

## Supplementary Information

### DNA cleavage with oxymyoglobin and cysteine-introduced metmyoglobin

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#### Contents

#### Experimental Section

<b>Protein preparation</b>	p. S2
<b>Spectroscopic measurements</b>	p. S3
<b>SDS-PAGE analysis</b>	p. S3
<b>DNA cleavage</b>	p. S3
<b>T4-ligase assay and cell transformation</b>	p. S4
<b>Figure S1.</b> Agarose gel electrophoresis of pUC19 DNA after incubation with horse oxyMb and metMb.	p. S5
<b>Figure S2.</b> T4-ligase assay and cell transformation.	p. S6
<b>Figure S3.</b> Structure of sperm whale Mb.	p. S7
<b>Figure S4.</b> Absorption spectra of K96C sperm whale metMb before and after incubation at low protein concentration under air.	p. S8
<b>Figure S5.</b> Absorption spectra of wild-type sperm whale metMb before and after incubation under air.	p. S9
<b>Figure S6.</b> SDS-PAGE analysis of K96C sperm whale metMb before and after incubation at high protein concentration under air.	p. S10
<b>Figure S7.</b> Agarose gel electrophoresis of pUC19 DNA after incubation with K96C sperm whale metMb in the presence of reagents.	p. S11
<b>Figure S8.</b> Agarose gel electrophoresis of pUC19 DNA after incubation with the monomer and dimer of K96C sperm whale metMb.	p. S12
<b>Figure S9.</b> Agarose gel electrophoresis of pUC19 DNA after incubation with V66C or K102C sperm whale metMb.	p. S13

## Experimental Section

**Protein preparation.** Recombinant sperm whale Mb was overproduced in *E. coli* TB-1 cells. MetMb was purified by the reported method with a few modifications.<sup>1,2</sup> Dithiothreitol (2 mM) was added during extraction of Mb from the cells for cysteine-introduced Mb (K96C, V66C, and K102C Mb). Hemin (0.05 mM) was added to the extracted Mb solution, and the obtained solution was stirred at 4 °C for 30 min. After purification with a DEAE column, Mb was completely oxidized with a small amount of potassium ferricyanide (Sigma Aldrich) dissolved in 20 mM potassium phosphate buffer, pH 6.0 buffer and purified with a CM-52 column (Whatman). The buffer for washing and eluting cysteine-introduced metMb in the CM column was bubbled with N<sub>2</sub> before use. MetMb was washed with N<sub>2</sub>-bubbled buffer thoroughly during purification with the CM column to avoid the effect of ferricyanide ion being introduced and ferrocyanide ion, which may be produced. The buffer of purified metMb was exchanged to 10 mM potassium phosphate buffer, pH 7.2, with a PD-10 column (Amersham Biosciences) before each measurement. The purity of the protein was confirmed by SDS-PAGE analysis and the absorbance (Abs) ratio between at 280 and 409 nm ( $Abs_{409}/Abs_{280} > 4.6$ ). The concentration of the protein was adjusted with the absorption spectrum.

Powders of horse skeleton muscle metMb (Sigma Aldrich) were dissolved in 10 mM potassium phosphate buffer, pH 7.2. The metMb solution was purified by size exclusion chromatography with a 10/300 Superdex 75 GL gel column (GE Healthcare, Buckinghamshire) using a fast protein liquid chromatography (FPLC) system (Biologic DuoFlow 10, Bio-Rad, CA) (flow rate, 0.4 mL/min; monitoring wavelength, 409 nm; solvent, 50 mM potassium phosphate buffer, pH 7.0; temperature, 4 °C). The metMb fractions were collected and the buffer was exchanged to 10 mM potassium phosphate buffer, pH 7.2, with a Centricon tube (Millipore, Bedford, USA).

For preparation of oxyMb, horse or sperm whale metMb solution (10 μM, 1ml) in 10 mM potassium phosphate buffer, pH 7.2, was introduced into the cuvette. The cuvette containing metMb was degassed and refilled with N<sub>2</sub> using a vacuum line. N<sub>2</sub> was introduced separately into the test tubes containing sodium dithionite powder (1.74 mg) and 10 mM potassium phosphate buffer, pH 7.2. N<sub>2</sub>-purged 10 mM potassium phosphate buffer, pH 7.2 (1.0 ml), was added to the test tube containing sodium dithionite powder (final sodium dithionite concentration, 10 mM). The sodium dithionite solution (50 μl, 10 mM) was added to the cuvette containing the horse or sperm whale metMb solution (10 μM, 1 ml). The mixed solution in the cuvette was degassed and purged with N<sub>2</sub>, which resulted in formation of deoxyMb. The deoxyMb solution was degassed and purged with O<sub>2</sub> to form oxyMb. The PD-10 column was used to remove sodium dithionite from the solution. The obtained oxyMb solution was concentrated using a centricon tube. The

concentration of the oxyMb was adjusted with the absorption spectrum.

Dimer of K96C sperm whale metMb was prepared by incubation of a concentrated solution (~100  $\mu$ M) of its monomer at 37 °C for 24 h under air. The solution containing the monomer and dimer was purified by size exclusion chromatography with the 10/300 Superdex 75 GL gel column (GE Healthcare) using the FPLC system (Biologic DuoFlow 10, Bio-Rad) (flow rate, 0.4 mL/min; monitoring wavelength, 409 nm; solvent, 50 mM potassium phosphate buffer, pH 7.0; temperature, 4 °C). The fractions containing the dimer were collected, and the buffer was exchanged to 10 mM potassium phosphate buffer, pH 7.2, with a Centricon tube (Millipore). The obtained Mb dimer was oxidized with of a small amount of potassium ferricyanide dissolved in 10 mM potassium phosphate buffer, pH 7.2, and the Mb solution was passed through the DEAE column (Whatman) to remove ferricyanide and ferrocyanide ions. The concentration of the K96C metMb dimer was adjusted with the absorption spectrum, and the dimer was used immediately for the DNA cleavage experiment.

**Spectroscopic measurements.** The absorption spectra of metMb was recorded on a Shimadzu UV-3100PC spectrophotometer at 37 °C. MetMb (8  $\mu$ M) in 10 mM potassium phosphate buffer, pH 7.2, was transferred into a quartz cell. The cell containing the Mb solution was degassed and refilled with N<sub>2</sub> using a vacuum line, and the initial absorption spectrum was measured before incubation.

**SDS-PAGE analysis.** K96C sperm whale metMb (120  $\mu$ M) in 10 mM potassium phosphate buffer, pH 7.2, was placed under air at 37 °C for 6 h, and subsequently diluted to 4  $\mu$ M with 10 mM potassium phosphate buffer, pH 7.2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis was performed for each sample using a 15 % separation gel with and without 2-mercaptoethanol. The obtained gel was stained with CBB R-250. For reduction of disulfide bonds, metMb was incubated with 2-mercaptoethanol (5 % v/v) at 95°C for 1 min.

**DNA cleavage.** Plasmid DNA (pUC19 DNA) was obtained by over-expression in *E-coli* DH5 $\alpha$  cells. The obtained DNA was purified with a QIAprep spin miniprep kit-250 (QIAGEN). Sterile ultra-pure water was used to prepare a DNA stock solution. The purity of DNA was checked by gel electrophoresis. The DNA solution was stored at -80 °C.

DNA cleavage was monitored by 1 % agarose gel electrophoresis, following the conversion of the supercoiled DNA form to the nicked circular and linear forms. MetMb in 10 mM potassium phosphate buffer, pH 7.2, was mixed with supercoiled pUC19 DNA (metMb, 8  $\mu$ M;

DNA, 0.02 µg/µL). DNA cleavage reaction was carried out by incubating the reaction mixture at 37 °C, and quenched at various time intervals by placing the sample on ice with an addition of loading buffer. The quenched samples were loaded to 1 % agarose gel, and electrophoresis was carried out for 45 min at 100 V using TAE buffer (Tris-acetate-EDTA, pH 8.0). After electrophoresis, the gel was stained with an ethidium bromide solution (0.25 µg/mL). The bands of the supercoiled, nicked circular, and linear DNA forms were visualized using the Bio-Rad gel documentation system (ChemiDoc XRS). 4',6-diamidino-2-phenylindole (DAPI) (Sigma Aldrich), methyl green (Sigma Aldrich), histidine (Wako, Osaka), sodium azide (Wako), ascorbic acid (Wako), potassium iodide (Wako), superoxide dismutase (SOD) from bovine erythrocytes (Cu/Zn type) (Wako), and catalase from bovine liver (Wako) were used to investigate their inhibitory effects on the DNA cleavage activity of Mb.

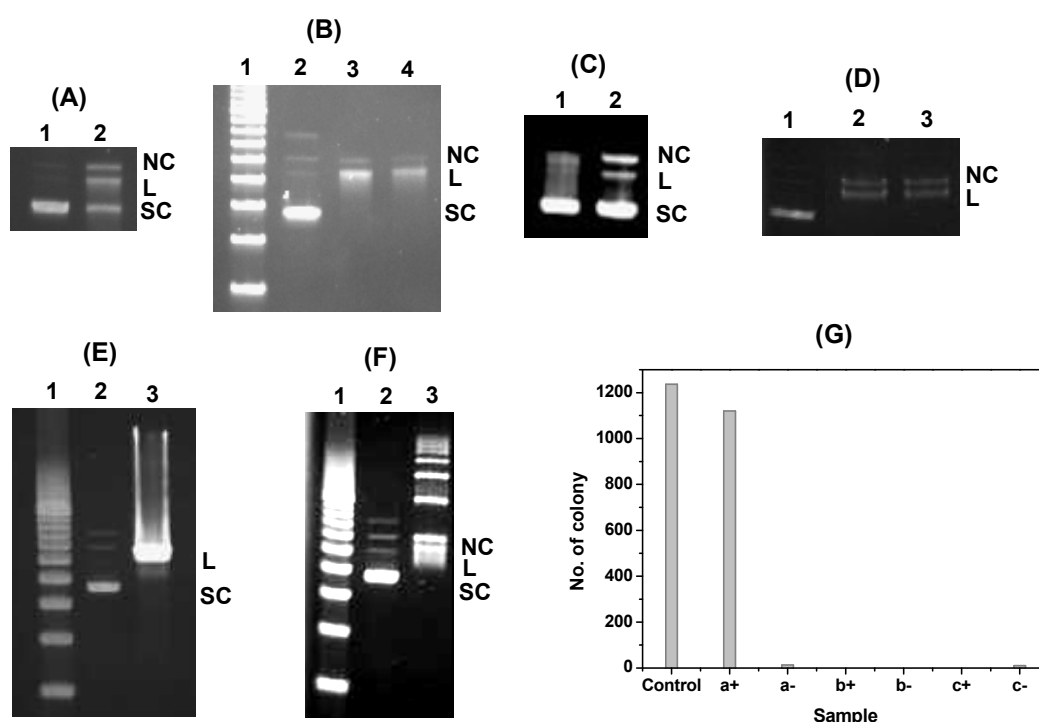
**T4-ligase assay and cell transformation.** Enzymatic assay was performed using T4 DNA ligase (Takara Bio, Otsu, Japan). The cleaved DNA products (nicked circular and linear forms) by WT sperm whale oxyMb and K96C sperm whale metMb were isolated from the gel after electrophoresis. Positive and negative controls were carried out using undigested pUC19 DNA and pUC19 DNA treated with the restriction enzyme, *Bam*HI (Takara Bio). The cleaved DNA was isolated from the reaction mixtures by using a purification kit (SV Gel and PCR Clean-Up System, Promega). The DNA concentration was measured using a BioSpec-nano spectrophotometer (Shimadzu, Kyoto). The isolated DNA solution was further used for T4-ligase assay. DNA products of pUC19 DNA (70 ng) obtained by incubation with K96C sperm whale Mb or digestion with *Bam*HI were incubated with T4 DNA ligase (final concentration: 4 units/µl) and ligation buffer (Takara Bio) at 16 °C for 18 h. The DNA (15 ng) solution after T4-ligation was transformed to DH5α competent cells. The cells were heat-shocked at 42 °C for 90 s and kept on ice for 2 min, followed by an addition of 100 µl SOC medium, and incubated at 37 °C for 2 h. The obtained samples were plated (size of plate, 90 × 15 mm) on LB-agar containing ampicillin (Wako) (100 mg/ml). The plates were incubated at 37 °C for 16 h, and the numbers of colonies were counted.

## References

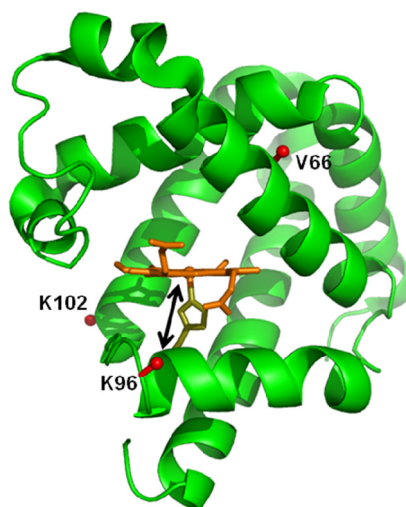
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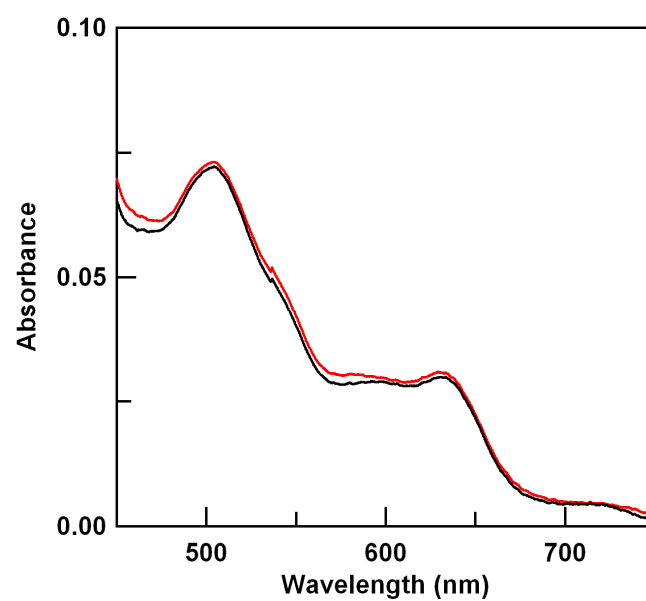
**Figure S1.** Agarose gel electrophoresis of pUC19 DNA after incubation with horse oxyMb and metMb. Incubation was performed under air at 37 °C for 5 and 20 min for oxyMb and metMb, respectively. SC, NC, and L represent supercoiled, nicked circular, and linear forms of DNA, respectively. Incubation of pUC19 DNA (0.02  $\mu\text{g}/\mu\text{L}$ ) with Mb (8  $\mu\text{M}$ ) was performed in 10 mM potassium phosphate buffer, pH 7.2.



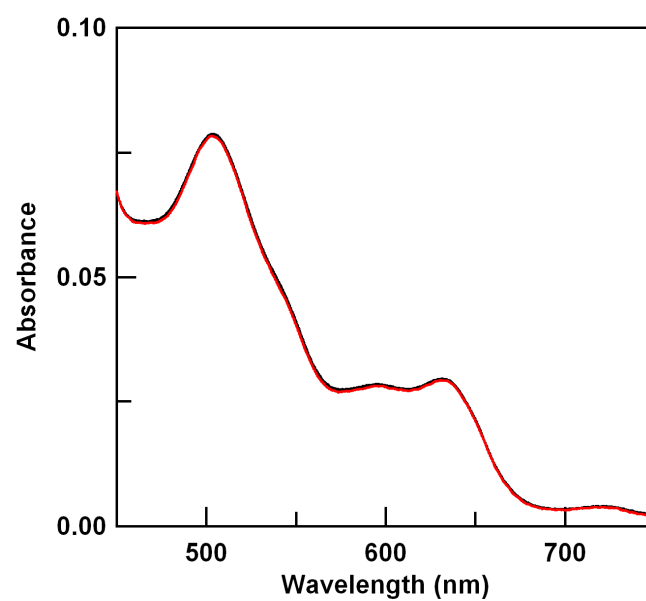
**Figure S2.** T4-ligase assay and cell transformation. (A) Agarose gel electrophoresis of pUC19 DNA after incubation with WT sperm whale oxyMb (8  $\mu$ M) at 37 °C for 20 min: Lane 1, pUC19 DNA (0.02  $\mu$ g/ $\mu$ L); lane 2, pUC19 DNA after incubation with oxyMb. (B) Agarose gel electrophoresis of DNA products after treatment with T4-ligase: Lane 1, DNA marker; lane 2, pUC19 DNA (0.02  $\mu$ g/ $\mu$ L); lane 3, isolated pUC19 DNA after incubation with WT sperm whale oxyMb; lane 4, DNA products after incubation with oxyMb, isolation, and ligation with T4 ligase (4 units/ $\mu$ L) at 16 °C for 18 h. (C) Agarose gel electrophoresis of pUC19 DNA after incubation with K96C sperm whale metMb (8  $\mu$ M) at 37 °C for 120 min: Lane 1, pUC19 DNA (0.02  $\mu$ g/ $\mu$ L); lane 2, pUC19 DNA after incubation with K96C metMb. (D) Agarose gel electrophoresis of pUC19 DNA after treatment with T4-ligase: Lane 1, pUC19 DNA (0.02  $\mu$ g/ $\mu$ L); lane 2, isolated pUC19 DNA after incubation with K96C metMb; lane 3, DNA products after reaction with K96C metMb, isolation, and ligation with T4 ligase (4 units/ $\mu$ L). (E) Agarose gel electrophoresis of pUC19 DNA after digestion with *Bam*HI: Lane 1, DNA marker; Lane 2, pUC19 DNA (0.02  $\mu$ g/ $\mu$ L); lane 3, DNA products after reaction with *Bam*HI. (F) Agarose gel electrophoresis of pUC19 DNA after treatment with T4-ligase: Lane 1, DNA marker; Lane 2, pUC19 DNA (0.02  $\mu$ g/ $\mu$ L); lane 3, DNA products after reaction with *Bam*HI, isolation, and ligation with T4 ligase (4 units/ $\mu$ L). The DNA solution after treatment with *Bam*HI or K96C metMb was incubated with T4-ligase at 16 °C for 18 h for ligation. (G) Ligation efficiency analysis of cleaved pUC19 DNA products. Numbers of colonies obtained after ligation and subsequent cultivation are shown. The cleaved DNA products obtained by treatment with (a) *Bam*HI, (b) WT oxyMb, and (c) K96C metMb were incubated (+) with and (-) without T4 ligase prior to transformation to DH5 $\alpha$  cells.



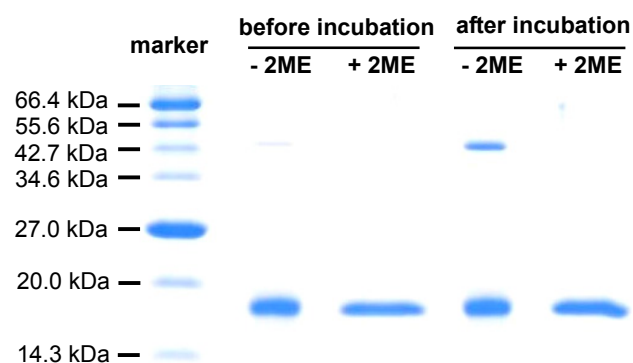
**Figure S3.** Structure of sperm whale Mb (PDB: 1DUK). The heme, modified residues (Val66, Lys96, and Lys102), and proximal histidine are shown as orange, red, and yellow stick models, respectively. K96 and the heme are located close to each other, less than 10 Å away in distance.



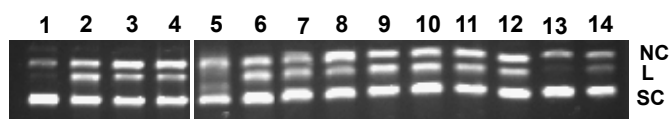
**Figure S4.** Absorption spectra of K96C sperm whale metMb before (black) and after (red) incubation in 10 mM potassium phosphate buffer, pH 7.2, under air at 37 °C for 6 h. K96C metMb was incubated at 8  $\mu$ M protein concentration. Absorption spectra were measured at 20 °C.



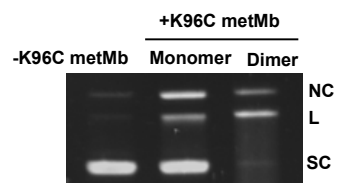
**Figure S5.** Absorption spectra of wild-type sperm whale metMb before (black) and after (red) incubation in 10 mM potassium phosphate buffer, pH 7.2, under air at 37 °C for 6 h. Wild-type metMb was incubated at 460  $\mu$ M protein concentration. Absorption spectra were measured at 20 °C.



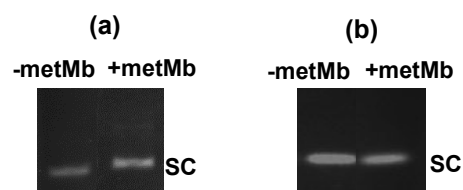
**Figure S6.** SDS-PAGE analysis of K96C sperm whale metMb before and after incubation at high protein concentration (120  $\mu$ M) in 10 mM potassium phosphate buffer, pH 7.2, under air at 37  $^{\circ}$ C for 6 h. The analysis was performed with and without 2-mercaptoethanol (2ME). The dimer band disappeared with an addition of 2-mercaptoethanol.



**Figure S7.** Agarose gel electrophoresis of pUC19 DNA after incubation with K96C sperm whale metMb in the presence reagents at 37 °C for 120 min: Lane 1, pUC19 DNA; lanes 2–11, pUC19 DNA after incubation with K96C metMb; lane 3, with 50 mM NaCl; lane 4, with 50 mM potassium iodide; lane 5, with 25  $\mu$ M ascorbic acid; lane 6, with 50 mM mannitol; lane 7, with 25 mM histidine; lane 8, with 25 mM sodium azide; lane 9, with SOD (15 units); lane 10, with SOD (30 units); lane 11, with catalase (15 units); lane 12, with catalase (30 units); lane 13, with 50  $\mu$ M DAPI; lane 14, with 50  $\mu$ M methyl green. SC, NC, and L represent supercoiled, nicked circular, and linear forms of DNA, respectively. Incubation of pUC19 DNA (0.02  $\mu$ g/ $\mu$ L) with K96C Mb (8  $\mu$ M) was performed in 10 mM potassium phosphate buffer, pH 7.2.



**Figure S8.** Agarose gel electrophoresis of pUC19 DNA after incubation with the monomer and dimer of sperm whale K96C metMb. The dimer of K96C metMb was formed with an intermolecular disulfide bond. Incubation of pUC19 DNA (0.02  $\mu\text{g}/\mu\text{L}$ ) with mutant Mb (8  $\mu\text{M}$ ) was performed in 10 mM potassium phosphate buffer, pH 7.2, at 37 °C for 120 min.



**Figure S9.** Agarose gel electrophoresis of pUC19 DNA after incubation with (a) V66C and (b) K102C sperm whale metMb. Incubation of pUC19 DNA (0.02  $\mu\text{g}/\mu\text{L}$ ) with mutant Mb (8  $\mu\text{M}$ ) was performed in 10 mM potassium phosphate buffer, pH 7.2, at 37  $^{\circ}\text{C}$  for 120 min.