Novel histidine-heme covalent linkage in a hemoglobin
Supporting Information

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Materials: All chemicals were purchased from Sigma Chemical Company, St. Louis, MO and endoproteinases from Promega Corporation, Madison, WI**.** Fe(III)-2,4 dimethyl-deuteroporphyrin IX was purchased from Porphyrin Products (Logan, UT) and Zn(II)-protoporphyrin IX was purchased from Mid-Century Chemicals (Posen, IL).

Protein expression and purification: Synechocystis sp. PCC 6803 rHb was prepared from inclusion bodies as reported previously.[1] The holoprotein (rHb-R) was formed by addition of hemin chloride in NaOH (10 mg per 200 μL), followed by chromatography using DEAE Sephacel anion exchange media to remove excess hemin. The protein was eluted using 0.2 M NaCl in 50 mM Tris, 1 mM EDTA buffer, pH 8, dialyzed versus ddH_2O, and lyophilized. The nature of the resulting material was ascertained by NMR spectroscopy prior to any reaction.

Fe(III) 2,4-dimethyldeuteroporphyrin S6803 rHb was prepared by the same procedure as described for the wild-type protein.[1] Zn-substituted S6803 rHb-R was prepared in the dark by adding 1.5 equivalents of Zn-PPIX (10 mg per 200 μL NaOH) to an apoprotein solution in 20 mM phosphate buffer, pH 7.2. The excess porphyrin was removed by size exclusion chromatography on a 0.7 × 5 cm Sephadex G25 column.

Controlled conversion to the alternate form (rHb-A) was achieved by addition of dithionite (Sigma, used without further purification) in 20 mM phosphate buffer, pH 7.2, in 1.5–fold excess to a phosphate (pH 7.2) buffered holoprotein solution. The reduced sample was run through a 0.7 × 5 cm Sephadex G25 desalting column immediately after reduction. The eluant was collected and subjected to mass spectrometry and NMR analysis.

Mass spectrometry: Digestion of rHb-A and rHb-R samples was carried out by adding 8 μL porcine gastric pepsin in 1% formic acid (1 mg/mL) to 10 μL of a 10 μM protein sample followed by incubation at 37 ºC for 24 hours. An additional 20 μL of 0.15 % formic acid was added to each sample before analysis by mass spectrometry.

Protein samples were analyzed on a Perseptive Biosystems Mariner mass spectrometer (Framingham, MA), using electrospray ionization in positive ion mode, equipped with a Hewlett-Packard model 1100 HPLC (Palo Alto, CA). Prior to ionization, peptide fragments were separated by reversed phase chromatography with a BetaBasic C-4 column (1.0 mm I.D. x 50 mm length, 3 μm packing, Keystone Scientific, Bellefonte, PA) at 25 ºC using a multi-step gradient over 45 minutes (solvent A, H_2O + 0.15% formic acid; solvent B, acetonitrile + 0.15% formic acid; solvent C, 2-propanol + 0.15% formic acid). The gradient was applied as follows: 0-30 minutes 95% A-5%B (0.5 mL/min), then changed to 5%A-95%B (0.5 mL/min) from 30-35 minutes and held from 35-40 minutes, then changed to 10%A-90%C (0.3 mL/min) from 40-45 minutes. HPLC-UV was achieved by coupling a Hewlett Packard 1100 series diode array detector to the HPLC instrument. Tandem mass spectrometry analyses were performed on a Quattro-II mass spectrometer (Micromass, Manchester, UK) interfaced to a LC-10ADvp HPLC (Shimadzu Instruments, Columbia, MD) using electrospray ionization, with collision induced dissociation effected using argon as collision gas (2 x 10-3 mbar) and 30 V offset of the collision cell.

Optical spectroscopy: Electronic absorption spectra were collected at 25 °C on an Aviv model 14 DS spectrophotometer.

NMR spectroscopy: All NMR spectra were collected at 600 MHz on a Bruker DRX spectrometer as described previously.[1-3] The ^{1}H-^{15}N HMQC data[4] were collected at 298K and 305K with the following parameters: ^{15}N, 9505.5 Hz, 100*; ^{1}H, 7002.8 Hz, 4096*. The ^{15}N carrier was at 210 ppm or 190 ppm. The HMQC delay was 22 ms to detect small $^2J_{NH}$ and $^3J_{NH}$ effects. A value of 15 ms was used in Figure 1

of the main text to emphasize possible coupling to the rapidly relaxing protons bound to the modified heme 2Cα and 2Cβ. Data were processed with squared sine bell of 90° in both dimensions. The water line was used as a reference for the ^{1}H chemical shifts with correction for temperature and the ^{15}N chemical shifts were referenced indirectly.[5] NOESY and TOCSY mixing times were 100 ms and 45 ms, respectively.

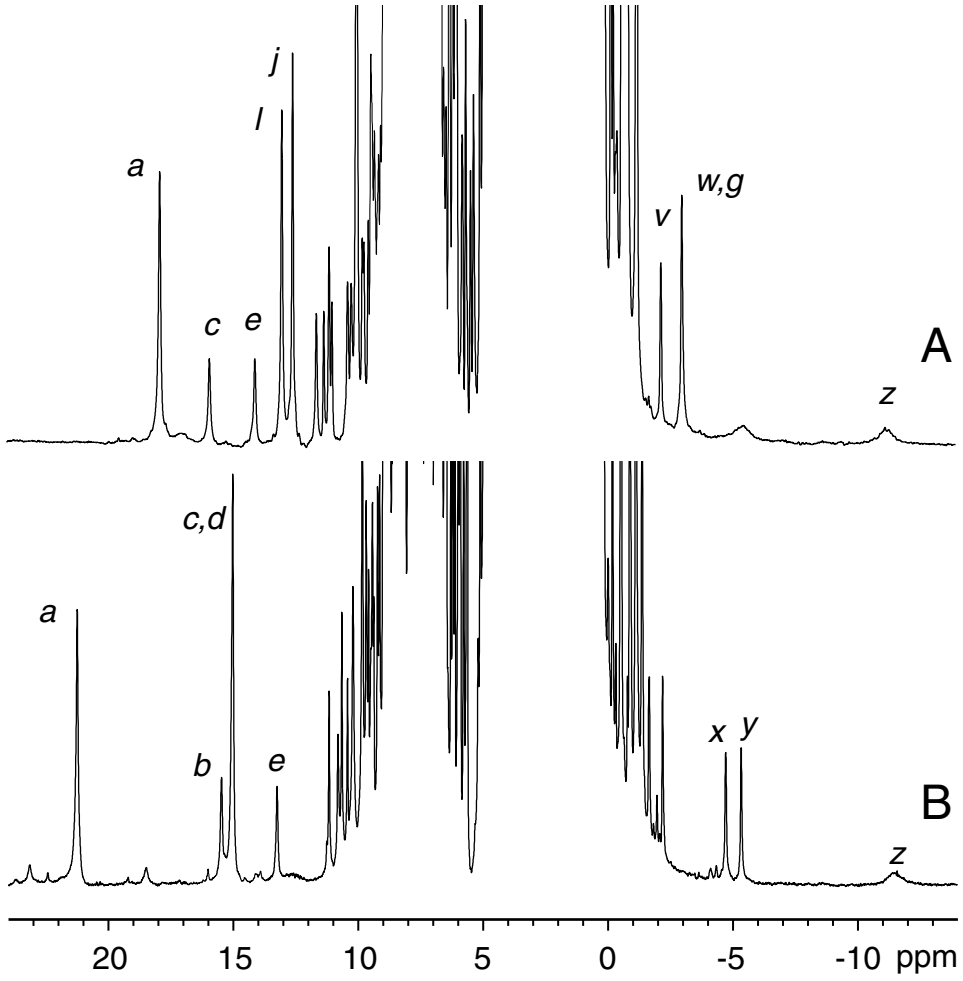

FIG. S1. 600 MHz ^{1}H NMR spectrum of S6803 hemoglobin in the ferric state at 25 °C. (A) rHb-A (~1 mM heme) in 95% H_2O / 5% $^{2}H_2O$, buffered at pH 7.3 with 20 mM phosphate; (B) rHb-R under comparable conditions. Both holoproteins exhibit narrow chemical shift dispersion and sharp lines typical of low-spin ferric heme proteins. In S6803 rHb-R (trace B), resolved methyl groups are labeled *a* (5-CH_3) and *d* (1-CH_3); the 2-vinyl signals appear in the –5 ppm region (*x* and *y*) and at 15.5 ppm (*b*). Small peaks arise from the minor heme isomer.[1] In rHb-A (trace A), the heme resonances have moved to new positions; resolved methyl groups are labeled *a* (5-CH_3), *l* (3-CH_3) and *j* (8-CH_3). The 4-β-vinyl resonances are labeled *v* and *w*. The spectra also contain the NδH resonances of His70 (*c*) and His46 (*e*). These protons gave rise to cross peaks in ^{1}H-^{15}N HSQC spectra and participated in a network of NOEs involving the CβHs, CαH and backbone NH of these histidines. Tables S1 and S2 list the chemical shifts.

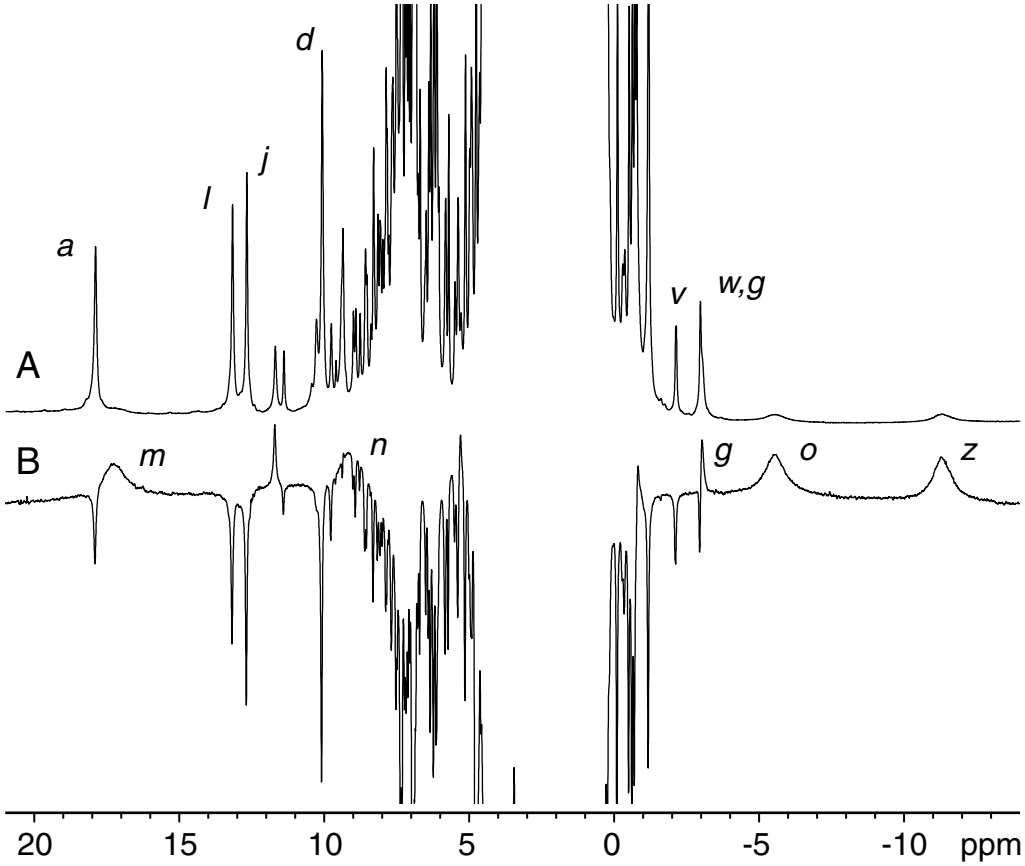

FIG. S2. ^{1}H WEFT NMR spectrum of ferric S6803 rHb-A. Data were collected at 600 MHz at a protein concentration of ~ 1mM on a per-heme basis. The solvent was $^{2}H_2O$, buffered at pH* 7.5 with 20 mM phosphate; the probe temperature was 25 °C. (A) Reference spectrum; (B) WEFT data with recovery delay of 55 ms and a total recycling time of 230 ms. Four broad resonances are observed: *m* (17.2 ppm), *n* (9.1 ppm), *o* (–5.6 ppm), and *z* (–11.3 ppm); these signals are tentatively assigned to the CδH and CεH of the two axial histidines as in rHb-R. The peak labeled *g* was identified as the γ meso proton through NOEs to the heme propionates.

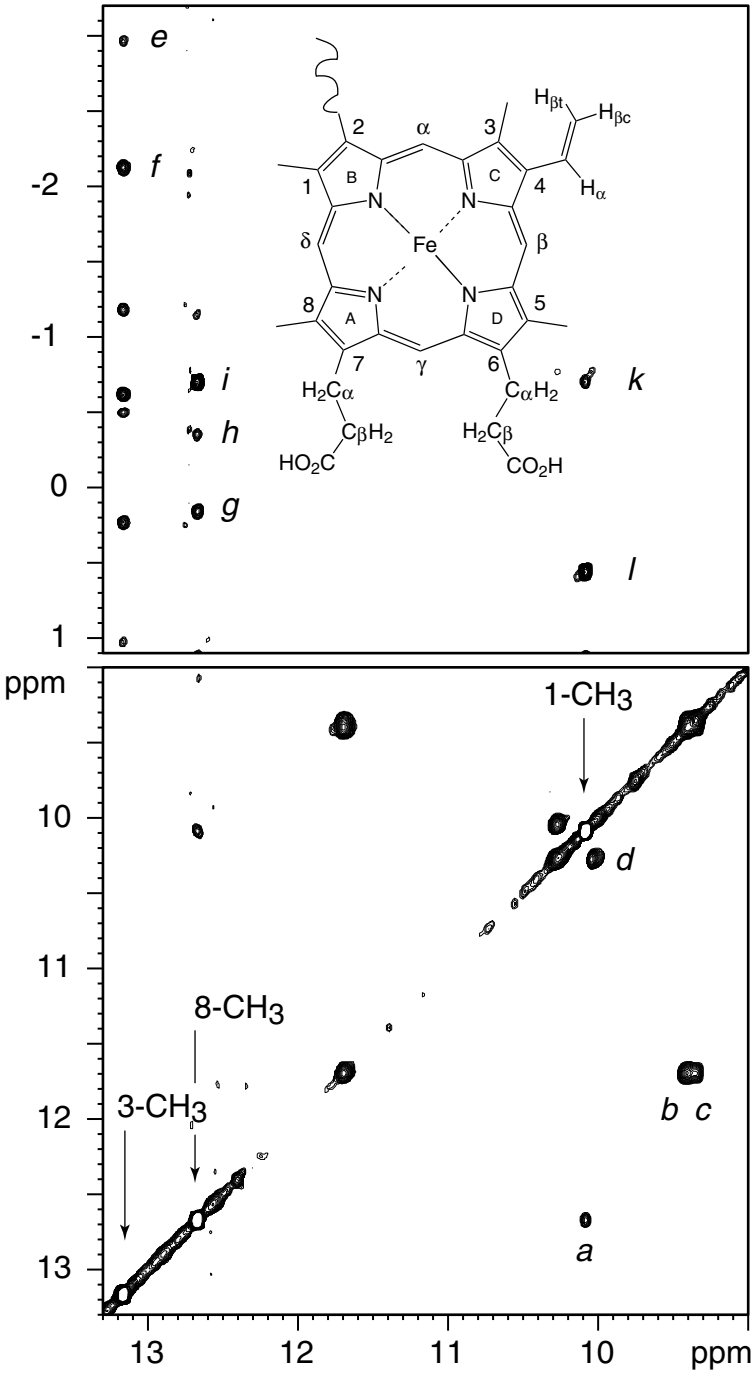

FIG. S3. Portion of the ^{1}H-^{1}H NOESY data for S6803 rHb-A in the ferric state. Conditions were as for Fig. S2. Key connectivities were identified in these regions: 8-CH_3 to 1-CH_3 (cross peak *a*), 3-CH_3 to 4-β-vinyl (*e*, *f*), 8-CH_3 to 7-β, propionates (*g*, *h*) and Met66 CεH_3 to 8-CH_3 (*i*) and to 1-CH_3 (*k*). Peaks *b* and *c* arise from His46 CαH–CβH_2 and *d* arises from His70 CβH_2. Chemical shifts are listed in Tables S1 and S2. Peak *l* connects the 1-CH_3 to the modified 2-vinyl group. The NOEs observed between the 4-vinyl and the protein in rHb-R (e.g., to Val87 and Phe34) were also present in rHb-A. Numerous other NOEs between the protein and the heme group (not shown) matched those observed in rHb-R and were consistent with a conserved orientation of the porphyrin ring within its cavity.

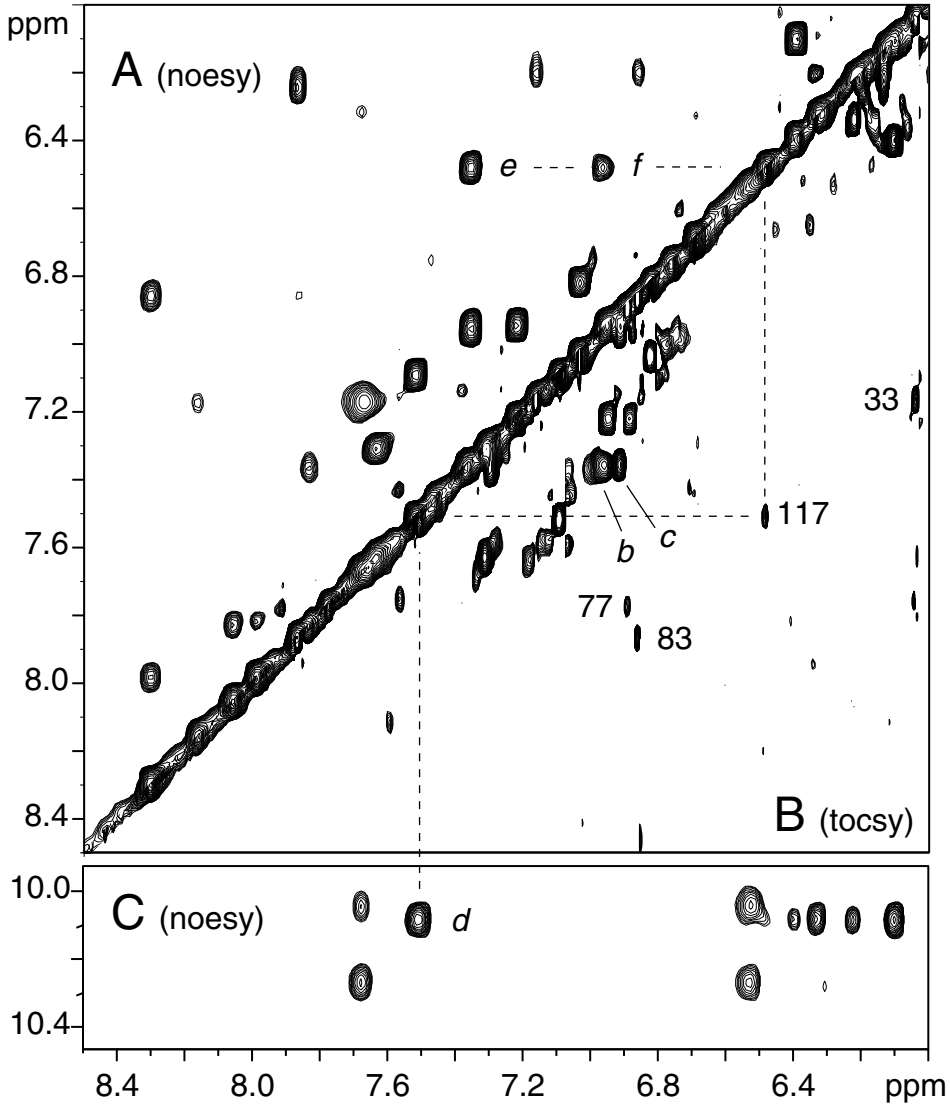

FIG. S4. Portions of the ^{1}H-^{1}H NOESY and TOCSY data for S6803 rHb-A in the ferric state. Conditions were as in Fig. S1. The upper section (A) is from a NOESY data set the lower section (B) is from a TOCSY dataset. The bottom panel (C) is from the same NOESY as (A). Panel B (TOCSY) contains weak CδH-CεH connectivities for each of the non-axial histidines, marked by their residue number. Peaks *b* and *c* arise from the ring of Phe84, assigned by analogy to rHb-R[3] and with NOEs to adjacent residues. Panel C (NOESY) shows that the CεH of His117 is in dipolar contact with the heme 1-CH_3 (cross peak *d*); Panel A (NOESY) also shows that the CδH of His117 (at 6.49 ppm) is in contact with the ring of Phe84 (cross peaks *e* and *f*). The His117/heme and His117/Phe84 interactions depicted here are not detected in rHb-R.

Table S1. *^{1}H NMR chemical shifts for heme resonances in the spectrum of ferric S6803 rHb-A*

Assignment	Signal	δ (ppm) rHb-A	δ (ppm) rHb-R
5-methyl	a	17.95	21.27
3-methyl	l	13.05	9.98
8-methyl	j	12.62	10.37
1-methyl	d	10.09	15.07
6-α'-propionate		7.74	
6-α-propionate		7.17	
4-α-vinyl		6.04	6.84
7-α-propionate		4.34	
7-α'-propionate		1.71	
6-β'-propionate		0.99	
6-β-propionate		0.31	
7-β-propionate		0.12	
7-β'-propionate		–0.37	
trans-4-β-vinyl		–2.12	–1.67
cis-4-β-vinyl		–2.96	–2.14
2-α-vinyl	b		15.54
trans-2-β-vinyl	x		–4.54
cis-2-β-vinyl	y		–5.15
γ-meso		-2.99	

In 95% $^{1}H_2O$/5% $^{2}H_2O$, at 25 °C and pH 7.3, with water resonance set at 4.76 ppm with respect to DSS. Assignments for rHb-R are from Ref. 3.

Table S2. *^{1}H NMR chemical shifts for axial histidine resonances in the spectrum of ferric S6803 rHb-A*

Assignment	Signal	δ (ppm) rHb-A	δ (ppm) rHb-R
His46 NH		11.15	10.71
His46 CαH		9.41	7.70
His46 CβH		11.65	10.82
His46 CβH		9.28	9.20
His46 NδH	e	14.13	13.2
His46 Cα		80.6	77.7
His46 N		123.3	123.0
His46 Nδ		142.2	135.6
His70 NH		9.75	9.90
His70 CαH		6.53	6.75
His70 CβH		10.26	9.62
His70 CβH		10.02	8.92
His70 NδH	c	15.9	15.0
His70 Cα		71.9	69.1
His70 N		113.3	113.0
His70 Nδ		138.4	133.8

In 95% $^{1}H_2O$/5% $^{2}H_2O$, at 25 °C and pH 7.3, with water resonance set at 4.76 ppm with respect to DSS. Assignments for rHb-R are from Ref. 3.

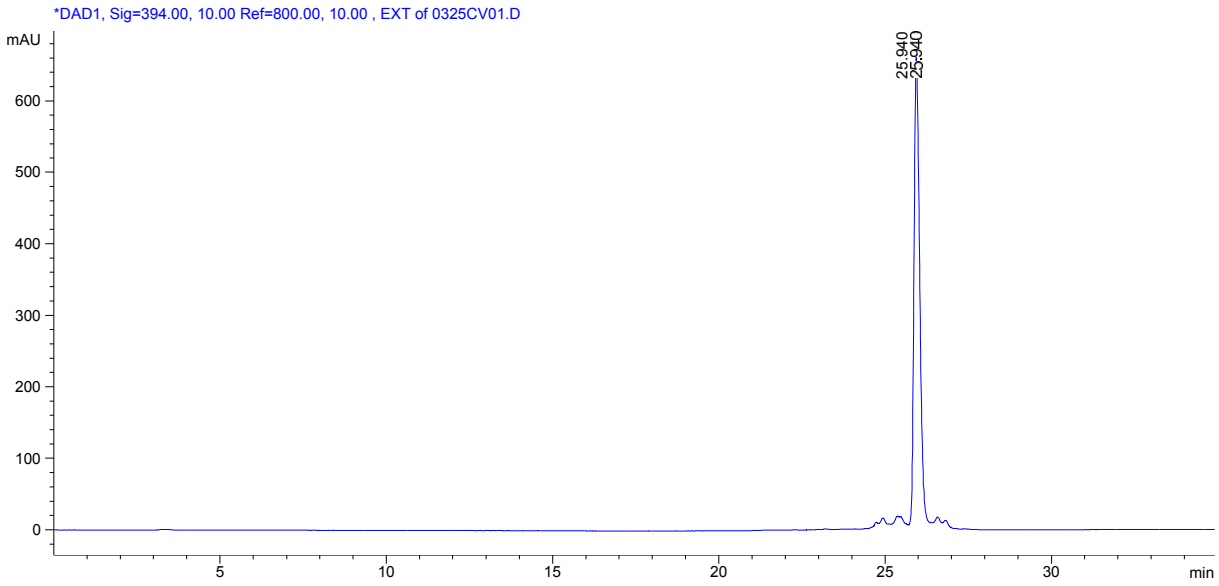

FIG. S5. HPLC-diode array chromatogram of products of pepsin digestion of rHb-A with detection at λ = 394 nm (bandwidth = 10 nm) showing a single heme-adducted peptide. No other chromatographic peaks with absorbance at $\lambda > 300$ nm were observed.

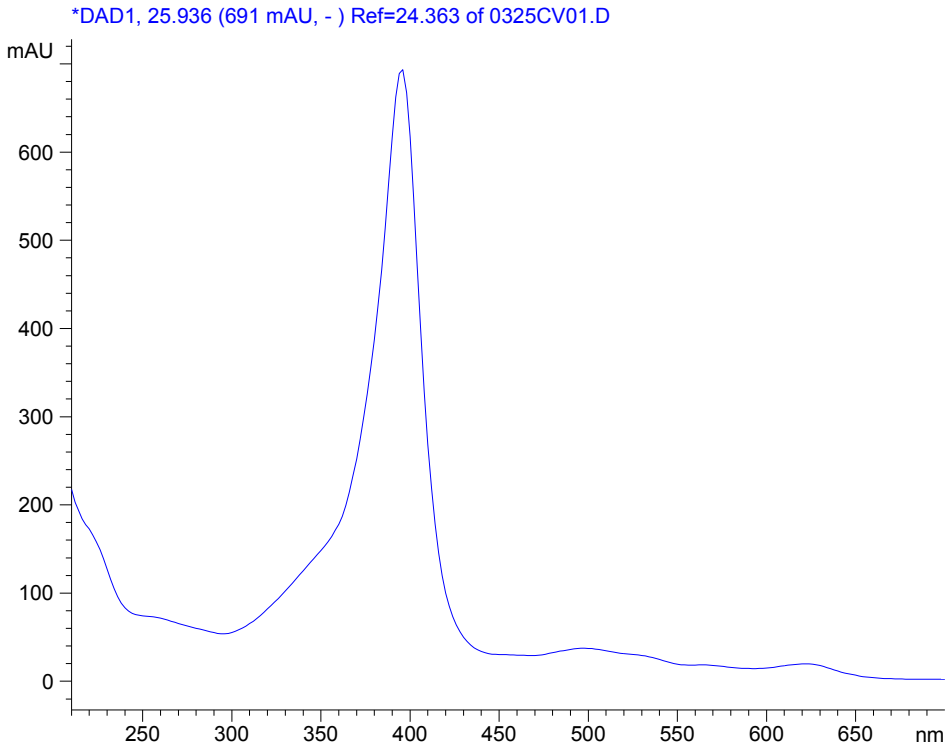

FIG. S6. UV-visible spectrum of peak with $^{t}_{R}$ = 25.94 minutes from pepsin digestion of rHb-A.

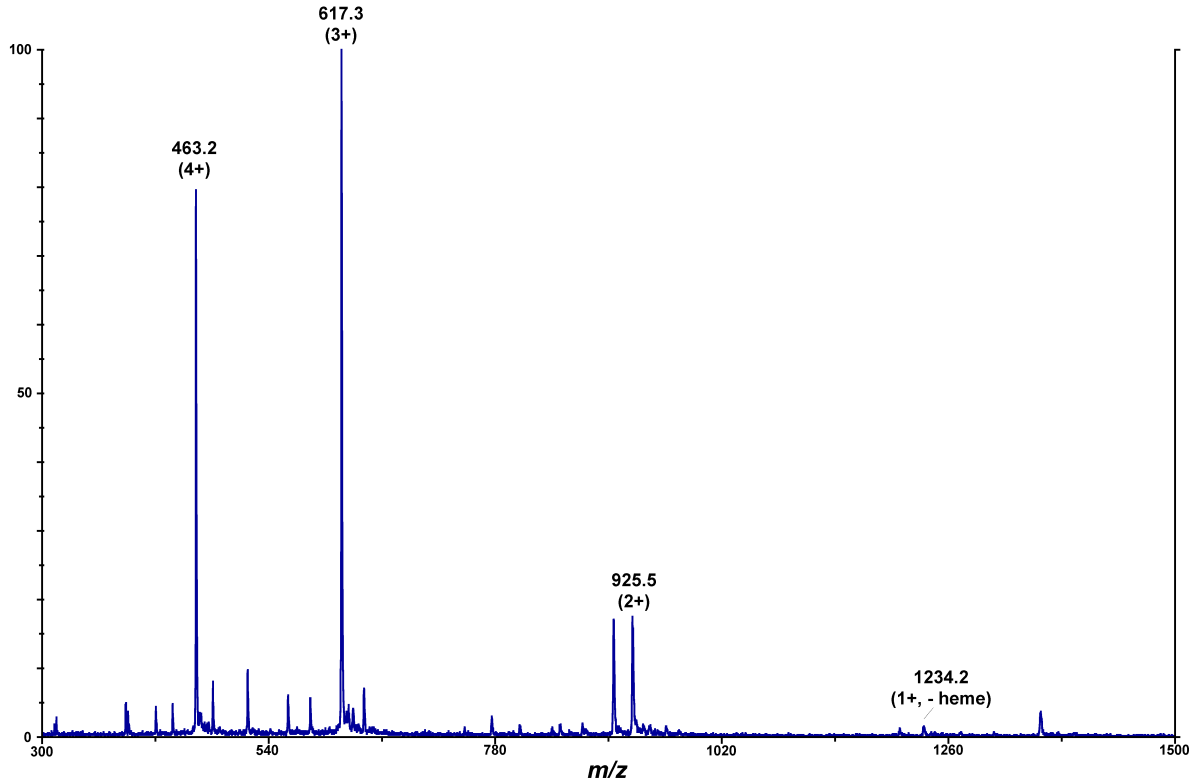

FIG. S7. Positive mode electrospray ionization mass spectrum of the heme-adducted peptide generated by pepsin digestion of rHb-A. The peak at m/z 1234.2 corresponds to the singly protonated peptide minus heme, and is attributed to collision induced dissociation in the electrospray ion source.

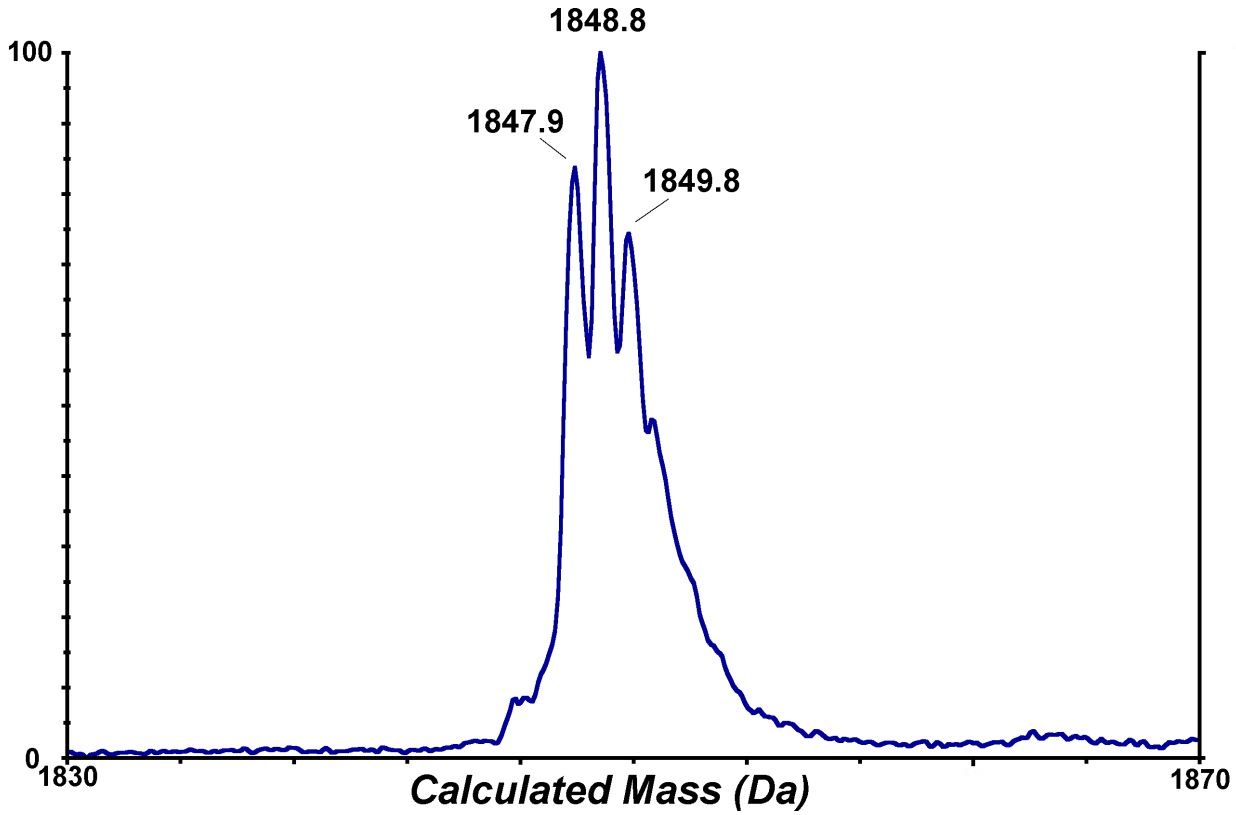

FIG. S8. Transformed "zero-charge state" spectrum calculated from the electrospray ionization mass spectrum in Fig. S7. The transform algorithm assumes that each positive charge arises from proton attachment, but this leads to a calculated monoisotopic mass (1847.9 Da) that is 1 Da less than the true zero-charge state mass (1848.9 Da) based on the following reasoning. Electrospray ionization of heme coordinated to Fe(III) yields M^+ (m/z 616) rather than $[M+H]^+$ ions owing to the +3 charge state of iron. This behavior is also expected in the adducted peptide, which already has one positive charge from the Fe(III) heme. Therefore, the doubly charged peak at m/z 925.5 (Fig. S7) is $[M+H]^{2+}$ in contrast to non-adducted peptides which typically form $[M+2H]^{2+}$ ions. In similar fashion, the triply charged peak at m/z 617 above corresponds to $[M+2H]^{3+}$ and m/z 463 corresponds to $[M+3H]^{4+}$.

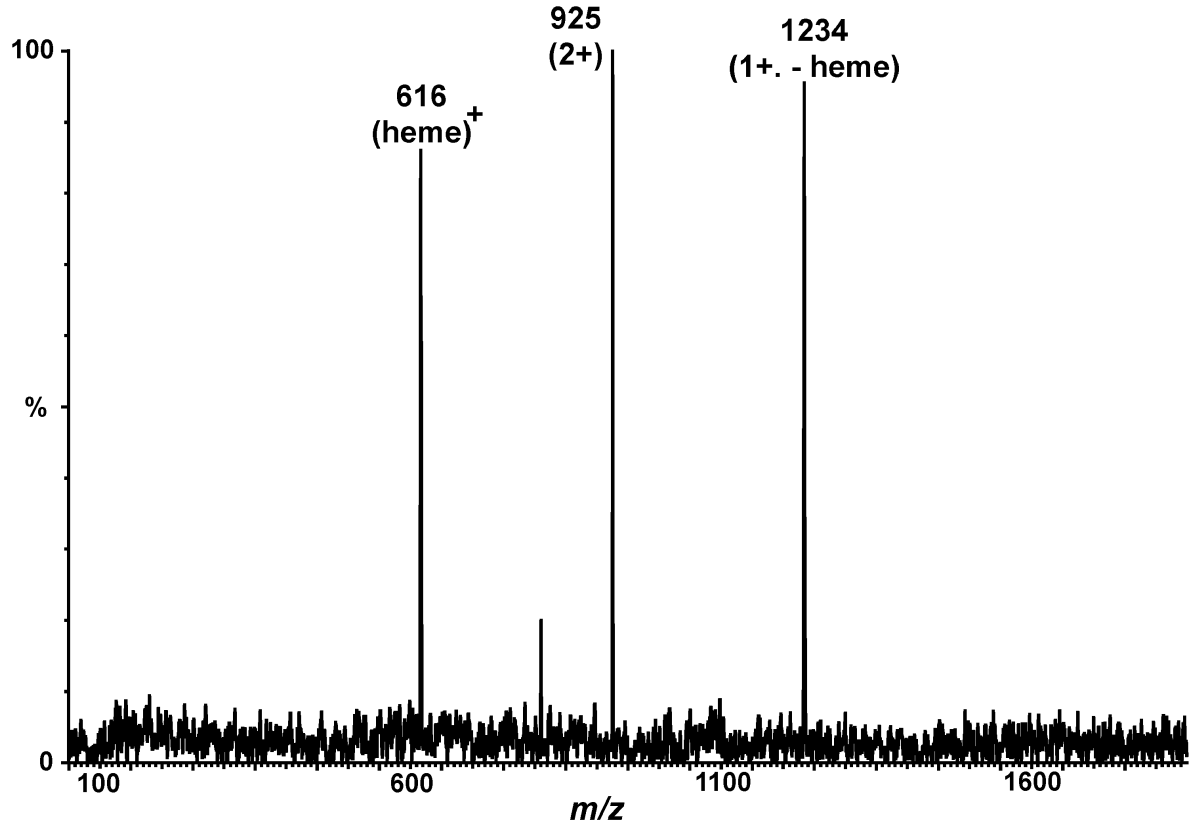

FIG. S9. Tandem mass spectrum of products derived from the doubly charged ion at m/z 925 generated from electrospray ionization of the heme-adducted pepsin digest product. Collision cell potential was 30 V and argon (2×10^{-3} mbar) was collision gas. This product ion spectrum shows formation of the singly protonated peptide (minus heme) at m/z 1234 and the singly charged heme at m/z 616. Conditions were not found that yielded heme-attached fragment ions that would indicate the position of attachment.

References

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