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Supporting Information

Co(II)/Co(I) reduction-induced axial histidine-flipping in myoglobin reconstituted with a cobalt tetradehydrocorrin as a methionine synthase model

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Instruments

UV-vis spectral measurements were carried out with a Shimadzu UV-3150 or UV-2550 double-beam spectrophotometer, or a Shimadzu BioSpec-nano spectrometer. ESI-TOF MS analyses were performed with a Bruker Daltonics micrOTOF mass spectrometer. ^{1}H spectra were collected on a Bruker BioSpin DPX400 (400 MHz) spectrometer. The ^{1}H chemical shift values are reported in ppm relative to a residual solvent peak. ICP-OES (inductively coupled plasma optical emission spectroscopy) was performed on a Shimadzu ICPS-8100 emission spectrometer. The EPR spectra were measured using a Bruker EMX Plus spectrophotometer at the Instrument Center of the Institute for Molecular Science (Okazaki, Japan). The equipment used for X-ray crystallographic analysis is described below. The pH measurements were made with an F-52 Horiba pH meter. Air-sensitive manipulations were performed in a UNILab glove box (MBraun, Germany).

Materials and Methods

All reagents of the highest guaranteed grade available were obtained from commercial sources and were used as received unless otherwise indicated. Distilled water was demineralized using a Barnstead NANOpure DIamond^{TM} apparatus. Dibenzyl *meso*-3,3′-bis(methoxycarbonylethyl)-4,4′-dimethyldipyrromethane-5,5′-dicarboxylate **1** and 2-formyl-3,4,5-trimethylpyrrole **2** were synthesized according to a previously published method.[S1,S2] A standard cobalt solution for ICP-OES was purchased from Wako Pure Chemical Industries.

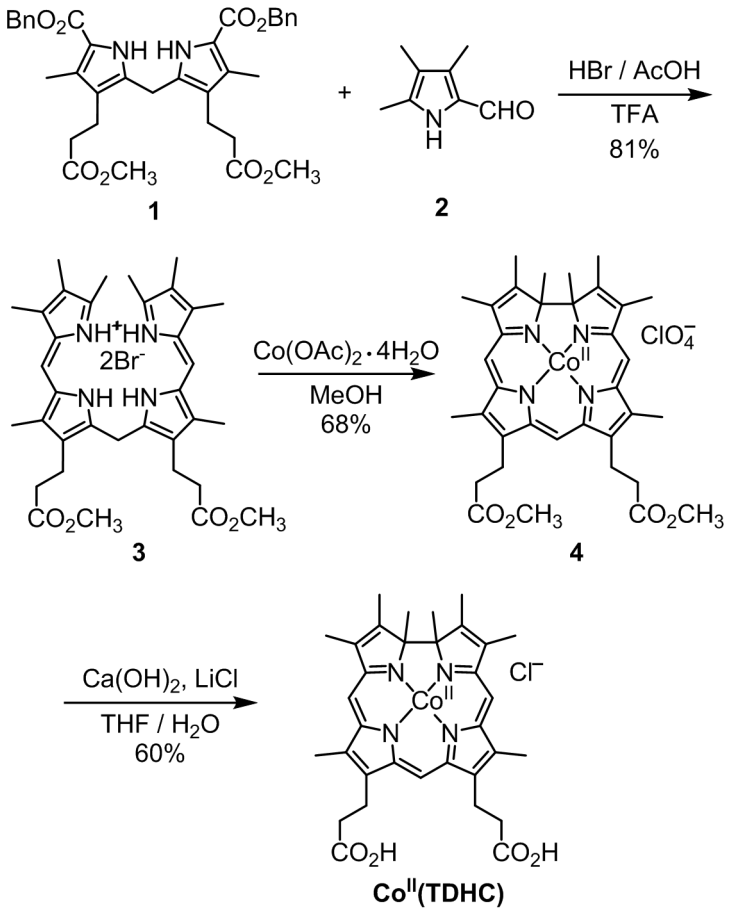
Preparation of Cobalt(II) Tetradehydrocorrin (Co^{II}(TDHC))

Dipyrromethane derivative **1** (1.4 g, 2.3 mmol) was dissolved in trifluoroacetic acid (TFA) (10 mL) under an N_2 atmosphere and then HBr/AcOH (25 wt.%, 8 mL) was added to the solution. After stirring for 8 h at room temperature, 2-formyl-3,4,5-trimethylpyrrole **2** (0.67 g, 4.8 mmol) in a methanol solution (20 mL) was added dropwise on an ice bath and stirred for 1 h at room temperature. Diethyl ether (200 mL) was added to the reaction mixture and the solution was cooled at –20 ˚C overnight. The obtained product was filtered and washed well with ether to yield a red-brown product of 1,19-dimethylbiladiene-*a,c* dihydrobromide, 8,12-bis(2′-methoxycarbonylethyl)-1,2,3,7,13,17,18,19-octamethylbiladience-*a,c* dihydrobromide **3** (1.4 g, 83%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 13.31 (br, 2H), 13.25 (br, 2H), 7.11 (s, 2H), 5.22 (s, 2H), 3.45 (s, 6H), 2.83 (t, J = 8 Hz, 4H), 2.70 (s, 6H), 2.28 (s, 6H), 2.25 (s, 6H), 2.03 (t, J = 8 Hz, 4H), 2.01 (s, 6H); HRMS (ESI, positive mode, m/z): $[M–HBr_2]^+$ calcd for $C_{35}H_{45}N_4O_4$, 585.3435; found, 585.3431.

The 1,19-dimethylbiladiene-*a,c* compound **3** (0.50 g, 0.67 mmol) and Co(OAc)•$4H_2O$ (1.7 g, 6.7 mmol) were dissolved in methanol (500 ml) and the solution was stirred for 1 h at room temperature. After removal of the solvent, methanol (15 mL) and water (3 mL) were added to the residue. The mixture was filtered, and 2 mL of aqueous solution of $NaClO_4$•H_2O (0.86 g, 6.1 mmol) was added to the filtrate. After 12 h, the precipitates were collected by filtration and recrystallized from

methanol/dichloromethane/hexane. The product of cobalt corrin derivative, 8,12-bis(2′-methoxycarbonylethyl)-1,2,3,7,13,17,18,19-octamethyltetradehydrocorrin cobalt(II) perchlorate **4**, was obtained as a dark red solid (0.34 g, 68%). HRMS (ESI, positive mode, *m*/*z*): $[M–ClO_4]^+$ calcd for $C_{35}H_{41}N_4O_4Co$, 640.2454; found, 640.2460; UV/Vis: λ_{max} (CH_2Cl_2, nm (abs.)) 274.5 (1.1), 351 (0.92), 489 (0.74), 543.5 (0.37); UV/Vis: λ_{max} (pyridine, nm (abs.)) 506 (0.25), 585 (0.085).

The dimethyl ester of the tetradehydrocorrin cobalt complex **4** (0.10 g, 0.14 mmol), $Ca(OH)_2$ (50 mg, 0.68 mmol) and LiCl (0.40 g, 9.5 mmol) were dissolved in THF (50 mL) and water (50 mL) and then stirred at room temperature for 12 h under an N_2 atmosphere. To the solution, citric acid (1.4 g, 7.3 mmol) was added and the solution was extracted with CH_2Cl_2 (200 mL × 2). The organic phase was dried over anhydrous Na_2SO_4 and the solvent was evaporated under reduced pressure. The residue was dissolved in a minimum amount of CH_2Cl_2 and reprecipitated with hexane to yield tetradehydrocorrin cobalt complex, Co^{II}(TDHC), as a black solid (52.6 mg, 60%). HRMS (ESI, positive mode, *m*/*z*): $[M–Cl]^+$ calcd for $C_{33}H_{37}N_4O_4Co$, 612.214132; found, 612.214126; UV/Vis: λ_{max} (CH_3OH, nm (abs.)) 279.5 (1.2), 495 (0.54), 571 (0.31).



Scheme S1. Synthetic pathway of Co^{II}(TDHC)

Incorporation of Co^{II}(TDHC) into Apomyoglobin

A Co^{II}(TDHC) solution (0.25 mM, 68 mL) in 0.1 M potassium phosphate buffer (pH 7.0) was added dropwise into 68 mL of a solution of apomyoglobin (0.1 mM) with gentle shaking on an ice bath. After equilibrating for 1 h at 4 ºC, the mixture was concentrated by ultrafiltration. To remove the excess cofactor, the protein containing Co^{II}(TDHC) was purified using 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 and ESI-TOF MS spectra of the obtained protein are shown in Fig. 2a and Fig. S1, respectively. The molar coefficient at 510 nm was determined to be 16.4 $mM^{-1}\cdot cm^{-1}$ by ICP-OES measurement.

Reduction of rMb(Co^{II}(TDHC))

The following procedures were performed in a glove box (O_2 < 0.1 ppm). A solution of sodium dithionite (10 mg/mL, 10 μL, 0.57 μmol) in 0.1 M potassium phosphate buffer (pH 7.0) was added to 1 mL of a rMb(Co^{II}(TDHC)) solution (25 μM) in the same buffer at 25 ºC.

EPR Measurements

The measurements of EPR spectra were carried out at the X-band (9.35 GHz) microwave frequency with 100 kHz field modulation and 10 G of modulation amplitude. During EPR measurements, the sample temperature was maintained at 10 K by an Oxford Instruments ESR900 cryostat equipped with a turbo pump to lower the vapor pressure of the liquid He. Each protein solution (0.5 mM) in 0.1 M potassium phosphate buffer (pH 7.0) was placed in a 5 mm tube. The sample was quickly frozen in a cold pentane bath chilled with liquid N_2.

Determination of Co(II)/Co(I) redox potentials

Spectroelectrochemical measurements were carried out at 25 ºC using an optically transparent thin-layer electrode cell (optical path length of 1 mm) in a glove box. A Pt wire counter electrode and a Pt mesh working electrode were used along with an Ag/AgCl reference electrode. The potentials of these electrodes were controlled and measured with a Hokuto Denko HA-301 potentiostat/galvanostat. A solution of rMb(Co^{II}(TDHC)) (0.3 mM) was prepared in 0.1 M potassium phosphate buffer (pH 7.0) containing 2-hydroxy-1,4-naphthoquinone (0.5 M) as an electron mediator. At each applied potential, the electronic absorption spectra were monitored until no further spectral changes were detected. The data were fitted to the Nernst equation (Fig. S6), and the resulting midpoint of the redox potential was determined with reference to the standard hydrogen electrode (SHE).

Protein Crystallization

Crystallization of rMb(Co^{II}(TDHC)) was performed at 25 ºC using the hanging-drop vapor-diffusion method. The drops were prepared by mixing of 5 μL of 10 mg/mL protein in 0.1 M Tris-HCl (pH 7.4) buffer with 5 μL of a reservoir solution containing 3.4 M ammonium sulfate and 0.1 M Tris-HCl (pH 7.4). Rosette-shaped crystals grew within a week and leaflets were separated for data collection. The crystal was soaked in a cryoprotectant solution (6–12% glycerol in a reservoir solution), fished with a standard nylon loop, and flash-cooled in an N_2 gas stream at 100 K.

The crystal of rMb(Co^{II}(TDHC)) was also obtained by the method described above with a reservoir solution containing 3.4 M ammonium sulfate, 0.1 M Tris-HCl (pH 7.4) and 10% trehalose. It was then soaked in the reservoir solution with 5 mg/mL sodium dithionite. A color change from dark red to pink was observed within 1 min and the crystal was then fished and flash-cooled in a stream of N_2 gas stream.

Crystal Structure Determination and Refinement

X-ray diffraction data were collected using the synchrotron radiation sources of the beamlines at SPring-8 (Harima, Japan) or at Photon Factory (Tsukuba, Japan). Diffraction data were processed with the program *HKL2000*. The structures were determined by the molecular replacement method using the program *MOLREP* 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 structures were visualized and modified using the program *COOT*. Since electron density indicated that two enantiomers (*RR* and *SS*) of TDHC are present in the heme pocket, we assigned an approximate *RR*:*SS* occupancy ratio of 0.65:0.35. Data collection and refinement statistics are summarized in Table S1. The final atomic coordinates and structure factor amplitudes were deposited in the Protein Data Bank (PDB). These have been assigned the following ID numbers: 3WFT for rMb(Co^{II}(TDHC)) and 3WFU for rMb(Co^{I}(TDHC)).

References

S1. T. Matsuo, A. Hayashi, M. Abe, T. Matsuda, Y. Hisaeda, T. Hayashi, *J. Am. Chem. Soc.* 2009, **131**, 15124–15125.

S2. K. L. Swanson, K. M. Snow, D. Jeyakumar, K. M. Smith, *Tetrahedron* 1991, **47**, 685–696.

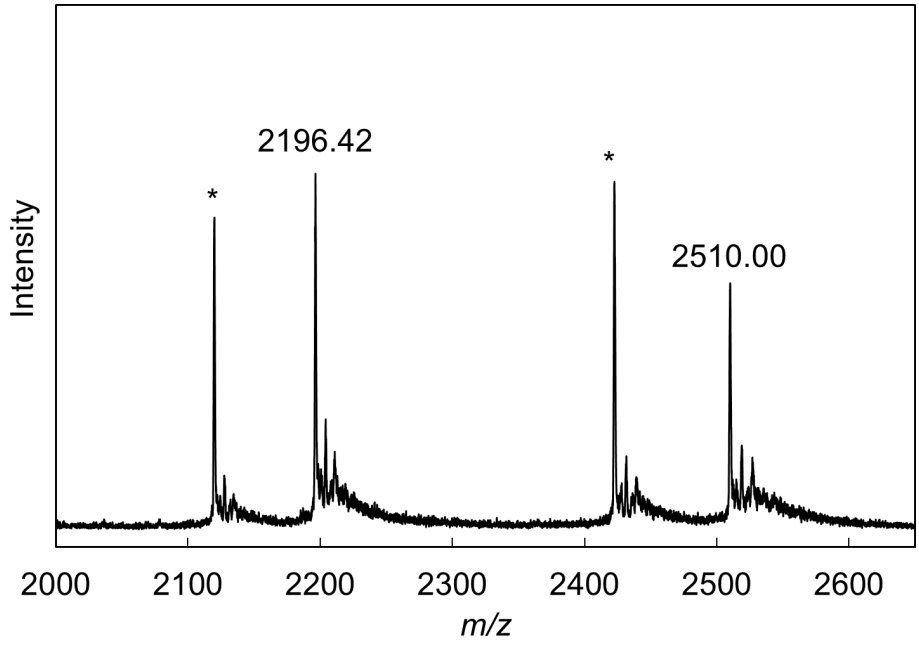


Fig. S1. ESI-TOF MS (positive mode) spectrum of rMb(Co^{II}(TDHC)). Two characteristic peaks are consistent with the calculated mass numbers as follows. *m*/*z* (*z*) for rMb(Co^{II}(TDHC)): 2196.38 (8+), 2510.18 (7+). Peaks with the asterisks were assigned as multiply ionized species of apomyoglobin.

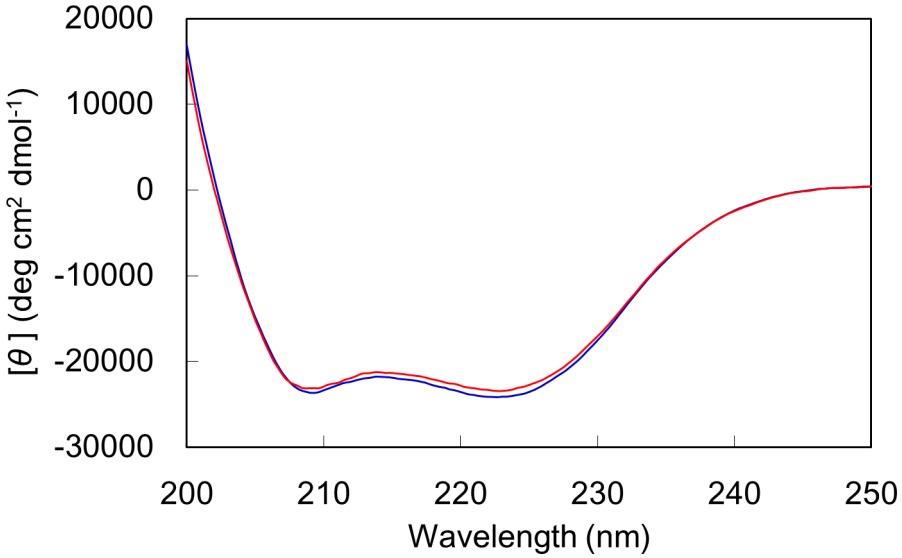


Fig. S2. Blue and red circular dichroism (CD) spectra represent rMb(Co^{II}(TDHC)) and rMb(Co^{I}(TDHC)), respectively. Conditions: [protein] = 0.018 mM in 0.1 M potassium phosphate buffer at pH 7.0, at 25 °C under N_2 atmosphere.

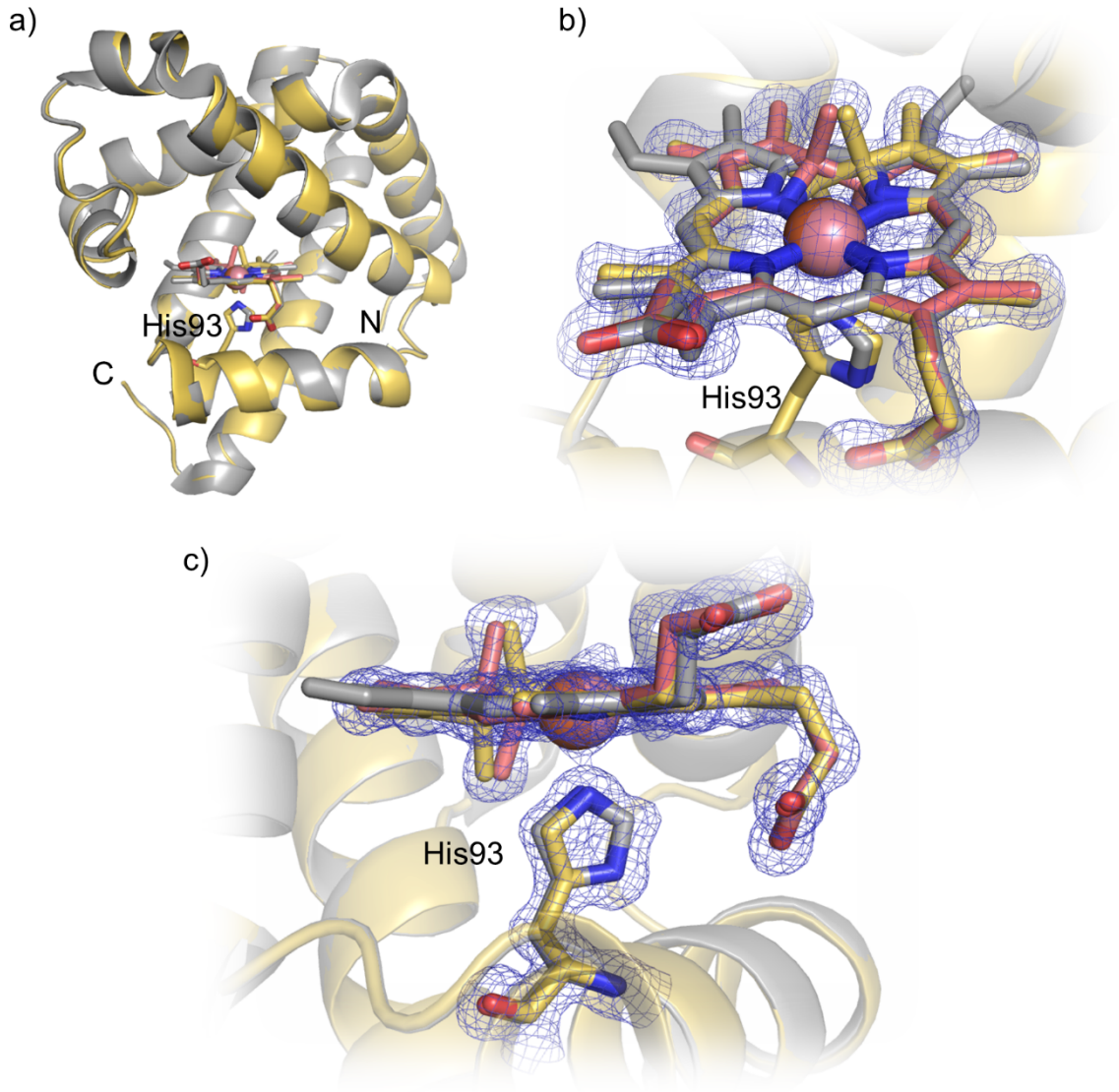


Fig. S3. Superimposed structure of rMb(Co^{II}(TDHC)) with native myoglobin. (a) Whole protein structures, rMb(Co^{II}(TDHC)) (yellow) and native myoglobin (gray) (PDB: 2V1K). (b) and (c) The heme pocket structures. Co^{II}(TDHC) (enantiomeric 1*R*,19*R*; pink, 1*S*,19*S*; yellow) and native heme (gray) are represented as stick models, respectively. The $2F_o - F_c$ electron density maps (σ = 1.0) around the cofactor and the cofactor with His93 residue of rMb(Co^{II}(TDHC)) are also shown in (b) and (c), respectively.

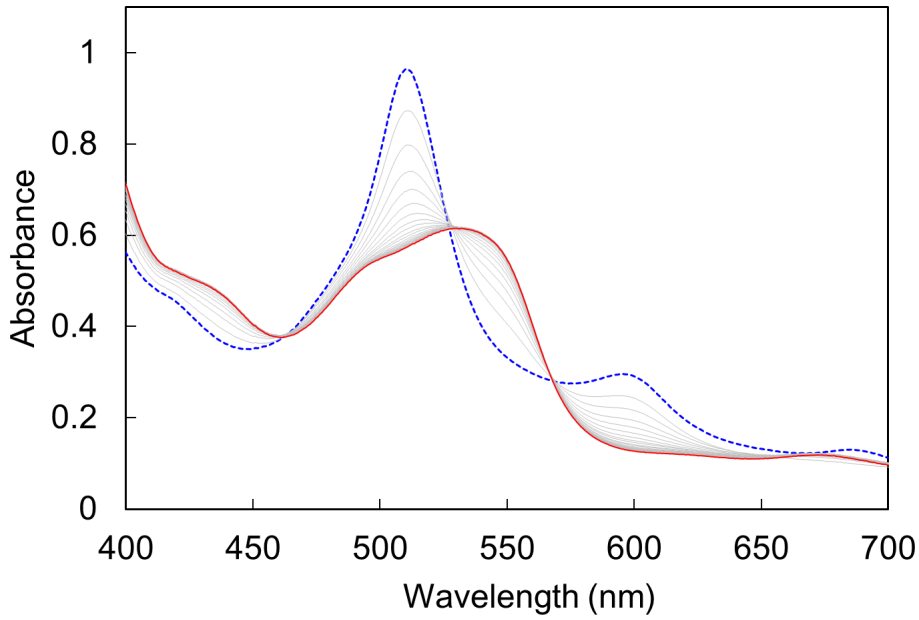


Fig. S4. UV-vis spectral changes of rMb(Co^{II}(TDHC)) upon addition of dithionite. Initial and final spectra are represented as dashed blue and red solid lines, respectively. The final spectrum (solid red line) is assigned as rMb(Co^{I}(TDHC)) after the addition of dithionite over 2 s. Conditions: [protein] = 0.12 mM in 0.1 M potassium phosphate buffer at pH 7.0, [dithionite] = 2.6 mM, at 25 °C under N_2 atmosphere.

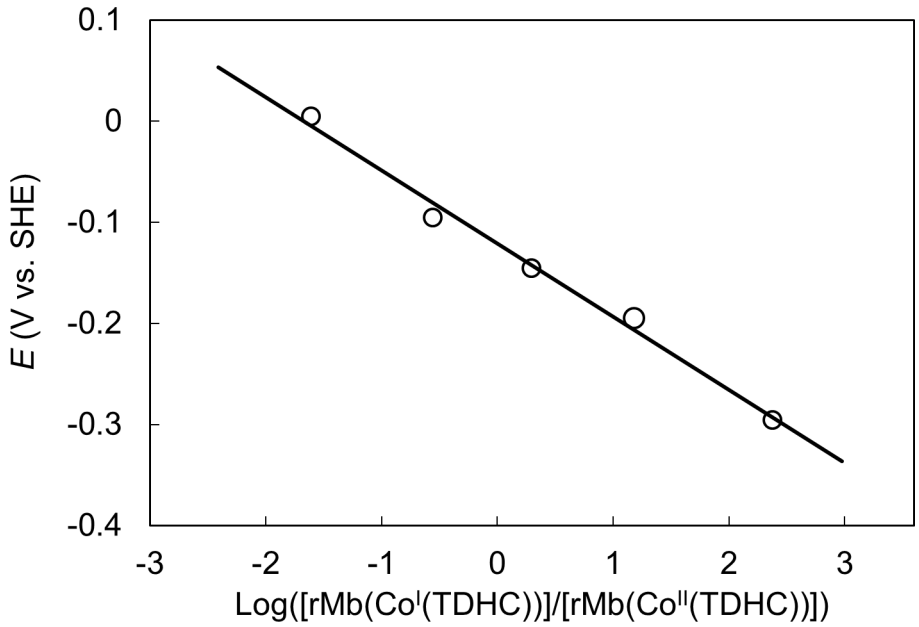


Fig. S5. Nernst plots obtained by spectroelectrochemistry of rMb(Co(TDHC)). The plots \obtained from the absorption changes at 600 nm of rMb(Co^{I}(TDHC))/rMb(Co^{II}(TDHC)) in spectroelectrochemical measurements.

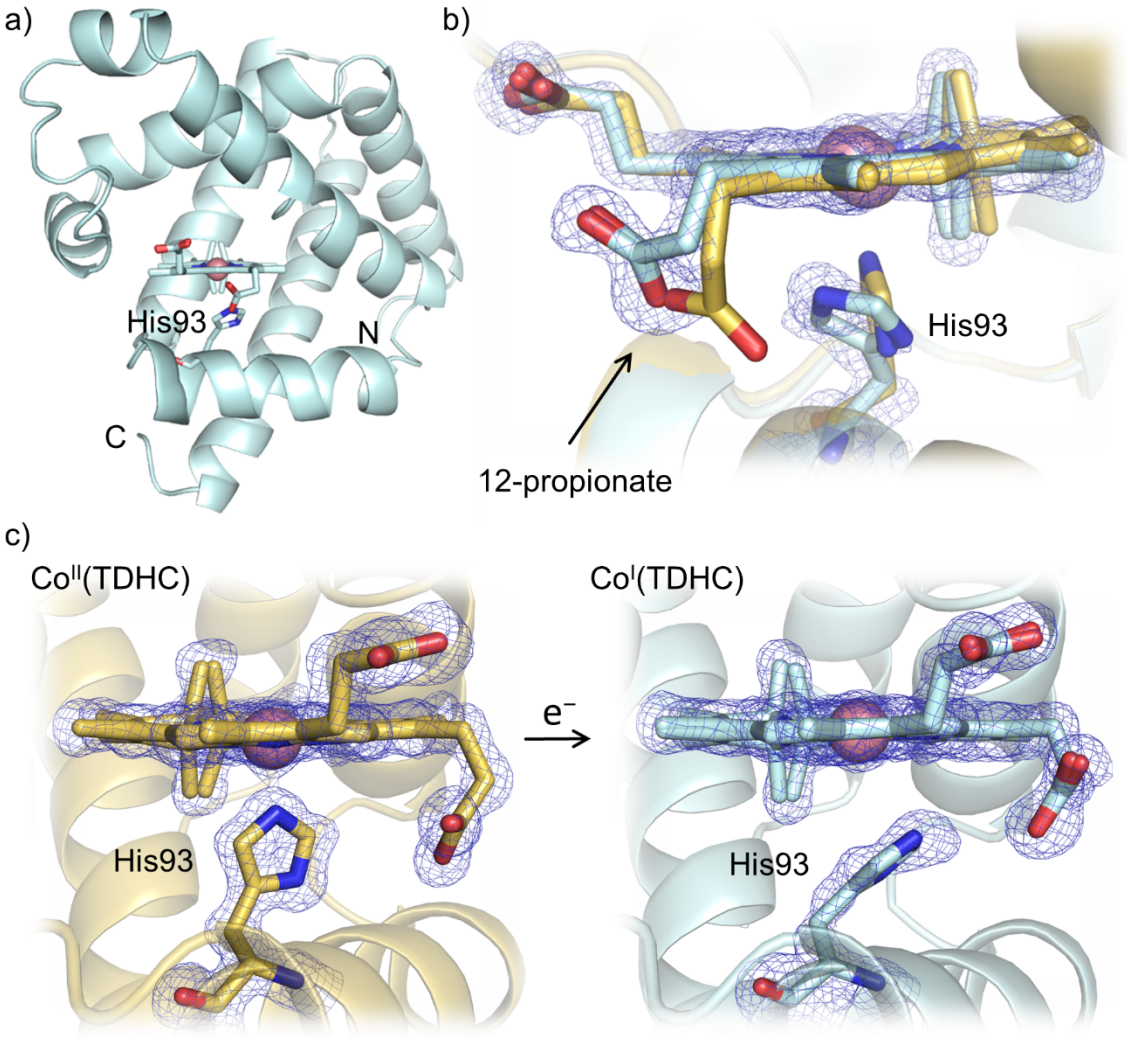


Fig. S6. Crystal structure of rMb(Co^{I}(TDHC)). (a) Whole protein structure of rMb(Co^{I}(TDHC)) and (b) superimposition of rMb(Co^{I}(TDHC)) (light blue) and rMb(Co^{II}(TDHC)) (yellow) in the heme pocket. The $2F_o - F_c$ electron density map (σ = 1.0) is shown in blue grid around the cofactor and the His93 residue of rMb(Co^{I}(TDHC)). (c) The structural changes by reduction of rMb(Co^{II}(TDHC)) to rMb(Co^{I}(TDHC)). The $2F_o - F_c$ electron density maps (σ = 1.0) are shown in blue grids around the cofactors and the His93 residues.

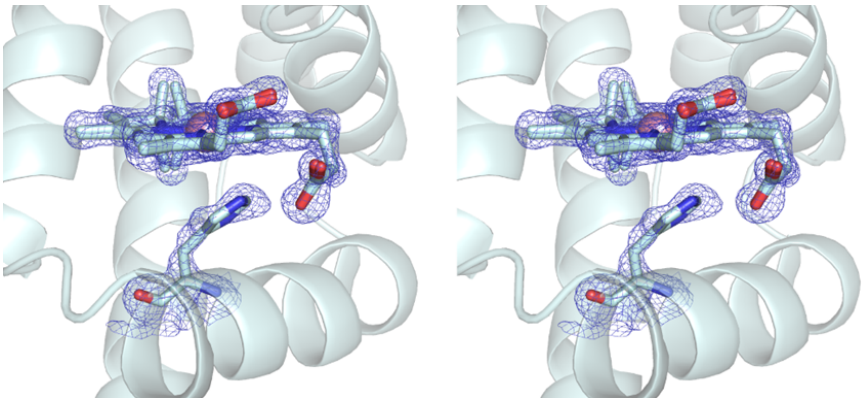


Fig. S7. Stereoview of the heme pocket in rMb(Co^{I}(TDHC)). The $2F_o - F_c$ electron density is shown in blue grid (contoured at 1.0 σ) around the cofactor and the His93 residue of rMb(Co^{I}(TDHC)).

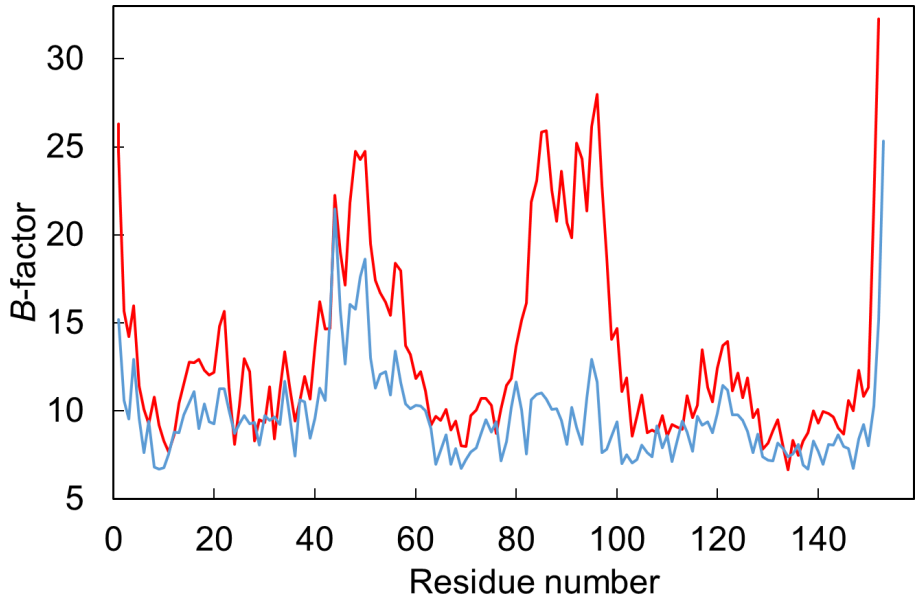


Fig. S8. *B*-factor of the reconstituted proteins. Summary of *B*-factor values of Cα atoms for rMb(Co^{II}(TDHC)) (blue line) and rMb(Co^{I}(TDHC)) (red line).

Table S1. Data collection and refinement statistics for reconstituted horse heart myoglobins

	rMb(Co^{II}(TDHC))	rMb(Co^{I}(TDHC))
Data collection		
X-ray source	Photon Factory BL-17A	SPring-8 BL44XU
Detector	ADSC Quantum 315r	Rayonix MX225HE
Wavelength (Å)	1.00000	0.90000
Resolution (Å) (outer shell)	50 – 1.30 (1.35 – 1.30)	50 – 1.35 (1.40 – 1.35)
Space group	$P2_1$	$P2_1$
Unit cell parameters (Å, deg.)	a = 34.98, b = 28.99, c = 63.21, β = 106.14	a = 34.84, b = 28.72, c = 63.35, β = 105.60
No. of total / unique reflections	78,300 / 28,032	69,170 / 25,415
Completeness (%)	92.8 (90.1)	95.2 (92.2)
R_{sym}†	4.1 (22.2)	3.7 (22.1)
$I/\sigma(I)$	26.9 (4.3)	28.9 (4.0)
Refinement		
Resolution (Å)	15 – 1.30	15 – 1.35
No. of reflections	26,544	24,026
R_{cryst} / R_{free} (%)	12.6 / 18.3	14.3 / 19.7
Mean B-factor (Å^2)	13.6	17.3
No. of non-H atoms	1,490	1,427
Rmsd from ideal		
Bond lengths (Å) / angles (deg.)	0.026 / 3.065	0.026 / 3.000
PDB ID	3WFT	3WFU

† $R_{sym} = \Sigma_{hkl}\Sigma_i |I_i(hkl) - \langle I(hkl)\rangle| / \Sigma_{hkl}\Sigma_i I_i(hkl)$, where $I_i(hkl)$ is the value of the ith measurement of the intensity of a reflection, $\langle I(hkl)\rangle$ is the mean value of the intensity of that reflection and the summation is over all measurements.