Supporting information

C(sp^3)–H Bond Hydroxylation Catalyzed by Myoglobin Reconstituted with Manganese Porphycene

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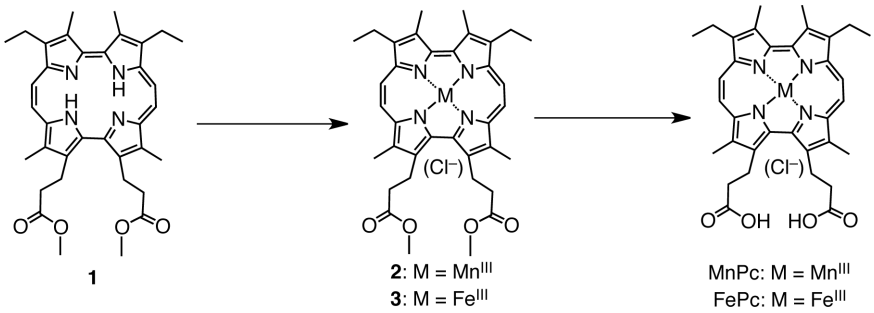
Instruments

UV-Vis spectral measurements were carried out with a Shimadzu UV-3150 or UV-2550 double-beam spectrophotometer. ESI-MS analyses were performed with a Bruker Daltonics micrOTOF mass spectrometer. ICP-OES (inductively coupled plasma optical emission spectroscopy) was performed on a Shimadzu ICPS-7510 emission spectrometer. The pH measurements were made with an F-52 Horiba pH meter. Ion exchange column chromatography was performed using an ÄKTApurifier system (GE Healthcare) at 4 ºC. The HPLC analyses were conducted with a Shimadzu HPLC Prominence system. The GC/FID measurements were made with a Shimadzu GC-2014 gas chromatography system. GC-MS(EI) was performed using a Shimadzu GCMS-QP2010 Ultra system.

Materials

Synthesis of porphycene diester **1** and iron porphycene, FePc, were reported in our previous paper.[S1] Manganese protoporphyrin IX, MnPor, was prepared according to procedures reported in literature.[S2] Native horse heart myoglobin was purchased from Sigma Aldrich and purified with a cation exchange CM52 cellulose column (Whatman). Reconstituted myoglobins were prepared by the same procedure described in the previous paper[S1] after the removal of native heme by Teale's 2-butanone method.[S3] The obtained reconstituted proteins were purified with a Sephadex G-25 column (100 mM potassium phosphate buffer, pH 7.0) and were concentrated by ultrafiltration prior to storage as frozen solutions. Unless mentioned otherwise, all protein solutions were prepared in 100 mM potassium phosphate buffer at pH 7.0. ^{18}O-labeled hydrogen peroxide was obtained from Taiyo Nippon Sanso Co. Other reagents and chemicals were purchased and used as received. Distilled water was demineralized using a Barnstead NANOpure DIamond^{TM} apparatus.

Experimental procedures



Scheme S1. Synthesis of metalloporphycenes, MnPc and FePc.

Synthesis of 2.

To a phenol solution (2.7 mL) of porphycene diester **1** (4.5 mg, 7.6 μmol), $Mn(OAc)_2$ (18 mg, 7.3 x 10^{-2} mmol) was added under an N_2 atmosphere and then the solution was refluxed for 30 min. After cooling, solvent was removed under reduced pressure and 10 mL of 1 M HClaq was added to the residue. The product was extracted with CH_2Cl_2. After the solution was dried over Na_2SO_4, the solvent was removed under reduced pressure. The residual solid was passed through a SiO_2 column (CH_2Cl_2 : CH_3OH = 9 : 1, v/v). The fraction was collected and evaporated *in vacuo* to obtain a green solid. Yield: 5.0 mg, quant.

UV-vis (CH_3CN): λ_{max}/nm (absorbance) = 621 (0.10), 562 (0.081), 423 (0.11), 346 (0.19). HR-ESI MS (positive mode): *m/z* 647.2440 $(M–Cl^–)^+$, calcd for $C_{36}H_{40}N_4O_4Mn^+$ 647.2430.

Synthesis of MnPc

To a solution (THF : MeOH = 1 : 1, v/v, 5 mL) of **2** (5.0 mg, 7.2 μmol) was added 2.5 mL of 0.2 M NaOH solution. After stirring for 4 h at ambient temperature, CH_2Cl_2 was added to the reaction mixture, followed by washing with 0.1 M HClaq. The organic phase was dried over Na_2SO_4 and solvent was removed under reduced pressure. Further HPLC purification (CH_3CN/H_2O eluent, C18 column) was performed to furnish green solid. Yield: 3.0 mg, 63%.

UV-vis (CH_3CN): λ_{max}/nm (absorbance) = 625 (0.25), 568 (0.27), 437 (0.33), 407 (0.25), 353 (0.45). HR-ESI MS (positive mode): *m/z* 619.2137 $(M–Cl^–)^+$, calcd for $C_{34}H_{36}N_4O_4Mn^+$ 619.2117.

Preparation of rMb(MnPc)

A MnPc solution (24 mM, 20 μL) in DMSO was added dropwise into 8.0 mL of apomyoglobin solution (50 μM) with gentle shaking on an ice bath. After equilibrating for 1 h at ambient temperature, the mixture was concentrated by ultrafiltration to remove excess cofactor. After removal of precipitates by centrifugation, the protein containing MnPc was purified by passing it through a Sephadex G-25 Fine (0.8 x 27 cm, GE Healthcare) column with 0.1 M potassium phosphate buffer (pH 7.0). The eluted fractions were concentrated and stored in the dark at –80 ºC. The UV-vis spectrum of the obtained protein is shown in Figure S1. The molar coefficient at 631 nm was determined to be 35.3 $mM^{-1}•cm^{-1}$ by ICP-OES measurements. The protein was identified by ESI-MS (positive mode): found *m/z,* 1953.4, calcd *m/z* (*z* = +9), 1953.3.

Crystallization of rMb(MnPc)

For crystallization, further purification of rMb(MnPc) was performed using an ion exchange column. To remove residual apomyoglobin, an SP (sulfopropyl) Sepharose column was employed. Eluent of 10 mM potassium phosphate buffer allowed pass of only reconstituted myoglobin and binding of apomyoglobin. After sufficient washing, the collected fractions were buffer-exchanged to 0.1 M imidazole, pH 7.0. The protein was crystallized at 298 K using the hanging-drop vapor diffusion method and cross-seeding with microcrystals of native myoglobin. The crystals were obtained using a reservoir solution containing 3.2 M ammonium sulfate and 0.1 M imidazole, pH 7.0, and a hanging drop solution containing 5 mg/mL of protein and 1.6 M ammonium sulfate and 0.1 M imidazole, pH 7.0.

Data collection, structure determination and refinement

The crystal was fished with a standard nylon loop and flash-cooled in an N_2 gas stream at 100 K. X-ray diffraction data were collected at the beamline BL44XU, SPring-8, Japan. The dataset was integrated and scaled with the *HKL2000* program. The structure was determined by the molecular replacement method using the program *PHASER* from the *CCP4* program suite. A crystal structure of horse heart myoglobin (Protein Data Bank (PDB) ID: 2V1F) was used as the search model. Refinement was carried out using the program *REFMAC*. The structure was visualized and modified with the program *COOT*. Data collection and refinement statistics are summarized in Table S1. The final atomic coordinates and structure factor amplitudes were deposited in the PDB with ID 3WI8.

Acid titration

To 3 mL of a 100 mM KCl solution was added 10 μL of a concentrated myoglobin solution (~0.4 mM) in 100 mM phosphate buffer (pH 7.0). The UV-vis spectrum and pH value of the protein solution were monitored for each addition of 10 μL of 100 mM HClaq at 25 ºC. The p$K_{1/2}$ value, the pH corresponding to a 50% loss of a cofactor, was determined by fitting the titration curve to the modified Henderson-Hasselbach equation.[S4]

Reaction of rMb(MnPc) with cumene hydroperoxide

A protein solution was treated with cumene hydroperoxide ([protein]=20 μM, [CHPO] = 10 mM) in potassium phosphate buffer (1 mL, 100 mM, pH 7.0) containing 1 mM of guaiacol at 25 ºC for 2 h. The reaction mixture was filtered using a Microcon concentrator (molecular weight cut off: 10,000 Da), and the filtrate was analyzed with an HPLC system equipped with a C18 column to determine the amounts of acetophenone and cumylalcohol formed by cleavage of CHPO based on the intensity ratios of these materials.

Sulfoxidation of thioanisole

The reactions were carried out in 100 mM potassium phosphate buffer, pH 7.0, containing 1% CH_3CN at 25 ºC. A buffer solution of protein and thioanisole was incubated prior to the addition of H_2O_2 to initiate the reaction. The final concentrations were: [protein] = 8 μM, [substrate] = 2.0 mM and [H_2O_2] = 10 mM. After the reaction period, benzyl alcohol as an internal standard and ether were added, and the reaction mixture was vigorously shaken using a vortex mixer to extract the organic materials. The separated organic phase was concentrated by evaporation with streaming N_2 gas, and the residue was analyzed with a GC/FID system equipped with a DB-1 column. To determine the turnover number, the reaction period was 16 h.

Determination of hydroxylase activity

The reactions were carried out in 100 mM potassium phosphate buffer, pH 8.5, containing 8% CH_3CN at 25 ºC. A buffer solution of protein and substrate (ethylbenzene, toluene or cyclohexane) was incubated prior to the addition of H_2O_2 to initiate the reaction. The final concentrations were: [protein or MnPc] = 20 μM, [substrate] = 8.0 mM and [H_2O_2] = 10 mM. In the case of MnPc, the solution contained 1% DMSO for complete dissolution of the complex. After the reaction period, benzyl alcohol (for

ethylbenzene and cyclohexane) or 1-phenylethanol (for toluene) as an internal standard and ether were added, and the reaction mixture was vigorously shaken using a vortex mixer to extract the organic materials. The separated organic phase was concentrated by evaporation with streaming N_2 gas, and the residue was analyzed with a GC/FID or GC/MS(EI) system equipped with a DB-1 column. To determine the turnover number, the reaction period was 3 h. The analyses of hydroxylation of deuterated substrates and *para*-substituted toluene were carried out using the same procedure.

References

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S2. Yonetani, T.; Asakura, T. *J. Biol. Chem.* **1969**, *244*, 4580–4588.

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S5. Matsuo, T.; Dejima, H.; Hirota, S.; Murata, D.; Sato, H.; Ikegami, T.; Hori, H.; Hisaeda, Y.; Hayashi, T. *J. Am. Chem. Soc.* **2004**, *126*, 16007–16017.

Table S1. Data collection and refinement statistics for rMb(MnPc)

	rMb(MnPc)
Data collection	
X-ray source	SPring-8 BL44XU
Detector	Rayonix MX300HE
Wavelength (Å)	0.90000
Resolution (Å) (outer shell)	50 – 2.20 (2.28 – 2.20)
Space group	$P2_1$
Unit cell parameters (Å, deg.)	a = 34.83, b = 28.66, c = 63.74, β = 105.18
No. of total reflections	24,226
No. of unique reflections	6,299
Completeness (%)	97.8 (95.5)
R_{sym}	5.5 (34.5)
$I/\sigma(I)$	24.9 (3.2)
Refinement	
Resolution (Å)	18 – 2.20
No. of reflections	5,939
R_{cryst} / R_{free} (%)	18.6 / 25.3
Mean B-factor (Å^2)	38.4
No. of non-H atoms	1,257
*R*msd from ideal	
Bond lengths (Å) / angles (deg.)	0.018 / 2.913

Table S2. Geometry of cofactors

	nMb(FePor)	rMb(MnPc)	rMb(FePc)	rMb(MnPor)
PDB code	1YMB	3WI8	2D6C	2O58
sample	aquomet	aquomet	imidazole ferric	aquomet
space group	$P2_1$	$P2_1$	$P2_1$	$P2_1$
resolution (Å)	1.9	2.2	2.3	1.6
pH of crystal	7.0	7.0	7.0	7.4
Rmsd of Ca atoms (Å) for rMb(MnPc)	0.42	—–	0.72	0.37
distance				
His93 Nε–Fe / Mn (Å)	2.26	2.58	2.28	2.22
Wat O–Fe / Mn (Å)	2.29	2.79	—	2.52, 3.96[a]
His64 Nε–Fe / Mn (Å)	4.33	4.72, 8.90[a]	7.93	4.47, 4.65[a]

[a]Two alternative conformations are existed.

Table S3. Comparison of p$K_{1/2}$ of myoglobins obtained by acid titration experiments

Protein	p$K_{1/2}$[a]
nMb(FePor)[b]	4.1
rMb(MnPc)[c]	5.1
rMb(FePc)[b]	3.1
rMb(MnPor)[c]	4.6

[a]A pH value corresponding to a 50% cofactor-dissociation from protein at 25 ºC. [b]ref. S5. [c]this work.

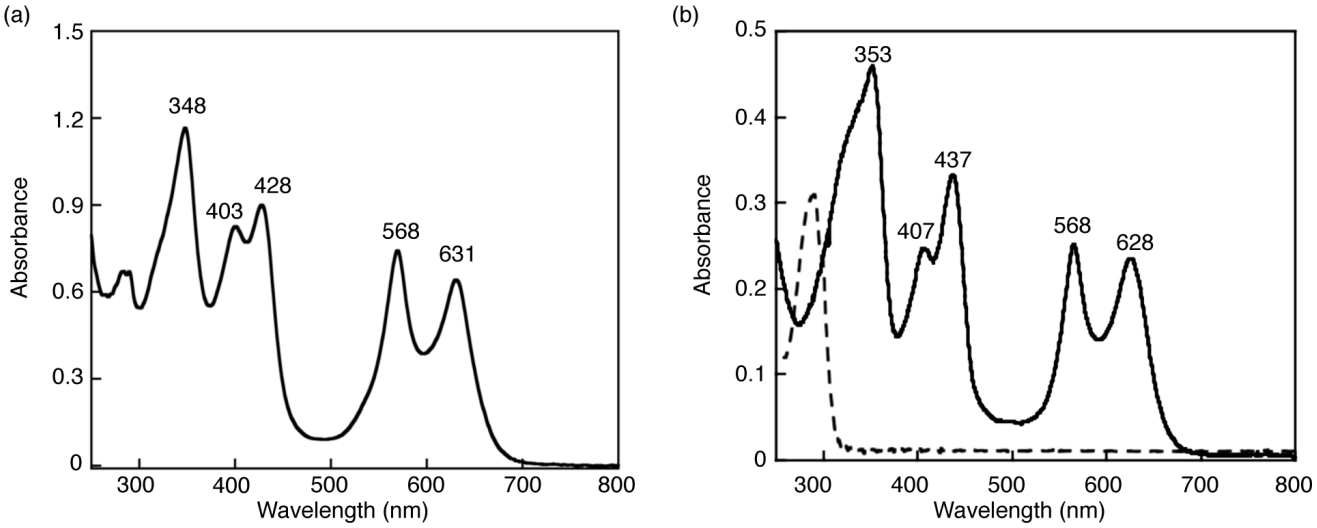

Figure S1. UV-vis absorption spectra at 25 ºC. (a) rMb(MnPc) in 100 mM potassium phosphate buffer, pH7.0. (b) solid line: MnPc in CH_3CN, broken line: apo-Mb in 100 mM potassium phosphate buffer, pH7.0.

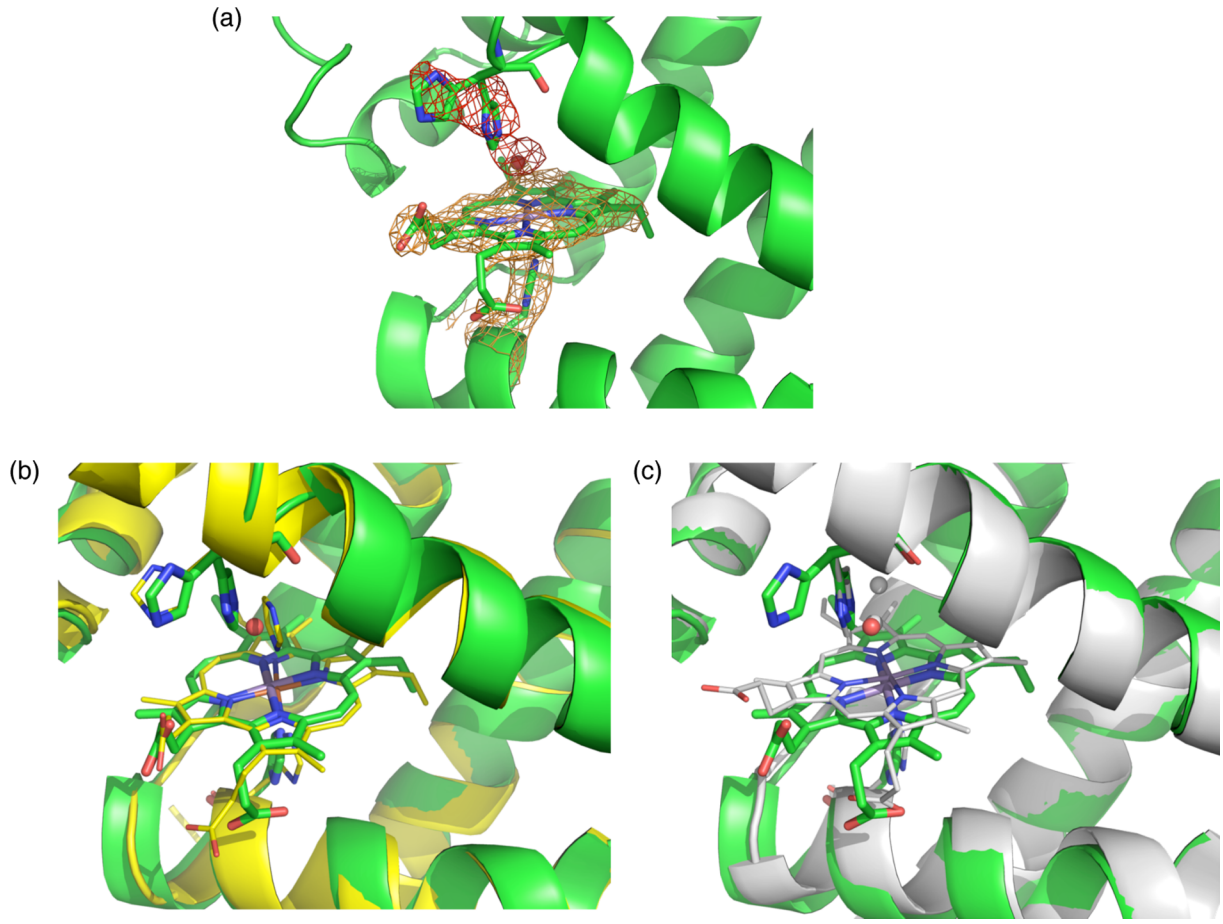

Figure S2. (a) Crystal structure of rMb(MnPc) with the $2F_{obs} - F_{calc}$ electron density (orange grid: 1.0 σ contours) in the vicinity of MnPc and His93 and the $F_{obs} - F_{calc}$ electron density (red grid: 3.0 σ contours) based on the refined coordinates excluding the side chain of His64 and the water molecule. (b, c) Superimposed structures of rMb(MnPc) (green) with rMb(FePc) (b, yellow, PDB ID: 2D6C) and with rMb(MnPor) (c, gray, PDB ID: 2O58).

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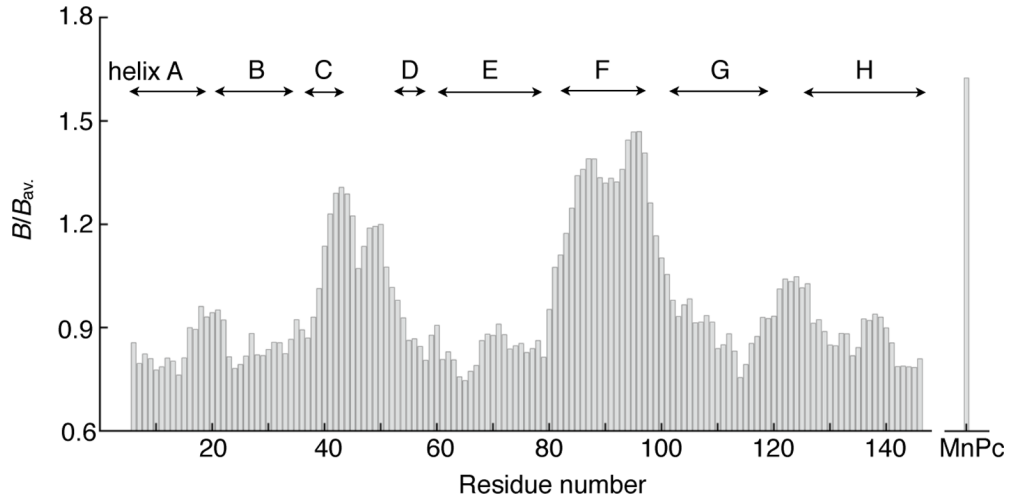

Figure S3. Plot of B/B_{av}. against the residue number and MnPc. B and B_{av} values respectively indicate the mean B-factor of Cα atoms of the main chain in the crystal structure and the averaged the B values between residues 5–146. For the B value of MnPc, an averaged B of the framework and metal atoms was utilized. Helix F and MnPc fluctuate more than CD loop, which is the most flexible region in native Mb.

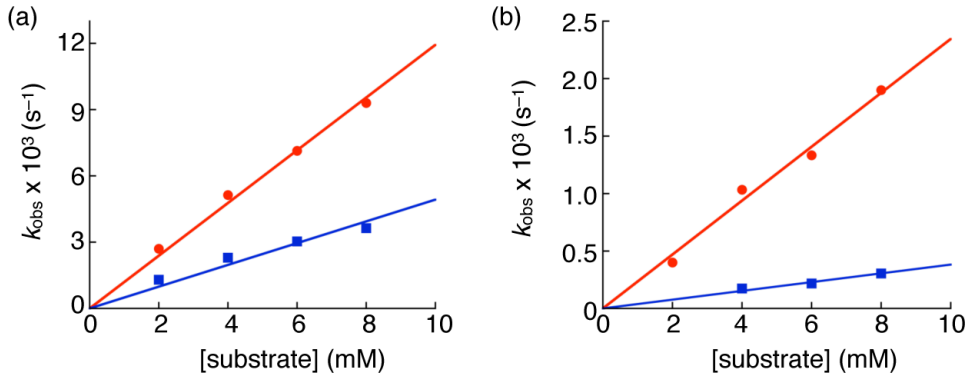

Figure S4. Plots of k_{obs}, initial turnover frequency at 5 min, against the concentration of substrate; (a) ethylbenzene (C_8H_{10}, red circles) and ethylbenzene-d_{10} (C_8D_{10}, blue squares), and (b) toluene (C_7H_8, red circles) and toluene-d_8 (C_7D_8, blue squares). The slopes of fitted red and blue lines show k_H and k_D, respectively.

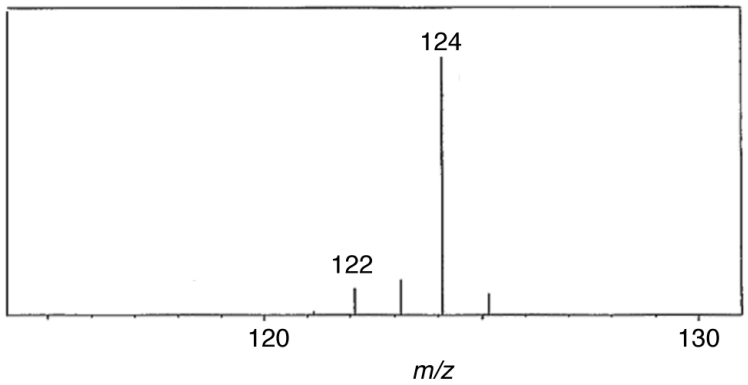

Figure S5. Mass spectrum of the product of hydroxylation of ethylbenzene with rMb(MnPc) and ^{18}O-labelled hydrogen peroxide. The main peak (124) and small peak (122) were assigned to ^{18}O-labeled and unlabeled 1-phenylethanol, respectively. This spectrum was derived from GC-MS analysis.