleavable N-terminal His_6 tag, which results in encoded full-length MhuD with an additional N-terminal Ala ($MhuD_{CH}$). The *M. tuberculosis* MhuD gene (*Rv3592*) was PCR amplified from the pET22a plasmid noted above using KOD-Hot Start DNA Polymerase (Novagen) with primers (5' – GGC CAT GGC CCC AGT GGT GAA GAT CAA CGC AAT CGA GGT GCC CGC C – 3') and (5' – GGA AGC TTA TTA TGC AGT CTT GCC GGT CCC ACC GAC GTC AAG CAC GAC – 3') containing the restriction sites *Nco*I and *Hind*III, respectively. The PCR product was gel purified (Qiagen) and ligated into a linearized blunt vector, pCR-BluntII-TOPO (Invitrogen), and transformed into One-Shot TOP10 *Escherichia coli* cells (Invitrogen). Restriction enzymes *Nco*I and *Hind*III were used to excise the MhuD insert from pCR-BluntII-TOPO as well as cut the empty pET30a (Kan^r) vector. The excised MhuD insert and cut pET30a were ligated together using T4 DNA ligase (New England Biolabs). The resulting DNA construct was verified by DNA sequencing (Retrogen).

Cells containing recombinant MhuD were lysed as described previously,[2] and the filtered supernatant was loaded onto a 5 mL Ni(II)-charged HiTrap chelating HP column (GE Healthcare) equilibrated with 50 mM Tris pH 7.8, 350 mM NaCl using an ÄKTA pure 25 L fast protein liquid chromatography (FPLC) system (GE Healthcare). The column was washed with a 2 mM/mL linear gradient from 0 to 100 mM imidazole in 50 mM Tris pH 7.8, 350 mM NaCl at a flow rate of 5.0

mL/min. Pure MhuD eluted during a subsequent 1.6 mM/mL linear gradient from 100 to 300 mM imidazole in 50 mM Tris pH 7.8, 350 mM NaCl at a flow rate of 5.0 mL/min (Figures S4-S5). FPLC fractions containing pure MhuD were pooled and the sample volume was reduced to 10 mL using Amicon stirred cells with 10 kDa ultrafiltration membranes (Millipore). Following overnight dialysis against 20 mM Tris pH 8.0, 50 mM NaCl, >99% pure MhuD was obtained as assessed by SDS-PAGE gel electrophoresis (Figure S6).

For recombinant expression of $MhuD_{CH}$, the pET30a (Kan^r) vector encoding $MhuD_{CH}$ was transformed into BL21-GOLD (DE3) cells (Stratagene). DNA sequencing at the Vermont Cancer Center DNA Analysis Facility confirmed the sequence of the $MhuD_{CH}$ gene for all cell lines used at the *University of Vermont* (Table S3). *E. coli* cells containing pET30a were grown in Luria-Bertani medium containing 30 μg/μL kanamycin at 37 °C using a MaxQ 5000 floor-model shaker (Thermo Scientific), and $MhuD_{CH}$ over-expression was induced with 1 mM isopropyl β-D-1-thiogalactopyranoside at an OD_{600} of 0.8 a.u. Cells were lysed in 50 mM Tris pH 7.8, 350 mM NaCl by sonication following the addition of 2.5 mg lysozyme and 0.01 M phenylmethane sulfonyl fluoride using a Branson S-450A Sonifier. The lysate was centrifuged at 15,000 x *g* for 45 mins using a Sorvall Legend XTR centrifuge (Thermo Scientific) and the supernatant was filtered through a 0.45 μm membrane (Millipore). The filtered lysate was loaded onto a 5 mL Ni(II)-charged HiTrap chelating HP column equilibrated with 50 mM Tris pH 7.8, 350 mM NaCl using an ÄKTA pure 25 L FPLC system. The column was washed with a 2.25 mM/mL linear gradient from 0 to 90 mM imidazole in 50 mM Tris pH 7.8, 350 mM NaCl at a flow rate of 5.0 mL/min. Pure, uncleaved $MhuD_{CH}$ eluted during a subsequent 1.48 mM/mL linear gradient from 90 to 275 mM imidazole in 50 mM Tris pH 7.8, 350 mM NaCl at a flow rate of 5.0 mL/min (Figures S7-S8). FPLC fractions containing pure, uncleaved $MhuD_{CH}$ were pooled and the sample volume was

reduced to 10 mL using Amicon stirred cells with 10 kDa ultrafiltration membranes. Uncleaved $MhuD_{CH}$ was dialyzed overnight against 20 mM Tris pH 8.0, 50 mM NaCl, 2 mM $CaCl_2$.

The His_6 tag of $MhuD_{CH}$ was removed by adding enterokinase (New England Biolabs) in a molar ratio of 1:250,000. The mixture was stirred for 36 h at 4 °C, then loaded onto a 5 mL HiTrap Q HP column (GE Healthcare) equilibrated with 20 mM Tris pH 8.0, 10 mM NaCl using an ÄKTA pure 25 L FPLC system. Pure, cleaved $MhuD_{CH}$ eluted during a 1.0 mM/mL linear gradient from 50 to 250 mM NaCl in 20 mM Tris pH 8.0 at a flow rate of 5.0 mL/min (Figures S9-S10). FPLC fractions containing pure, cleaved $MhuD_{CH}$ were pooled and the sample volume was reduced to 2.5 mL using Amicon stirred cells with 10 kDa ultrafiltration membranes. Following exchange into 50 mM Tris pH 7.4, 150 mM NaCl using a PD-10 desalting column (GE Healthcare), >95% pure, cleaved $MhuD_{CH}$ was obtained as assessed by SDS-PAGE gel electrophoresis (Figure S11).

Electrospray ionization mass spectrometry (ESI-MS) was used to assess the $MhuD_{CH}$ product. A 30 μM sample of cleaved $MhuD_{CH}$ in 50 mM Tris pH 7.4, 150 mM NaCl was loaded onto a C_{18} guard column equilibrated with 2% acetonitrile with 0.1% formic acid (*v*/*v*) in water (*v*/*v*) using a QTRAP 4000 LCMS/MS system (Sciex) with an M/z from 600-2000. The column was washed using 2% acetonitrile with 0.1% formic acid (*v*/*v*) in water (*v*/*v*) for 1 min at a flow rate of 100 μL/min. $MhuD_{CH}$ eluted during a 13.7%/min linear gradient from 2 to 98% acetonitrile with 0.1% formic acid (*v*/*v*) in water (*v*/*v*) at a flow rate of 100 μL/min. The spectrum was deconvoluted using BioAnalyst 1.5 software. The observed molecular weight of 11,327 Da was in perfect agreement with the expected molecular weight of 11,327 Da (Figure S12).

Spectroscopic characterization. The MhuD–heme extinction coefficient was determined previously,[2] and the extinction coefficients for MhuD–diheme, heme-bound $MhuD_{CH}$ ($MhuD_{CH}$–heme), and diheme-bound $MhuD_{CH}$ ($MhuD_{CH}$–diheme) were determined using a similar

procedure. Samples of MhuD–diheme, $MhuD_{CH}$–heme, and $MhuD_{CH}$–diheme were prepared in 50 mM Tris pH 7.4, 150 mM NaCl as described previously,[1] and their room temperature UV/Vis absorption (Abs) spectra were acquired from 900 to 200 nm with a scan rate of 600 nm/min, a 1.0 nm data interval, and a 0.1 s integration time using a Cary 100 Bio UV/Vis Spectrophotometer. The following extinction coefficients were determined using the pyridine hemochrome assay:[3] MhuD–diheme (ε_{410} = 165.3 $mM^{-1}cm^{-1}$), $MhuD_{CH}$–heme (ε_{407} = 87.9 $mM^{-1}cm^{-1}$), and $MhuD_{CH}$–diheme (ε_{395} = 162.3 $mM^{-1}cm^{-1}$).

Fluorescence-detected heme titrations into MhuD and $MhuD_{CH}$ were completed by slightly modifying a previously described procedure.[4] Briefly, 100 nM samples of MhuD and $MhuD_{CH}$, plus 17.5 μM heme solutions, were prepared in 50 mM Tris pH 7.4, 150 mM NaCl as previously described.[2] Heme was titrated into MhuD and $MhuD_{CH}$ in 16 nM increments, and allowed to equilibrate for 5 min prior to fluorescence characterization. Fluorescence emission spectra were acquired for 285 nm excitation using a Photon Technology International QuantaMaster 4 spectrofluorometer equipped with a Xenon arc lamp connected to an LPS-220b power supply, an ASOC-10 electronics interface, an MD-4000 motor driver control, and a model 814 photomultiplier detection system. Emission spectra were acquired in the 410 to 310 nm range with a step size of 1 nm, an integration time of 1 s, and slit widths of 3 nm.

Abs-detected titrations were carried out using similar protein samples. 5 μM samples of MhuD and $MhuD_{CH}$, and a 500 μM heme solution, were prepared in 50 mM Tris pH 7.4, 150 mM NaCl as described before.[2] Heme was titrated into MhuD and $MhuD_{CH}$ in 1 μM increments, and allowed to equilibrate until no further spectral changes were observed. Abs spectra were acquired using the equipment and parameters described above.

Spectral Analysis. The fluorescence-detected titrations of heme into MhuD and $MhuD_{CH}$ were analyzed in order to determine K_{d1} for MhuD and $MhuD_{CH}$. The Trp66 fluorescence intensity for a mixture of MhuD, MhuD–heme, and heme depends upon equation (1):

$$F = \frac{([MhuD]_T + [heme]_T + K_{d1}) - \sqrt{([MhuD]_T + [heme]_T + K_{d1})^2 - 4[MhuD]}}{2} X\left(\frac{F_{min} - F_{max}}{[MhuD]_T}\right) + F_{max} \quad (1)$$

where $[MhuD_T]$ is the total MhuD concentration, $[heme_T]$ is the total heme concentration, F_{max} is the fluorescence intensity in the absence of heme, and F_{min} is the fluorescence intensity for fully heme-bound MhuD. The emission intensity at 336 nm as a function of $[heme_T]$ was fit to equation (1) using GraphPad Prism 7.0 to determine K_{d1} and its standard error. The complete derivation of equation (1) has been reported previously for *Staphylococcus aureus* IsdG,[4] and a similar equation can be derived for $MhuD_{CH}$.

The Abs-detected titrations were analyzed to extract K_{d2} for MhuD and $MhuD_{CH}$. The Abs intensity at 410 nm for a mixture of MhuD, MhuD–diheme, MhuD–heme, and heme depends upon equation (2):

$$A_{410} = \frac{\varepsilon_{MhuD-diheme}[MhuD_T][heme]^2 + \varepsilon_{MhuD-heme}K_{d2}[MhuD_T][heme]}{[heme]^2 + K_{d2}[heme] + K_{d1}K_{d2}} - \quad (2)$$

where $\varepsilon_{MhuD–diheme}$, $\varepsilon_{MhuD–heme}$, and ε_{heme} are the molar extinction coefficients for MhuD–diheme, MhuD–heme, and heme, respectively, at 410 nm. The Abs intensity at 410 nm as a function of $[heme_T]$ was fit to equation (2) using Graph Pad Prism 7.0 to determine K_{d2} and its standard error. K_{d1} was constrained to the value determined above from analysis of the fluorescence-detected

titrations. A similar equation can be derived for $MhuD_{CH}$. The complete derivation of equation (2) is shown below.

The Abs-detected heme titrations intoWT MhuD were performed to extract the dissociation constant (K_{d2}) of binding for the second heme substrate. The Abs intensity at 410 nm for a mixture containing MhuD–diheme, MhuD–heme, MhuD and heme depends on equation (3):

$$A_{410} = \varepsilon_{MhuD-diheme}[MhuD-diheme] + \varepsilon_{MhuD-heme}[MhuD-heme] + \varepsilon_{heme}[heme] \quad (3)$$

where, $\varepsilon_{MhuD\text{-}diheme}$, $\varepsilon_{MhuD-heme,}$ and ε_{heme} are the extinction coefficients of these species at 410 nm. The K_{d1} for the first heme binding to MhuD is given by equation (4):

$$K_{d1} = \frac{[MhuD][Heme]}{[MhuD-heme]} \quad (4)$$

The K_{d2} for the second heme binding to MhuD is given by equation (5):

$$K_{d2} = \frac{[MhuD-heme][Heme]}{[MhuD-diheme]} \quad (5)$$

The total amount of MhuD ($MhuD_T$) is defined by equation (6):

$$[MhuD_T] = [MhuD] + [MhuD-diheme] + [MhuD-heme] \quad (6)$$

Rearranging equation (6):

$$[MhuD] = [MhuD_T] - [MhuD-diheme] - [MhuD-heme] \quad (7)$$

The total amount of heme ($heme_T$) is defined by equation (8):

$$[heme_T] = [heme] + 2[MhuD - diheme] + [MhuD - heme] \quad (8)$$

This equation can be rearranged to give equation (9)

$$[heme] = [heme_T] - 2[MhuD - diheme] - [MhuD - heme] \quad (9)$$

Next, we have introduced a dummy variable, α, which is given by equation (10)[5]

Let $$\alpha = \frac{[MhuD - diheme]}{[heme]^2} \quad (10)$$

By dividing [*MhuD–diheme*], [*MhuD–heme*] and [*MhuD*] by α, we get equations (11) - (13) respectively:

$$\frac{[MhuD - diheme]}{\alpha} = [heme]^2 \quad (11)$$

$$\frac{[MhuD - heme]}{\alpha} = K_{d2}[heme] \quad (12)$$

$$\frac{[MhuD]}{\alpha} = K_{d1}K_{d2} \quad (13)$$

Dividing equation (6) by α using equations (11), (12) and (13) results in equation (14):

$$\frac{[MhuD_T]}{\alpha} = [heme]^2 + K_{d2}[heme] + K_{d1}K_{d2} \quad (14)$$

Dividing equation (9) by α and substituting with equations (11) and (12) gives equation (15):

$$\frac{[heme_T]}{\alpha} = 2[heme]^2 + K_{d2}[heme] + \frac{[heme]}{\alpha} \quad (15)$$

Dividing equation (13) by equation (14) gives equation (16):

$$[MhuD] = \frac{[MhuD_T]K_{d1}K_{d2}}{[heme]^2 + K_{d2}[heme] + K_{d1}K_{d2}} \quad (16)$$

Dividing [*heme*] by α and using equation (10), we get equation (17):

$$\frac{[heme]}{\alpha} = \frac{[heme]^3}{[MhuD - diheme]} \quad (17)$$

Multiplying equation (4) and (5), we get equation (18):

$$K_{d1}K_{d2} = \frac{[heme]^2[MhuD]}{[MhuD - diheme]} \quad (18)$$

Rearranging equation (18) results in equation (19):

$$[MhuD - diheme] = \frac{[heme]^2[MhuD]}{K_{d1}K_{d2}} \quad (19)$$

Substitution of equation (19) into equation (17) gives equation (20):

$$\frac{[heme]}{\alpha} = \frac{[heme]K_{d1}K_{d2}}{[MhuD]} \quad (20)$$

Substitution of equation (16) into equation (20) gives equation (21):

$$\frac{[heme]}{\alpha} = \frac{[heme]^3 + [heme]^2K_{d2} + [heme]K_{d1}K_{d2}}{[MhuD_T]} \quad (21)$$

Substitution of equation (21) in equation (15), gives equation (22)

$$\frac{[heme_T]}{\alpha} = \frac{2[MhuD_T][heme]^2 + [MhuD_T][heme]K_{d2} + [heme]^3 + [heme]^2K_{d2}}{[MhuD_T]}$$

(22)

Dividing equation (22) by equation (14) and solving, gives cubic equation (23):

$$[heme]^3 + [heme]^2\left(2[MhuD_T] + K_{d2} - [heme_T]\right)$$

$$+ [heme]\left([MhuD_T]K_{d2} + K_{d1}K_{d2} - [heme_T]K_{d2}\right) - [heme_T]K_{d1}K_{d2} = 0 \quad (23)$$

The above equation (23) is in the form $x^3 + ax^2 + bx + c = 0$, where:

a = $2[MhuD_T] + K_{d2} - [heme_T]$

b = $\left([MhuD_T]K_{d2} + K_{d1}K_{d2} - [heme_T]K_{d2}\right)$

c = $-[heme_T]K_{d1}K_{d2}$

x = $[heme]$

Solving and simplifying the cubic equation for x gives three real roots which is given by equation (24.a), (24.b) and (24.c) :[6]

$$x = 2\sqrt{-Q}\cos\left(\frac{\emptyset}{3}\right) - \frac{a}{3} \quad (24.a)$$

$$x = 2\sqrt{-Q}\cos\left(\frac{\emptyset}{3} + 120^o\right) - \frac{a}{3} \quad (24.b)$$

$$x = 2\sqrt{-Q}\cos\left(\frac{\emptyset}{3} + 240^o\right) - \frac{a}{3} \qquad (24.b)$$

where,

$$Q = \frac{3b - a^2}{9} \qquad (25.a)$$

$$\emptyset = cos^{-1}\left(\frac{R}{\sqrt{-Q^3}}\right) \qquad (25.b)$$

$$R = \frac{9ab - 27c - 2a^3}{54} \qquad (25.c)$$

Equation (24.a) is the relevant solution of the cubic equation for this expreiment. Other solutions: (24.b) and (24.c) did not provide best fits for the experiment and hence would not be used to fit the data to calculate K_{d2}. Therefore, equation (24.a) will be used for rest of the analysis. Dividing equation (1) by α yields equation (26):

$$\frac{A_{410}}{\alpha} = \frac{\varepsilon_{MhuD-diheme}[MhuD - diheme]}{\alpha} + \frac{\varepsilon_{MhuD-heme}[MhuD - heme]}{\alpha} + \frac{\varepsilon_{heme}[heme]}{\alpha} \qquad (26)$$

Substituting the values from equation (11), (12) and (21) to equation (26) gives equation (27):

$$\frac{A_{410}}{\alpha} = \varepsilon_{MhuD-diheme}[heme]^2 + \varepsilon_{MhuD-heme}K_{d2}[heme] + \varepsilon_{heme}\left(\frac{[heme]^3 + }{}\right) \qquad (27)$$

Dividing equation (27) by equation (14) and solving for A_{410} gives equation (28):

$$A_{410} = \frac{[MhuD_T]\varepsilon_{MhuD-diheme}[heme]^2 + [MhuD_T]\varepsilon_{MhuD-heme}K_{d2}[heme]}{[heme]^2 + K_{d2}[heme] + K_{d1}K_{d2}} + \quad (28)$$

Equation (28) and equation (24.a) was rewritten in Graph Pad Prism 7.0 to fit the data collected from UV/Vis spectrophotometer and extract K_{d2} as equation (29):

$$A = 2 * P + Kd2 - X$$

$$B = (P * Kd2) + (Kd1 * Kd2) - (X * Kd2)$$

$$C = -(X * Kd1 * Kd2)$$

$$Q = ((3 * B) - (A * A))/9$$

$$R = ((9 * A * B) - (27 * C) - (2 * A * A * A))/54$$

$$T = arccos(\frac{R}{sqrt(-(Q * Q * Q))})$$

$$H = ((2 * (sqrt(-Q)) * cos(\frac{T}{3})) - (\frac{A}{3})$$

$$Y = \frac{(P * E * H * H) + (F * P * Kd2 * H) + (G * ((H * H * H) + (Kd2 * H *}{(H * H) + (Kd2 * H) + (Kd1 * Kd2)} \quad (29)$$

where, E, F and G are extinction coefficient of *MhuD–diheme, MhuD–heme* and *heme*, P is the total concentration of the protein. Abs data was used to plot abosrobance at 410 nm (A_{410}) as a function of total heme concentration ($heme_T$).

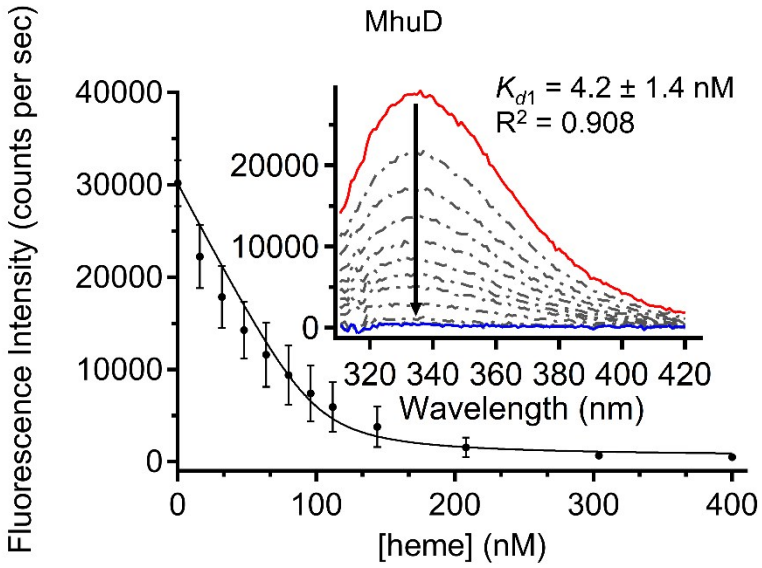

Figure S1. Fluorescence-detected titration of heme into 100 nM MhuD in 50 mM Tris pH 7.4, 150 mM NaCl. The error bars represent the standard deviation of three independent trials. The emission intensity was fit to equation (i) yielding a K_{d1} of 4.2 ± 1.4 nM. Inset: Emission spectra with 0 (solid red), 4 (solid blue), and intermediate (dashed gray) equivalents of heme

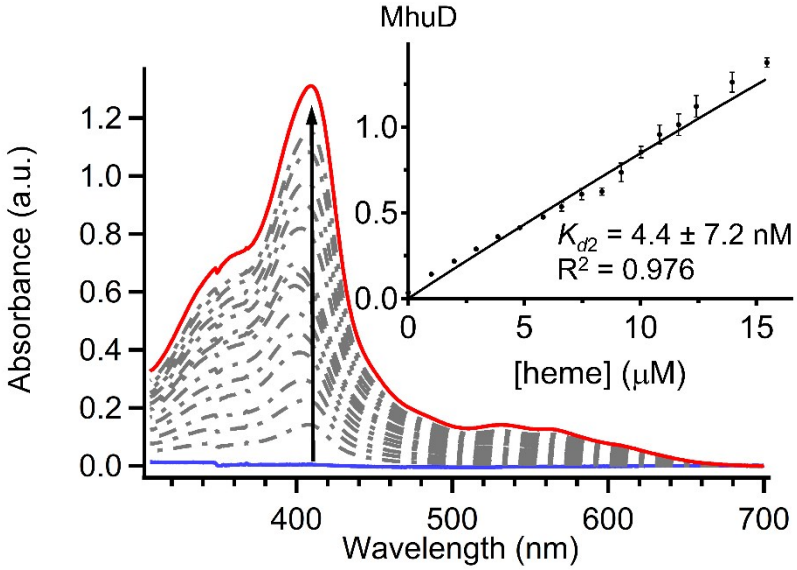

Figure S2. Abs-detected heme titration into 5 μM MhuD in 50 mM Tris pH 7.4, 150 mM NaCl. The spectra represent MhuD with 0 (solid blue), 3 (solid red), and intermediate (dashed gray) equivalents of heme. Inset: The error bars represent the standard deviation of three independent trials. The Abs-detected heme titration was fit to equation (ii) yielding a K_{d2} of 4.4 ± 7.2 nM.

Table S1: Soret band wavelength upon addition of 0.2, 1, 2 and 3 equivalents of heme to MhuD.

	Soret band, λ_{max} (nm)			
Heme Equivalents	**Trial 1**	**Trial 2**	**Trial 3**	**Average**
0.2	407	408	407	407
1	401	401	402	401
2	401	403	400	401
3	409	410	410	410

Table S2: Soret band wavelength upon addition of 0.2, 1, 2 and 3 equivalents of heme to $MhuD_{CH}$.

	Soret band, λ_{max} (nm)			
Heme Equivalents	**Trial 1**	**Trial 2**	**Trial 3**	**Average**
0.2	408	408	408	408
1	401	399	399	400
2	395	395	395	395
3	394	393	394	394

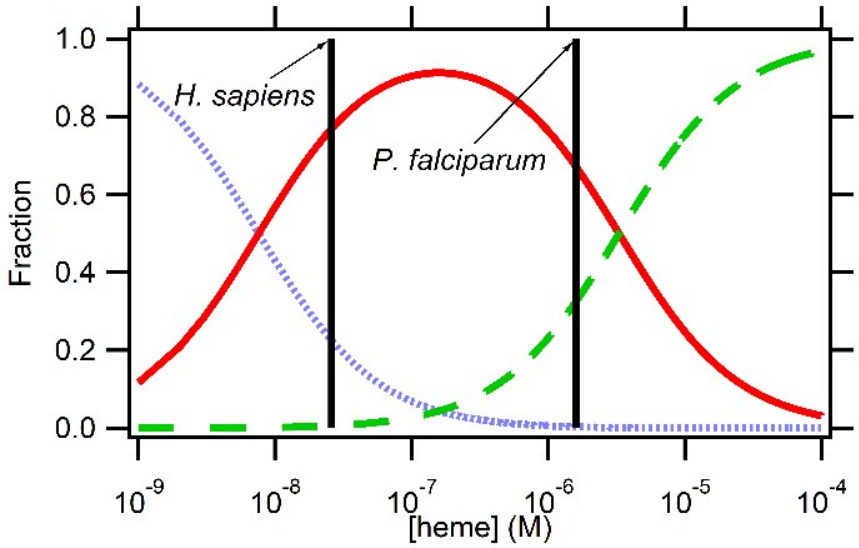

Figure S3. Fractionation of MhuD as a function of heme concentration. The curves represent the fraction of MhuD (dotted blue), MhuD–heme (solid red), and MhuD–diheme (dashed green) present for heme concentrations between 1 nM and 100 μM. Under typical conditions, the primary form of MhuD is MhuD–heme, but significant amounts of MhuD–diheme can be formed under heme replete conditions.

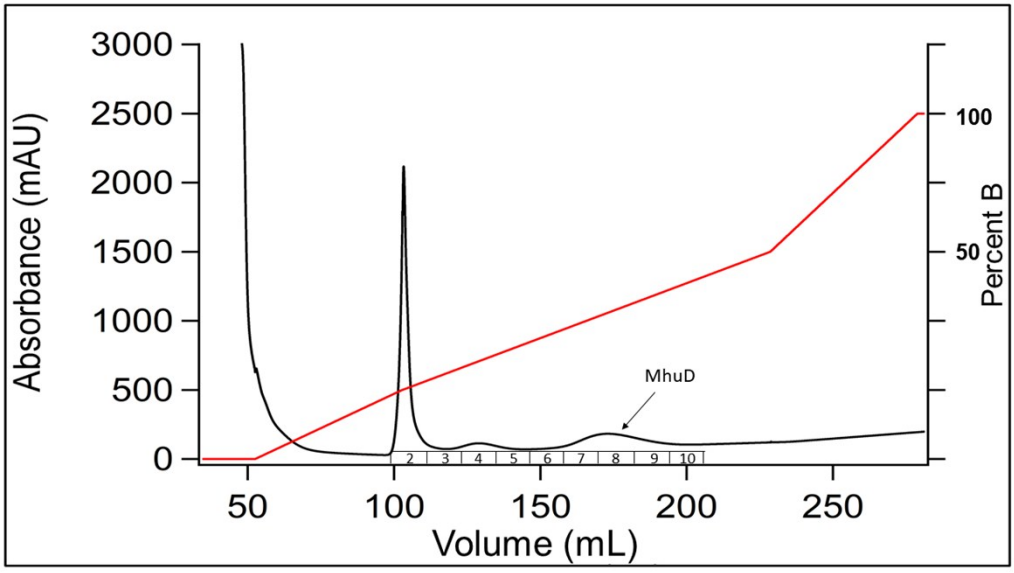

Figure S4: FPLC chromatograph for purification of MhuD. The 280 nm absorbance (*black trace*) and percentage of Buffer B (50 mM Tris, 350 mM NaCl, 500 mM Imidazole pH 7.8, *red trace*) are plotted as function of buffer run through the column. Numbers in boxes on top of the x-axis refer to fractions collected during FPLC.

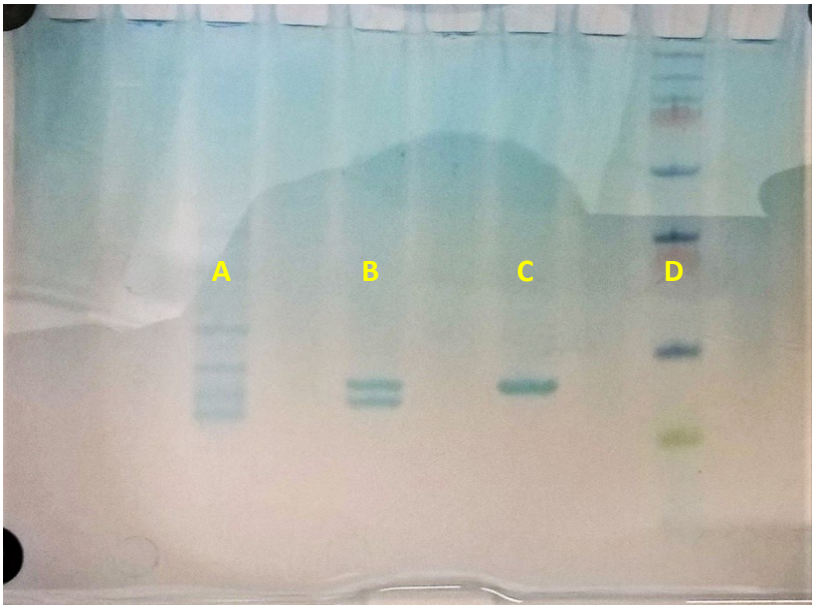

Figure S5: MhuD was obtained as assessed by SDS-PAGE gel electrophoresis. From left to right, the lanes correspond to: (A) FPLC fraction 2, (B) FPLC fraction 4, (C) FPLC fractions 7-9, and (D) PageRuler Plus prestained protein ladder (Pierce).

Table S3: $MhuD_{CH}$ gene sequence

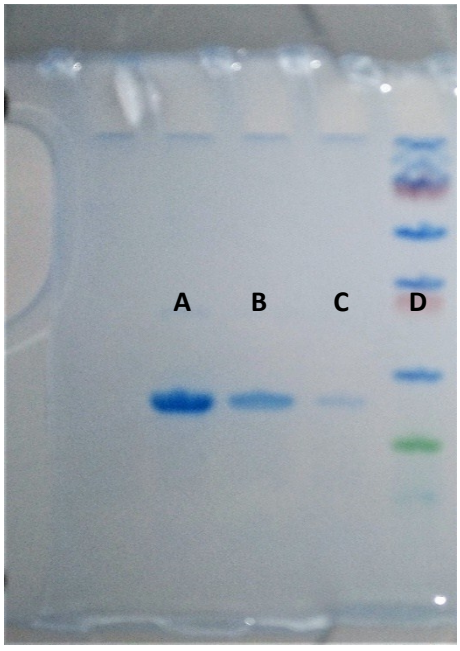

Figure S6: Purity of MhuD assessed by SDS-PAGE. (A) MhuD, (B) 1/10 dilution of MhuD, (C) 1/100, dilution of MhuD, and (D) PageRuler Plus prestained protein ladder (Pierce)

M-46	H-45	H-44	H-43	H-42	H-41	H-40	S-39	S-38	G-37	L-36	V-35
ATG	CAC	CAT	CAT	CAT	CAT	CAT	TCT	TCT	GGT	CTG	GTG
P-34	R-33	G-32	S-31	G-30	M-29	K-28	E-27	T-26	A-25	A-24	A-23
CCA	CGC	GGT	TCT	GGT	ATG	AAA	GAA	ACC	GCT	GCT	GCT
K-22	F-21	E-20	R-19	Q-18	H-17	M-16	D-15	S-14	P-13	D-12	L-11
AAA	TTC	GAA	CGC	CAG	CAC	ATG	GAC	AGC	CCA	GAT	CTG
G-10	T-9	D-8	D-7	D-6	D-5	K-4	A-3	M-2	A-1	P2	V3
GGT	ACC	GAC	GAC	GAC	GAC	AAG	GCC	ATG	GCC	CCA	GTG
V4	K5	I56	N7	A8	I9	E10	V11	P12	A13	G14	A15
GTG	AAG	ATC	AAC	GCA	ATC	GAG	GTG	CCC	GCC	GGC	GCT
G16	P17	E18	L19	E20	K21	R22	F23	A24	H25	R26	A27
GGC	CCC	GAG	CTG	GAG	AAG	CGG	TTC	GCT	CAC	CGC	GCG
H28	A29	V30	E31	N32	S33	P34	G35	F36	L37	G38	F39
CAC	GCG	GTC	GAG	AAC	TCC	CCG	GGT	TTC	CTC	GGC	TTT
Q40	L41	L42	R43	P44	V45	K46	G47	E48	E49	R50	Y51
CAG	CTG	TTA	CGT	CCG	GTC	AAG	GGT	GAA	GAA	CGC	TAC
F52	V53	V54	T55	H56	W57	E58	S59	D60	E61	A62	F63
TTC	GTG	GTG	ACA	CAC	TGG	GAG	TCC	GAT	GAA	GCA	TTC
Q64	A65	W66	A67	N68	G69	P70	A71	I72	A73	A74	H75
CAG	GCG	TGG	GCA	AAC	GGG	CCC	GCC	ATC	GCA	GCC	CAT
A76	G77	H78	R79	A80	N81	P82	V83	A84	T85	G86	A87
GCC	GGA	CAC	CGG	GCC	AAC	CCC	GTG	GCG	ACC	GGT	GCT
S88	L89	L90	E91	F92	E93	V94	V95	L96	D97	V98	G99
TCG	CTG	CTG	GAA	TTC	GAG	GTC	GTG	CTT	GAC	GTC	GGT
G100	T101	G102	K103	T104	A105						
GGG	ACC	GGC	AAG	ACT	GCA						

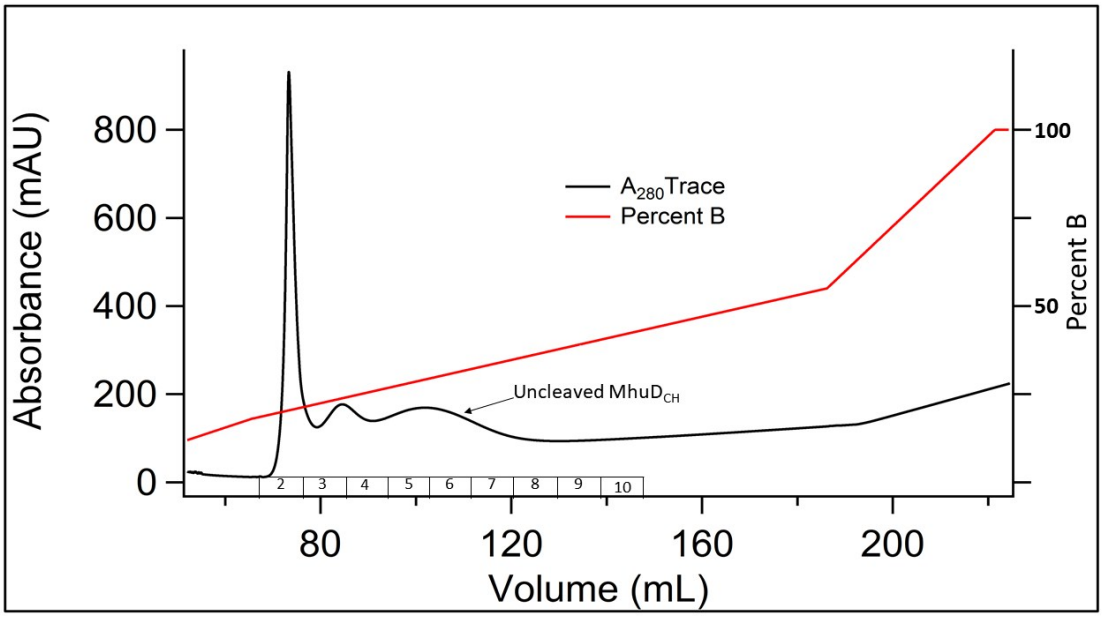

Figure S7: FPLC chromatograph for purification of uncleaved $MhuD_{CH}$. The 280 nm absorbance (*black trace*) and percentage of Buffer B (50 mM Tris, 350 mM NaCl, 500 mM Imidazole pH 7.8, *red trace*) are plotted as a function of buffer run through the column. Numbers in boxes on top of the x-axis refer to fractions collected during FPLC.

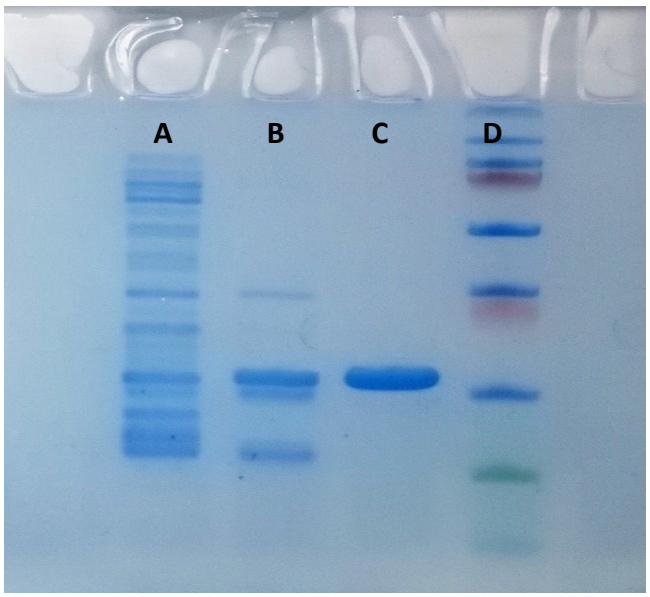

Figure S8: Uncleaved $MhuD_{CH}$ was obtained as assessed by SDS-PAGE gel electrophoresis. From left to right, the lanes represent: (A) FPLC fraction 2, (B) FPLC fraction 3, (C) FPLC fractions 5-7, and (D) PageRuler Plus prestained protein ladder (Pierce).

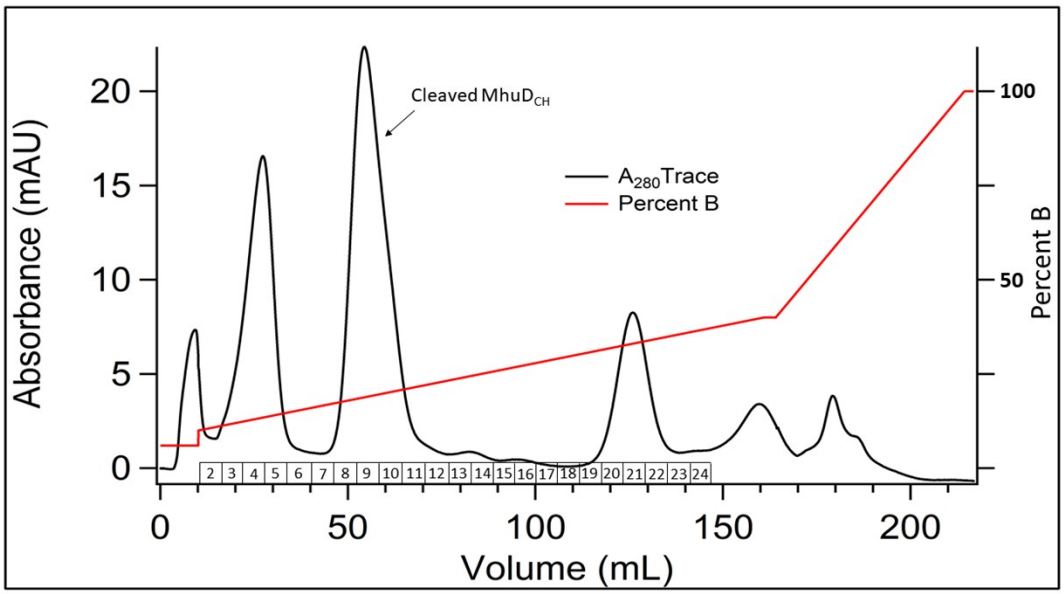

Figure S9: FPLC chromatograph for purification of cleaved MhuD$_{CH}$ (*Black Trace*). The 280 nm absorbance (black trace) and percentage buffer B (20 mM Tris, 500 mM NaCl pH 8.0, red trace). Numbers in boxes on top of the x-axis refer to fractions collected during FPLC.

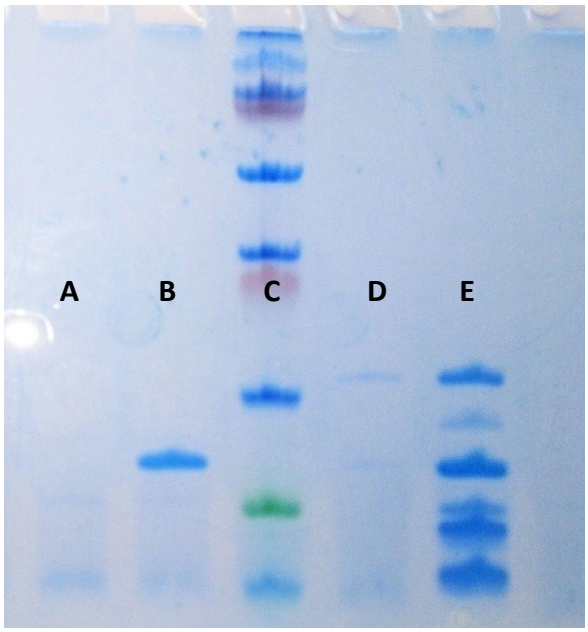

Figure S10: Cleaved MhuD$_{CH}$ was obtained as assessed by SDS-PAGE gel electrophoresis. From left to right, the lanes correspond to: (A) FPLC fractions 3-5, (B) FPLC fractions 8-11, (C) PageRuler Plus prestained protein ladder (Pierce), (D) FPLC fractions 20-22, and (E) MhuD$_{CH}$ enterokinase reaction mixture prior to anion-exchange chromatography.

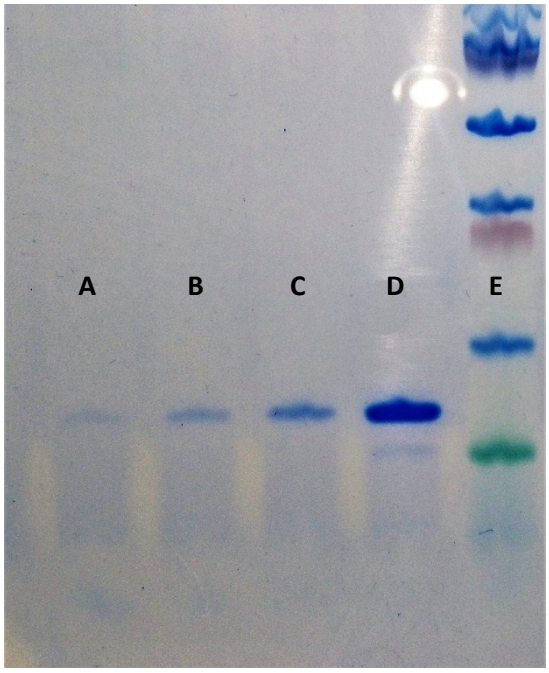

Figure S11: Purity of $MhuD_{CH}$ assessed by SDS-PAGE. (A) 1/100 dilultion of $MhuD_{CH}$, (B) 1/20 dilution of $MhuD_{CH}$, (C) 1/10, dilution of $MhuD_{CH}$, (D) Cleaved $MhuD_{CH}$, and (E) PageRuler Plus prestained protein ladder (Pierce)

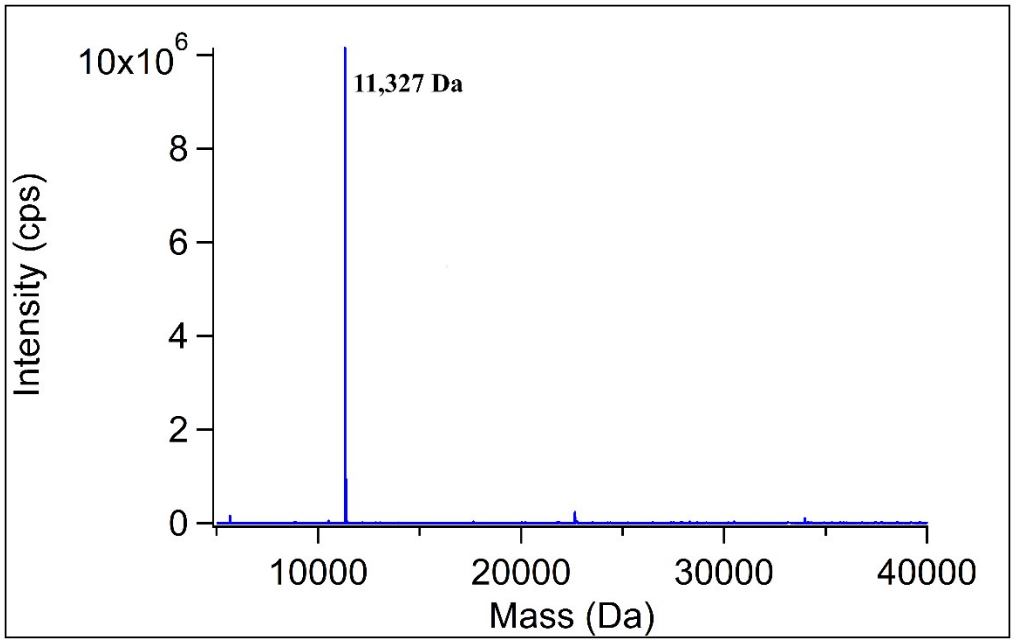

Figure S12: ESI-MS of 30 uM cleaved $MhuD_{CH}$ in 50 mM Tris pH 7.4, 150 mM NaCl. The observed molecular weight of 11,327 Da is in excellent aggrement with the expected molecular weight 11,327 Da.

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