

# **Heme binding by *Corynebacterium diphtheriae* HmuT: Function and heme environment**

Supporting Information

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Cd          -----MKSLLRAC---MSVVCACALVGCVGQGTVDSTK--DLRESLPKAGDVKDPRS -47
CU          -----MNKFVRVA---ASVACALSLSICGVQGSYDSTK--ELRESLPT--DVKDPRS
Cjk        MSIVLNRTVRLAFRTC---VLFICTASIAACGVKGAYESADAALRNDIKNAADLQDPRS
Cglut      -----MNNAFRRTLTSVVLAASLALTACASWDSPTASSNGDLIEEQASSTSTDPRT
Curea      -----MRTPPQRVCLPLYLAAALALSSCGAFTSGPTPTA----EQSQAASA EKAATA
           :. : : : : * . : : : :
           :. : : : : * . : : : :

Cd          FTGVSDVRDFDDVFPVSESVSPSL---PVHLTDADGFDVEVTDVSR I IALDIYGTYTKT -103
CU          FKGVSEVKNFDDVQPVADSVSPKL---FVKLTDADGHEVEVTDVSR I IALDIYGTYTKT
Cjk        FEGVSEVKDFTDVEPVTKHPSPKL---FVELTDADGHDVKNKLDRI LALDLYGTYTKT
Cglut      FTGLSIVEDIGDVPVTDNASPAL---FVSLTDADGNDVVVEDVSR I LPLDLYGTYSKT
Curea      RHGESSP----ASSPAGHVSARLPSRDPEVLVDKQTVESQSPARDAR I LTLDRAGALSRT
           * * : * * * * * : * * * * : * * : * *
           H136

Cd          LEGLGLADKIVGRTVSSTENVLKDV PVVTEGGHNINVEAVLSLHPSLLIVDHS IGPRDAI -163
CU          LEGLGLTKNIVGRTVSSTENALKDL PVVTEGGHTINVEAVLNLRPSLIVDHS IGPRDRI
Cjk        LTGLGLADRIVGRTVSSTENILADR PVVTEGGHNINVEAVLSLEPDI VIVDHS IGPRDAI
Cglut      IAGLGLVDNIVGRTVSSTEPALADTEVVTTGGHTLNAEA I LNLHPTLV I IDHS IGPREVI
Curea      VWALGMGENLIGRDTASDFPGVKDLPLVTPGGHSINAE T VLSLRPDI V L TDGS IGPSRVM
           : * * : * * * * * : * * * * * : * * * * * : * * * * * :

Cd          DQIRNAGVTTVMEPTRTIDSVAEDIKTLG SVVGLSDEASILAERSVHEI SAAREIAAAI -223
CU          DQIRAAAGVTTVMEPTRTIDSVAEDIK NLGGVVGLNDEAAKLAERSINEINSARETI KNI
Cjk        DQIRQAGVTTVMEPTRTIDSVAEDITTLGAVVGLPEDAEKLADRTVEEINLDKET I KKM
Cglut      DQIRAAAGVATVIMSPQRSIASIGDDIRDIASV VGLPEEGEKLAERSVAEVEEASTV VDEL
Curea      KKLRAATGVKVIDITAERTPETIGTLVEEVAAG I GLEQAADHVTEKINAKLDQASASAR--
           : * * * * : * : * : * : * : * : * : * : * : * : * : * : * : * :
           Y235

Cd          APSDFMRVAFLYARGNGGVFFIMGEGTGAKDLIEGVGAKDMGAEYKLS-YAEPANAEALA -282
CU          APKDFMRMAFLYARGNGGVFFIMGDGTGAKDLDEGLSAVDLAEHKLS-YAEPANAEALA
Cjk        VPSTFMRVAFLYARGNGGVFFIMGEGTGAKDLIEGVGAVDVGTENNLS-YIEPANAE SLA
Cglut      TPEDPLKMVFLYARGTGGVFFILGDAYGGRDLIEGLGGVDMAAEKGIM-DLAPANAEALA
Curea      SRADGRSMVLYVRGTG-VAMIAGPESGGRSLIERLGGTDAGVKLGDIGSFTPLTPEALI
           : * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * *
           M292

Cd          KINPEAIIMM TAGLESTGGIDG LLARPGVAQT IAGKNRRVITIPDGQSLAFGPMTGQTLL -342
CU          KINPEAIIMMSGGLESTGGIDG LLSRPGVAQT TAGKNRRVITIPDGQSLAFGPMTGQTLL
Cjk        RLNPDAFIMMTGGLESTGGIEGLLRPGIAQT TAGQKRRVITIPDGQSLAFGPMTGQTLL
Cglut      ELNPDV FVMSEGLVSTGGIDGLMERPGIAQT TAGQNQRV LALPDGQSLAFGAQTGELL
Curea      EAAPDTLIVMSSGLESVGGVDG LLKVPVSQT PAGKNRSVLDVPSSELLSFGPNTPGVID
           . * * : * * * * * : * * : * * * * : * * : * * : * * * * * :
           Y349

Cd          RTAQALYDPQV -353
CU          RTAQALYAPQT-
Cjk        RTAKALYDPHG-
Cglut      RASRELYVQGG-
Curea      AMAEALYGD---
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**Figure S1.** Alignment of the amino acid sequence of HmuT from various *Corynebacterium* species. Species are designated as follows: Cd: *C. diphtheriae* 1737/NCTC13129; CU: *C. ulcerans* 712; Cjk: *C. jeikeium* k411-jk0316; Cglut: *C. glutamicum* ATCC 13032; Curea: *C. urealyticum* DSM 7109. Conserved residues that were subjected to site-directed mutagenesis are indicated above the sequence alignment; asterisks indicate sequence identity and colons and periods show sequence similarity.

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PhuT      -----MRIDRLFNGLALGI----- 14
ShuT      -----MNRRLYFIYNSNDNHDSQFDKSSHIMPRIITRPFLFTPLTLCISAVAS 49
Yp-HmuT   -----MRLRLLSLPFILSL----- 14
Cd-HmuT   MKSLLRACMSVVCACALVGCQVQGTYDSTKDLRESLPAKGDVKDPRSFTGVSDVRDFDDV 60
IsdE      -----MRIIKYLTILVISVILTS 19
          :
          .

PhuT      -----LLGTGMAQAAELPQRWVSAG--GSLSEWVVALGGESKLVGVDITS 57
ShuT      -ASKSTVKRKKLFTAVLALSWAFSVTAAERIVVAG--GSLTELIYAMGAGERVVGVDETT 106
Yp-HmuT   -----CAPLLPLNTLAAERIVTIG--GDVTEIAYALGAGDEIVARDSTS 56
Cd-HmuT   RPFVSESVSPSLPFVHLTDADGFDVEVTDVSRIIALDIYGTYKTLLEGLGLADKIVGRIVSS 120
IsdE      -----CQSSSSQESTKSQGEFRIVPTTVALTMTLTKLDLP--IVGKPTSY 61
          .
          :
          :
          :
          :

PhuT      Q-HPQALKQLPSVYRQLAAEGVLALRPDILIGTEEMGPPFVLKQLEGAGVVRVETLS-A 115
ShuT      S-YPPETAKLPHIENKQLSSEGILSLRPDSVITWQDAGPQIVLDQLRAQKVNVTLPV 165
Yp-HmuT   Q-QPQAAQKLPDVFYRRTLNAEGILAMKPTMLLVSELAQPSVLVTQIASSGVNVTVP-G 114
Cd-HmuT   T-ENVLKDVPVVTGESHINVEAVLSLHPSLLIVDHSIGPRDAIDQIRNAGVITVVM-E-P 178
IsdE      KTLPNRYKDVPEIGQMPINVEAVKKLKPTHVLSVSTIKD---EMQPFYKQLNMKGIFYD 118
          *.: :.* :
          :

PhuT      KPDLLEALESNLKKLGDWLVGVPQRAEAAELDYRQLRRQADWIAAAQKSQPAPGVLLVIGN 175
ShuT      PATLEQMYANIRQLAKTLQVPEQGDALVTQINQRLERVQQNVAKKAP---VKAMFILSA 222
Yp-HmuT   QTTPEVSAMKINAVATALHQTEKGQKLIEDYQQR-----LAAVNKTPLPVKLVFVMSH 167
Cd-HmuT   TRTIDSVAEDIKTLGSVVGLSDEASILAERSVHEISAAREAIAAAPS DPMRVAFYLRG 238
IsdE      FDSLKGMQKSIITQLGDQFNRKAQAKELNHDHLSNVKQKIENKAAKQKKHP---KVLILMGV 175
          . : .: .: .
          . . .
          *
          :

PhuT      AGGQLLVAGRNTGGDWLVNRAGARNLAT---HEGYKPI SVEALAALDFVAVVIADRSLEG 232
ShuT      GGSAPQVAGKGSVADAILSLAGAEENVAT---HQQYKSYSAESLIAANPEVIVVISQMDVG 279
Yp-HmuT   GGLTPMAAGQNTAADAMIRAAAGGSNAMQG--FSRYRPLSQEGVIASAPDLLLITTDGVKA 225
Cd-HmuT   NGGVFFIMGEGTGAKDLIEGVGAKDMGAEYKLSYAE PANAEALAKINPEAIIIMAGLES 298
IsdE      PG-SYLVATDKSYIGDLVKIAGGENVIKVK-DRQYISSNTENLLNINPDIILRLPHMPE 233
          * : : : .*. :
          . . * : * : :

PhuT      DAARAAL--LKQNPGLAATRAARDGRLLVLDPTLLVGGGLPRLPDGLAALSAAFYPSAKP 290
ShuT      DINR----LRSIAGITHTAAWKNQRIITVDQNLILG-MGPRIADVVESLHQQLWPFQ--- 330
Yp-HmuT   LGSSEN---IWKLPGMALT PAGKHKRLLVDDMALLG-FGLETPQVLAQLREKMEQMQ-- 279
Cd-HmuT   TGGIDG---LLARPGVAQTIAGKNRRVITIPDGQSLA-FGPMTGQTLRLTAQALYDPQV- 353
IsdE      EVKRMFQKEFKQNDIWKHFKAVKNNHVYDLEEVFPGITANVDADKAMTQLYDLFYKDKK- 292
          :
          * : .: : :
          . . : :

PhuT      LSTEAAH 297
ShuT      -----
Yp-HmuT   -----
Cd-HmuT   -----
IsdE      -----

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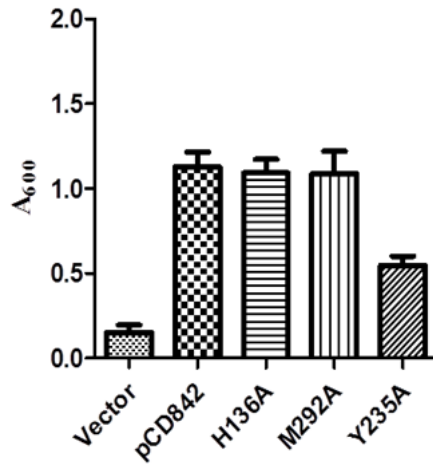
PhuT: Y71  
ShuT: Y67

IsdE:  
M78, H229

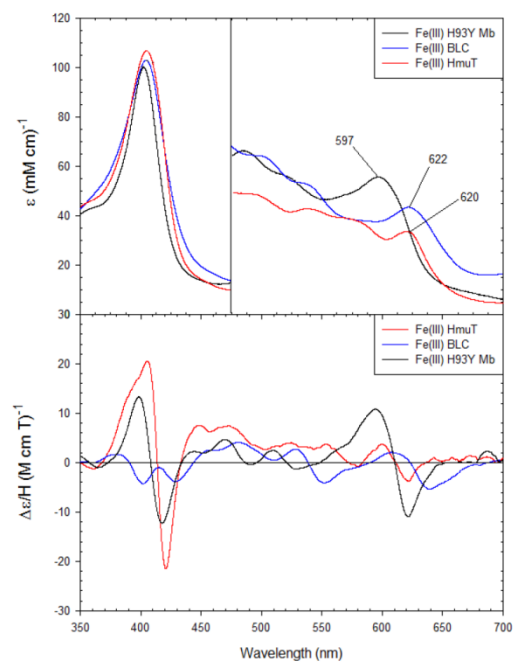
YpHmuT:  
Y70, H167

CdHmuT:  
H136, Y235

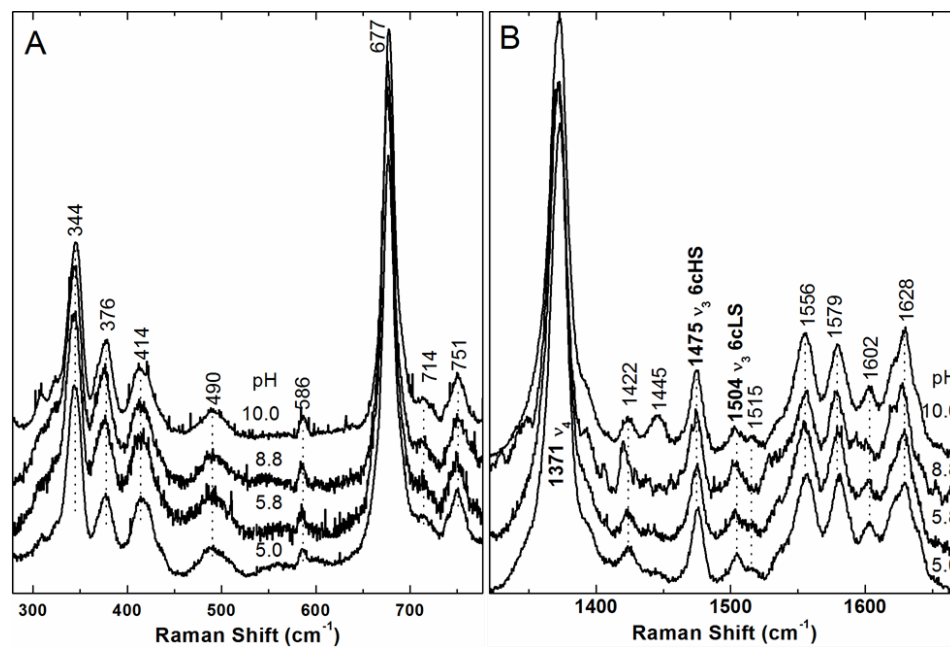
**Figure S2.** Alignment of the amino acid sequence of CdHmuT with four HBPs with known crystal structures. Square boxes indicate the known axial ligands. Orange: *P. aeruginosa* PhuT (1) and *S. dysenteriae* ShuT (1). Green: *S. aureus* IsdE (2). Blue: *Y. pestis* HmuT (3). Red: *C. diphtheriae* HmuT. For CdHmuT, M292 is also shown.



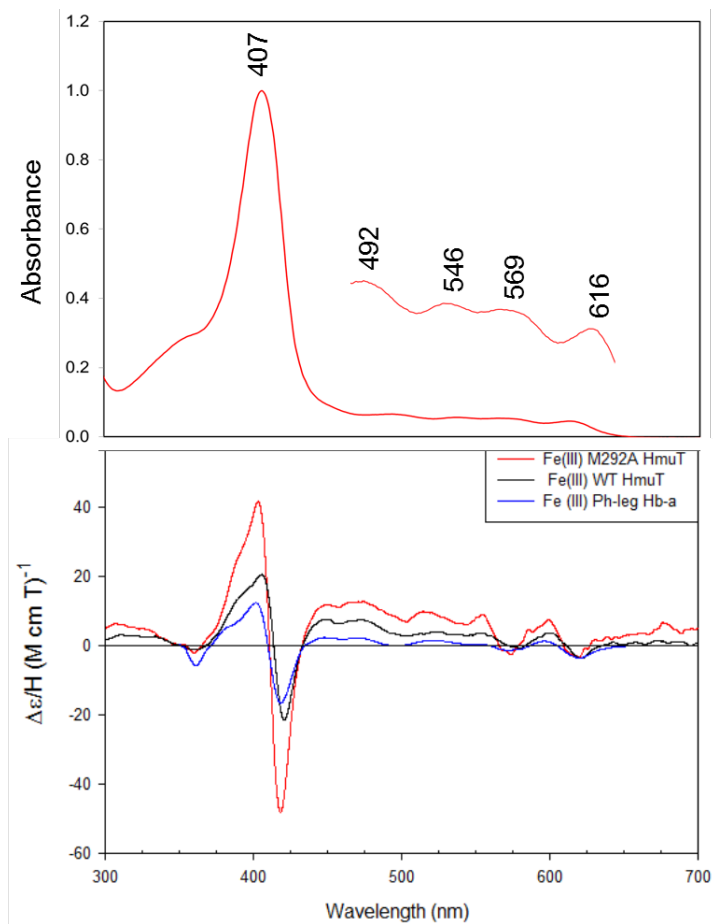
**Figure S3.** Hb-iron utilization assay. *C. ulcerans* CU77 (*hmuT*) carrying plasmids that encode the wild type (pCD842) and various mutants of the *hmuT* gene were assessed for their ability to use Hb as the sole iron source for growth in low-iron mPGT medium. Cultures were grown for 36 hours at 37 °C in the presence of 25 µg/ml Hb supplemented with 10 µM EDDA, and then cell density was measured by absorbance at A<sub>600</sub>. Results are the mean of three independent experiments ± standard deviation. The growth difference between WT (pCD842) and Y235A is significant at p < 0.01.



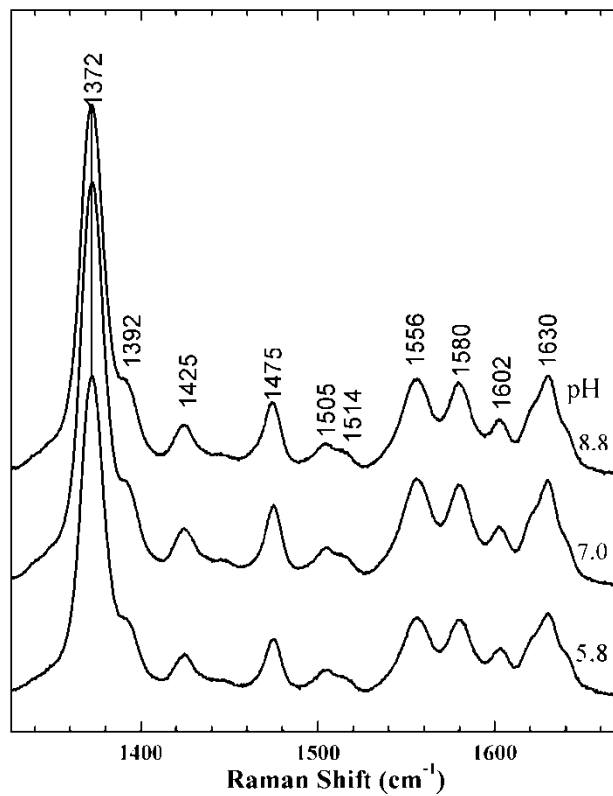
**Figure S4.** Comparison of the UV-visible absorption and MCD spectra for Fe(III) WT *CdHmuT* at pH 6.5 with Fe(III) bovine liver catalase (BLC) and H93Y myoglobin. The samples were taken in 50 mM phosphate buffer. Spectra were slightly dependent on buffer conditions. The spectra of BLC and H93Y myoglobin were replotted from (4-6) and (7), respectively.



**Figure S5.** The rR spectrum of ferric WT *CdHmuT* as a function of pH. A) Low frequency window. B) High frequency window. Protein concentration was 40  $\mu\text{M}$ ; excitation frequency of 413.1 nm was used with 9.4 mW laser power at the sample. The pH values are as indicated with the buffers described in the experimental section.

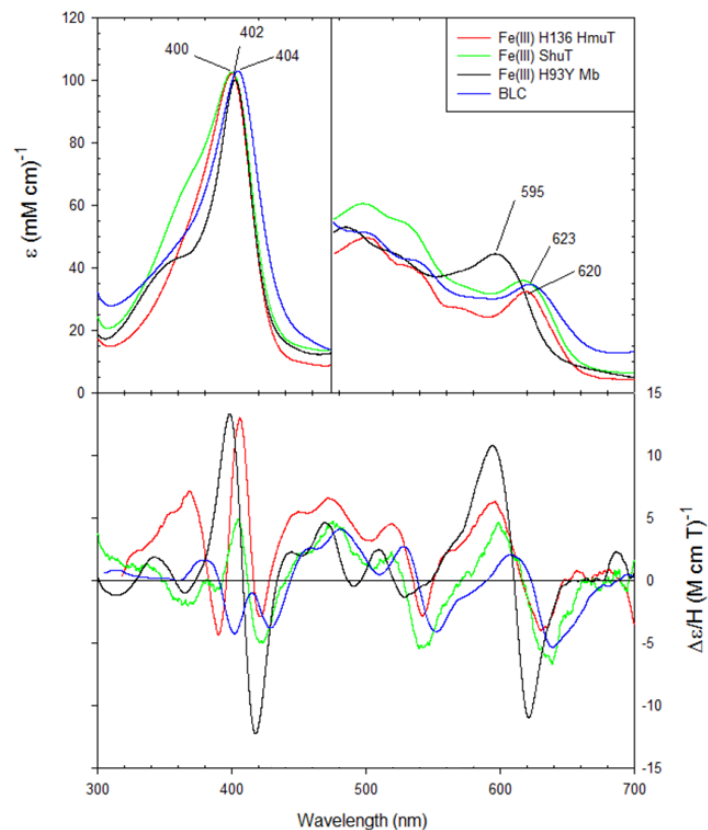


**Figure S6.** Top panel: UV-visible spectrum of M292A *Cd*HmuT. The sample was taken in 50 mM Tris-Cl, pH 7.0. Bottom panel: Comparison of the MCD spectra for Fe(III) M292A *Cd*HmuT at pH 6.5 with Fe(III) WT *Cd*HmuT and Fe(III) phenol-leg Hb *a*. The samples were taken in 50 mM phosphate buffer. The spectrum of phenol-leg Hb *a* was replotted from (8).

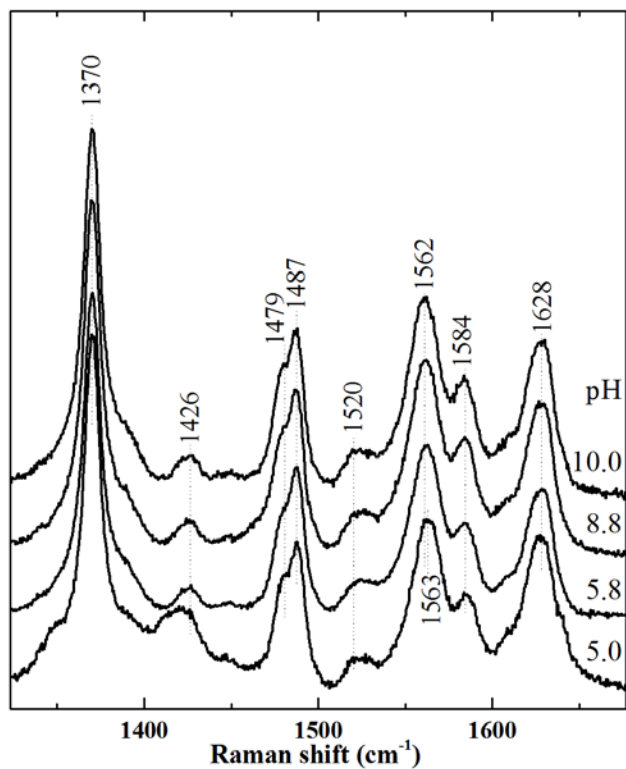


**Figure S7.** The rR spectrum of ferric M292A as a function of pH. Protein concentration was 36  $\mu\text{M}$ ; 406.7-nm excitation with 11 mW at the sample was used.

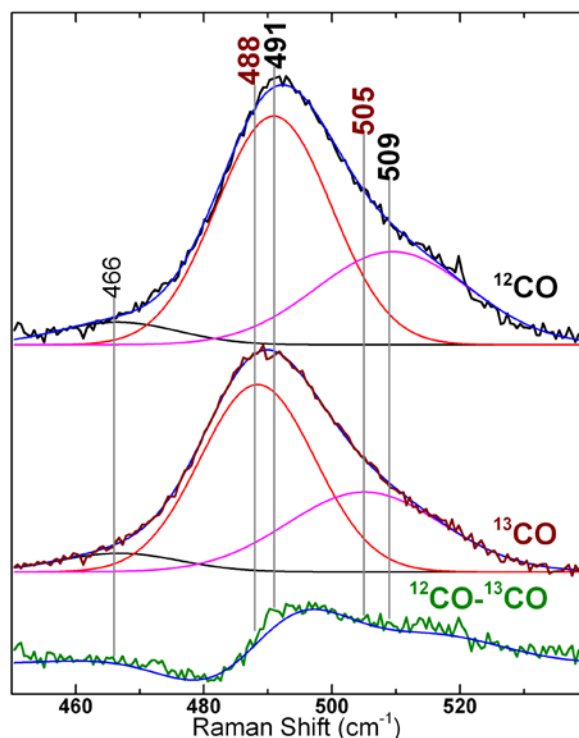




**Figure S8.** The UV-visible and MCD comparison spectra for Fe(III) H136A *CdHmuT* at pH 6.5. Bottom panel: Comparison of the MCD spectra for Fe(III) H136A *CdHmuT* with Fe(III) WT *CdHmuT*, Fe(III) *ShuT*, Fe(III) H93Y Mb, and Fe(III) BLC. All samples in the work were taken in 50 mM phosphate buffer. Spectra of H93Y, *ShuT*, and BLC were replotted from (7),(9), and (4-6), respectively.



**Figure S9.** The rR spectrum of ferric H136A as a function of pH. Protein concentration was 25  $\mu$ M; 406.7-nm excitation with 11 mW at the sample was used.



**Figure S10.** The Fe–C stretching region of the Y235A-CO rR spectrum. The experimental data for the natural abundance CO (black) and  $^{13}\text{C}$  (burgundy) complexes are shown with the peak fitting analysis of the 509/505 (magenta) and 491/488  $\text{cm}^{-1}$  bands (red). Band widths are 24 and 18  $\text{cm}^{-1}$ , respectively. The 466  $\text{cm}^{-1}$  band is not  $^{13}\text{C}$  sensitive. The simulated spectra are shown in blue; they are the sums of the fit peaks. The difference spectrum, obtained by subtraction of  $^{13}\text{C}$  spectrum from the natural abundance CO spectrum, is shown in green. The simulated  $^{12}\text{C}^{13}\text{C}$  difference spectrum (blue) is the difference between the simulated spectra for the  $^{12}\text{C}$  and  $^{13}\text{C}$  complexes.

**Table S1.**  $pK_a$  values of water *trans* to histidine in selected ferric heme proteins. The  $pK_a$  of ferrous microperoxidase 8 is reported as 10.9 (10).

<b>Class</b>	<b>Protein</b>	<b>Fe(III)</b>	<b>Reference</b>
CCOx	Cytochrome <i>c</i> oxidase	9.0	(11)
CID	GR-1 chlorite dismutase	8.2	(12)
CID	<i>Ideonella dechloratans</i> chlorite dismutase	8.5	(13)
CID	<i>Dechloromonas aromatica</i> chlorite dismutase	8.7	(14)
FixL	<i>Rhizobium meliloti</i> FixL	9.3	(15)
FixL	<i>Bradyrhizobium japonicum</i> FixL	9.3	(15)
FixL	<i>Rhizobium meliloti</i> FixL	10	(15)
Hb	Leghemoglobin	8.3	(16)
Hb	Hemoglobin I (clam)	9.6	(17)
H-NOX	<i>Thermoanaerobacter tengcongensis</i> H-NOX	6.8	(18)
H-NOX	<i>Thermoanaerobacter tengcongensis</i> H-NOX I5L	7.9	(18)
H-NOX	<i>Thermoanaerobacter tengcongensis</i> H-NOX I5L/P115A	>10	(18)
H-NOX	<i>Thermoanaerobacter tengcongensis</i> H-NOX P115A	>10	(18)
HO	Heme oxygenase	7.6	(19;20)
HO	Mammalian HO-1	7.6	(20)
HO	Rat heme oxygenase-1	7.6	(20)
HO	<i>Pseudomonas aeruginosa</i> heme oxygenase	8.3	(21)
HO	Mammalian HO-2	8.5	(22)
HO	Bacterial heme oxygenase HmuO	9.0	(23)
HO	<i>Neisseriae meningitidis</i> heme oxygenase	9.3	(24)
HRP	Horseradish peroxidase	10.9	(25) (26)
IsdI	<i>Staphylococcus aureus</i> IsdI	7.1	(27)
Mb	Porcine myoglobin H64V/V68H/H93A/H97F	7.17	(28)
Mb	Aplysia myoglobin	7.6	(25)
Mb	Porcine myoglobin H64V/V68H/H93G/H97F	7.74	(28)
Mb	<i>Dolabella auricularia</i> myoglobin	7.8	(29)
Mb	Sperm whale myoglobin	8.95	(25)
MP	Microperoxidase 8	9.6	(10;30)

**Table S2.** Selected His/Tyr and Tyr heme-binding proteins with corresponding residues which are hydrogen-bonded to the axial tyrosine ligand.

<b>Protein</b>	<b>Axial Ligation</b>	<b>Residue Hydrogen Bonding to the Axial Tyrosine</b>	<b>Motif<sup>a</sup></b>	<b>Reference</b>
<i>S. aureus</i> IsdA	Y166	Y170	<b>YxxxY</b>	(31)
<i>S. aureus</i> IsdB-N2	Y440	Y444	<b>YxxxY</b>	(32)
<i>S. aureus</i> IsdC	Y132	Y136	<b>YxxxY</b>	(33)
<i>S. aureus</i> IsdH-N3	Y642	Y646	<b>YxxxY</b>	(34)
<i>B. anthracis</i> IsdX1	Y136	Y140	<b>YxxxY</b>	(35)
<i>B. anthracis</i> IsdX2-N5	Y108	Y112	<b>YxxxY</b>	(36)
<i>P. aeruginosa</i> HasA	H32/Y75	H83	<b>YxxxxxxxxH</b>	(37)
<i>S. marcesans</i> HasA	H32/Y75	H83	<b>YxxxxxxxxH</b>	(38)
<i>Y. pestis</i> HasA	Y75	H81	<b>YxxxxxH</b>	(39)
<i>M. tuberculosis</i> Rv0203	Y59/ H89	H63	<b>YxxxH</b>	(40)
<i>Y. pestis</i> HmuT	Y70/H167	R72 <sup>b</sup>	<b>YxR</b>	(3)
<i>P. aeruginosa</i> PhuT	Y71	R73	<b>YxR</b>	(1)
<i>S. dysenteriae</i> ShuT	Y67	K69 <sup>b</sup>	<b>YxK</b>	(1)
<i>P. homomalla</i> cAOS	Y353	R349	<b>RxxxY</b>	(6)
Human catalase	Y358	R354	<b>RxxxY</b>	(41)
<i>M. avium</i> ssp. <i>paratuberculosis</i> MAP	Y294	R290	<b>RxxxY</b>	(6)

<sup>a</sup> Residues in bold represent the amino acid hydrogen bonded to the axial ligand.

<sup>b</sup> Predicted that the residue could hydrogen bond the axial ligand, but is not directly observed in the crystal structure.

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