

Metal-substituted protein MRI contrast agents engineered for enhanced relaxivity and ligand sensitivity

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SUPPORTING INFORMATION

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SUPPLEMENTARY FIGURES

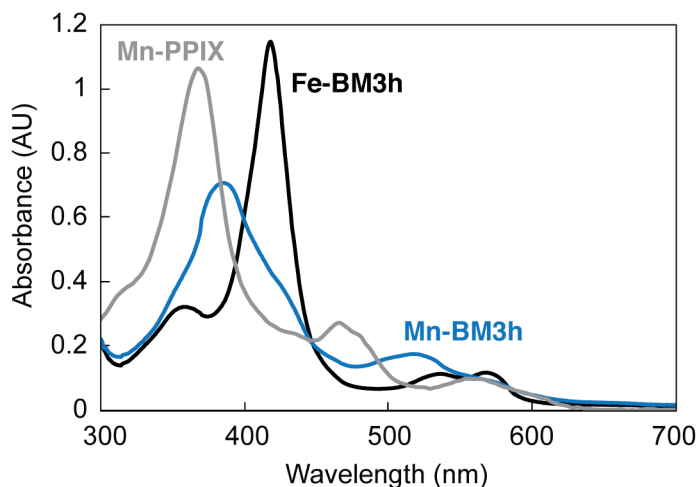


Figure S1. Optical absorbance spectra of purified wild-type manganic BM3h (blue), ferric BM3h (black), and free Mn(III)-protoporphyrin IX (gray) at 23 °C in phosphate buffered saline (PBS), pH 7.4. The primary Soret bands have maxima at 368 nm, 386 nm, and 418 nm, respectively.

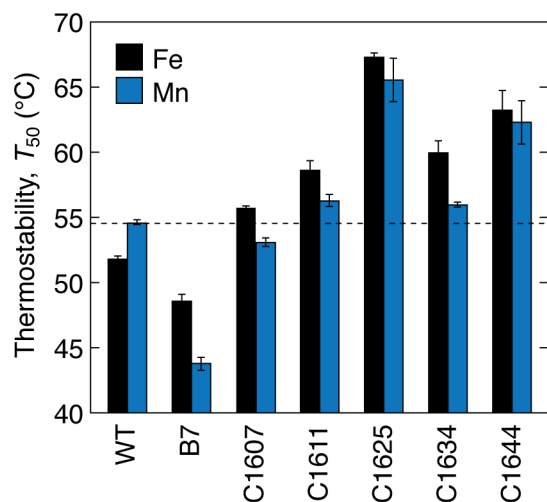


Figure S2. Comparison of the thermostabilities of ferric (black) and manganic (blue) BM3h variants. Purified protein at 5 μ M in PBS was incubated for 10 min at a range of temperatures and clarified by centrifugation. The amount of remaining soluble folded protein in the supernatant was estimated using the absorbance at the Soret band maxima. The reported thermostability, T_{50} , was obtained by fitting a two-state denaturation model to the absorbance difference as a function of incubation temperature. The horizontal dotted line corresponds to the stability of manganic wild-type BM3h, and is provided as a visual aid. Error bars denote the standard error of the mean ($n = 4$).

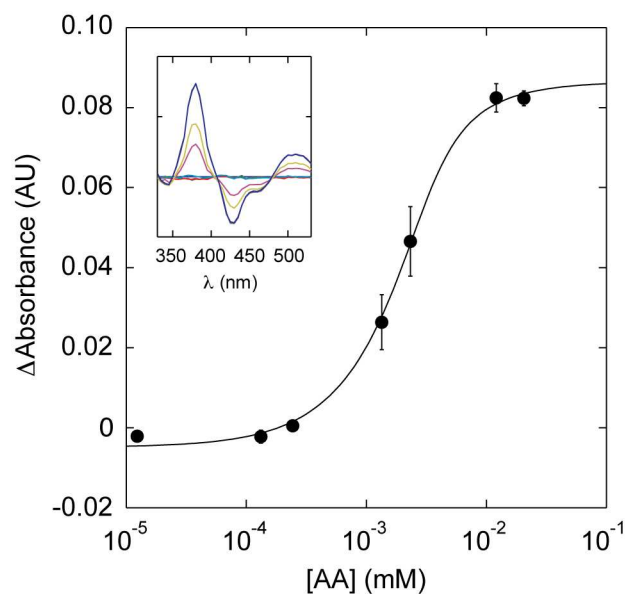


Figure S3. Titration of AA vs. wild-type MnBM3h observed by UV-Vis absorbance difference. Titration was performed as in Figure 2C. The solid curve was obtained by fitting the data to a two-component binding model. Inset: Representative absorbance difference spectra observed during titration; the greatest difference (dark blue spectrum) was observed at the highest AA concentration.

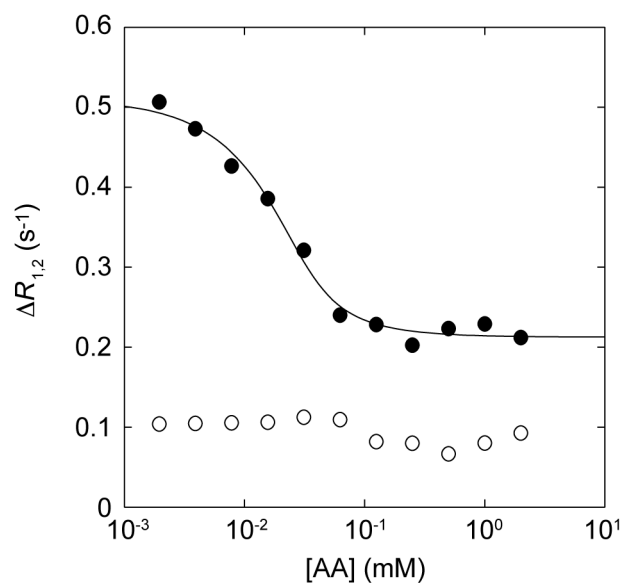


Figure S4. AA dependence of Mn-C1634 T_1 and T_2 relaxation rates. Titration of AA and data analysis was performed as in Figure 2D, except that ΔR_1 (solid circles and fitted line) and ΔR_2 (open circles) were calculated as a difference in relaxation rates with respect to background observed in PBS buffer.

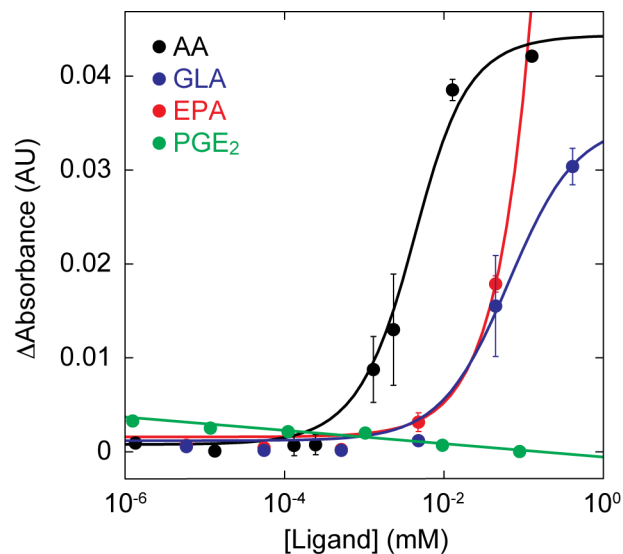


Figure S5. Titration of Mn-C1634 with AA and three biologically-relevant AA competitors observed by UV-Vis absorbance difference. Data are presented for AA (black), γ -linolenic acid (GLA, blue), cis-5,8,11,14,17-eicosapentaenoic acid (EPA, red), and prostaglandin E₂ (PGE₂, green). GLA and EPA are both bioavailable polyunsaturated fatty acids chemically similar to AA; PGE₂ is a downstream metabolite of AA in inflammatory signaling. Titrations were performed as in Figure S3. AA and GLA titration data could be fit to two-component binding curves with K_d values of $2.5 \pm 0.7 \mu\text{M}$ and $64 \pm 18 \mu\text{M}$, respectively. Dissociation constants could not be obtained from data for EPA and PGE₂, and plotted curves for these ligands are presented as visual aids only.

SUPPLEMENTARY TABLES

Table S1.

Porphyrin Metal^a	Soret maximum, λ (nm)	Q maxima, λ (nm)
Co(III)	427	544, 571
Fe(III)	418	537, 567
Mn(III)	386	516
Zn(II)	448	569, 602, 643

^aUV-Vis absorbance spectra of wild type BM3h reconstituted with the designated metalloporphyrin species were obtained at 23 °C in PBS, pH 7.4.

Table S2.

Variant	Composition^a
C1607	21311231
C1611	21312233
C1625	22313333
C1634	21313313
C1644	22312333

^aChimeric cytochrome P450 variants are composed of recombined sequence fragments derived from homologues CYP102A1 (BM3h, 1), CYP102A2 (2), and CYP102A3 (3), as described previously².

SUPPLEMENTARY METHODS

Plasmid construction. A ChuA-encoding plasmid, pChuA, was constructed by directional cloning of the complete open reading frame of ChuA from a genomic isolate of O157:H7 *E. coli* (ATCC) between the NcoI/XhoI sites on the high copy plasmid pRSFDuet-1 (Novagen). Wild-type and variant cytochrome P450 BM3 heme (BM3h) domains were expressed from previously described pCWori(+) plasmids^{1,2}. All BM3h variants lacked the reductase domain of the wild-type holoenzyme, and were therefore incapable of undergoing the canonical P450 catalytic cycle.

Protein expression and purification. pChuA and pCWori plasmids were cotransformed into chemically competent BL21 AI cells (Invitrogen). This strain carries an arabinose-inducible construct expressing T7 polymerase under control of an araBAD operator. Cotransformants were selected on Luria-Bertani (LB) agar plates containing 50 µg/mL kanamycin and 100 µg/mL carbenicillin. All liquid cultures were inoculated from fresh colonies and grown in M9 minimal media supplemented with 0.2 µm-filtered 0.5% glucose, 0.2% casamino acids, 340 µg/mL thiamine hydrochloride, 2 mM MgSO₄, 0.1 mM CaCl₂, 100 µg/mL kanamycin, and 200 µg/mL carbenicillin. Overnight cultures grown at 30 °C were inoculated at 1:100 into fresh media in 2 L flasks and grown at 37 °C with vigorous shaking for approximately 4 hours until early log phase, such that OD_{600nm} = 0.4-0.6 (1 cm path length). Batch cultures were induced for 12-16 hours at 30 °C by addition of 0.5% L-arabinose and 1 mM IPTG, and cultures were simultaneously supplemented with 30 µg/mL Mn(III), Fe(III), Zn(II), or Co(III)-coordinated protoporphyrin IX (Frontier Scientific) from fresh 3 mg/mL stocks prepared in 0.1 M NaOH.

Harvested bacterial pellets were lysed at room temperature in Bugbuster reagent (Novagen) containing the enzyme reagent lysonase (containing recombinant lysozyme and benzonase, 1000-fold diluted into lysate) and protease inhibitors (Protease Inhibitor Cocktail Set III, Novagen, 1000-fold diluted). Lysates were clarified by centrifugation and incubated with batch Ni-NTA Superflow agarose resin (Qiagen) for 2 hours at 4 °C. The resin was washed and eluted in gravity columns using the manufacturer's recommended protocol. Eluate was purified by anion exchange chromatography (HiTrap Q XL, Amersham) in 100 mM Tris-Cl pH 7.8 using a linear 1M NaCl gradient, concentrated in a 30 kD molecular weight cutoff centrifugal filtration device (Amicon Ultra, Millipore), and buffer exchanged into 1X PBS pH 7.4 using a G-25 sepharose column (PD-10, Amersham). Purity was assayed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) following a standard protocol. For initial assessments of native folding, ferric and manganic protein samples were loaded onto a precast 4%/7.5% polyacrylamide gel equilibrated in 50 mM imidazole, 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH 7.6 running buffer, separated in this buffer at 4 °C, and observed directly by their native coloring.

Spectroscopic characterization. Ultraviolet-visible (UV-vis) absorbance spectra of purified proteins in PBS were obtained on a SpectraMax M2e spectrophotometer (Molecular Devices) in cuvettes of 1 cm path length at 23 °C. Circular dichroism was measured in an Aviv Model 202

circular dichrometer (Aviv Biomedical) at 25 °C in 10 mM potassium phosphate, pH 7.4, and 100 mM (NH₄)₂SO₄, in a 1 mm path length quartz cuvette. Native iron-containing BM3h protein concentrations were determined as the absorbance difference between 450 and 490 nm of the dithionite reduced ferrous-CO adduct by the method of Omura and Sato³, with molar extinction coefficient 91 mM⁻¹ cm⁻¹ in 100 mM Tris-HCl, pH 8.3. Purified manganese substituted BM3h protein concentrations were determined as the manganic species using the molar extinction coefficient 78 mM⁻¹ cm⁻¹, determined by atomic emission spectroscopy of acid hydrolyzed samples in an Avisa ICP-AES (Horiba Jobin Yvon).

Relaxivity measurements. For MRI relaxation measurements at 4.7 T, protein samples were diluted in PBS, pH 7.4 into 384-well microtiter plate wells (BD Falcon) in a linear range of concentrations. Peripheral plate wells were filled with buffer alone. Plates were scanned in a 4.7 T Bruker Avance MRI scanner with a 40 cm bore, and measurements were performed at ambient temperature (23 °C) for comparability with earlier results. Images were taken in a 2-mm slice across each plate using a T_1 -weighted spin echo pulse sequence. Time to echo (TE) was 10 ms for all scans. Repetition time (TR) ranged from 73 ms to 5000 ms, and averaging was correspondingly adjusted from 64 to 4 scans per TR . Data matrices were 256 x 128 points, with field of view of 5 x 5 cm. Longitudinal relaxation rates (R_1) were fit from reconstructed image data using custom routines implemented in Matlab (Mathworks). Transverse relaxation rates (R_2) were determined at 4.7 T by fitting exponential decay curves to image data obtained from samples in microplates, using a Carr-Purcell-Meiboom-Gill acquisition pulse sequence with echo times of 30 ms. For relaxometry measurements at lower field, T_1 relaxation times were fit from saturation recovery pulse sequence experiments of concentrated protein samples in PBS, pH 7.4 in a custom-built variable field relaxometer (Southwest Research Institute) at 23 °C. Reported relaxivity (r_1 and r_2) values were computed as the slope of relaxation rate vs. concentration for the various protein samples.

Thermostability assay. Thermostability of manganic and ferric BM3h variants was assayed as described previously². Briefly, 125 μ L samples of 5 μ M purified protein in PBS were incubated for 10 minutes in a gradient thermocycler (MyCycler, Bio-Rad) at a range of temperatures and then cooled rapidly to 4 °C. Protein precipitate was removed by centrifugation at 4 °C, and 100 μ L of the remaining supernatant was transferred into a clear-bottom 384-well plate. Absorbance spectra were measured in a spectrophotometer (SpectraMAX M2e, Molecular Devices) at 23 °C. Difference spectra were generated by subtracting untreated from thermally treated spectra. The denaturation midpoint, T_{50} , was obtained by fitting a two-state denaturation model to the difference between the maximum and minimum deflections in the difference spectra at the Soret maxima, plotted versus incubation temperature.

Measurements of ligand-dependent absorbance and relaxivity. Absorbance and relaxivity effects of ligand binding were measured essentially as described previously.¹ Briefly, fresh 5 mg

aliquots of porcine liver arachidonic acid (AA), sodium salt (Sigma-Aldrich, St. Louis, MO) were dissolved in ice cold degassed deionized water as stock solutions for titrations and kept on ice. UV-vis absorbance difference spectra were measured from 5 μ M protein solutions titrated with increasing concentrations of AA in degassed PBS at 23 °C in multiwell plates by using unbound protein spectra as a spectral baseline. Titration curves were obtained from the maximum and minimum deflections in difference spectra at the Soret maxima for both manganic and ferric BM3h proteins. NMR relaxation effects of ligand binding were assessed as above by acquiring T_1 -weighted MRI scans of multiwell plates containing mixtures of 50 μ M protein with AA at a range of concentrations in PBS. T_1 relaxation rates averaged over each well (R_1) were extracted using custom routines written in Matlab. Both optical and relaxivity measurements were fit to a quadratic two-component binding model to obtain equilibrium dissociation constants. Absorbance changes due to binding of three potential AA competitors to Mn-C1634 were measured in similar fashion. For these experiments, γ -linolenic acid (GLA), cis-5,8,11,14,17-eicosapentaenoic acid (EPA), and prostaglandin E₂ (PGE₂) were purchased from Sigma-Aldrich; GLA and EPA were formulated in ice cold ethanol, while PGE₂ was formulated in cold degassed deionized water.

REFERENCES

- (1) Shapiro, M. G.; Westmeyer, G. G.; Romero, P. A.; Szablowski, J. O.; Küster, B.; Shah, A.; Otey, C. R.; Langer, R.; Arnold, F. H.; Jasanoff, A. *Nat. Biotechnol.* **2010**, 28, 264-70.
- (2) Li, Y.; Drummond, D. A.; Sawayama, A. M.; Snow, C. D.; Bloom, J. D.; Arnold, F. H. *Nat. Biotechnol.* **2007**, 25, 1051-6.
- (3) Omura, T.; Sato, R. *J. Biol Chem.* **1964**, 239, 2379-85.