Supporting Information for

Bioinspired design of artificial peroxidase: Introducing key residues of native peroxidases into F43Y myoglobin with a Tyr-heme cross-link

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1. Materials and Methods

1.1 Protein preparation

WT sperm whale Mb was expressed using the Mb gene of pMbt7-7 and purified using the procedure described previously [1]. The gene of F43Y/T67R/F138W Mb were constructed using the QuickChange Site Directed Mutagenesis Kit (Stratagene) using F43Y/T67R Mb as a template [2]. The mutation was confirmed by DNA sequencing assay. F43Y/T67R/F138W Mb was expressed in BL21(DE3) host cells and purified by using the procedure described previously for F43Y Mb single mutant.

1.2 UV-Vis spectroscopy

UV-Vis spectra were recorded in 100 mM KH_2PO_4 (pH 7.0) on a Hewlett-Packard 8453 diode array spectrometer. The pyridine hemochrome spectrum was obtained by using 10 μM proteins in 19 % (vol/vol) pyridine and 0.15 M NaOH, and the protein was reduced by a small amount of sodium dithionite. Protein concentration was determined with an extinction coefficient of ε_{404} = 150 ± 5 mM^{-1} cm^{-1}, as calculated using the standard hemochromagen method [3].

1.3 Mass spectrometry

Protein mass spectrum measurement was carried out on an G2-XS QTOF mass spectrometry (Waters). The F43Y/T67R/F138W Mb sample was diluted with 0.1 M acetic acid (pH 3.0) to ~20 μM. The protein solution was mixed with 1% formic acid, which was transferred into the mass spectrometer chamber for measurement under positive mode. The multiple m/z peaks were transformed to the protein molecular weight by using software MaxEnt1.

1.4 Acid titration study

Acid titration study of F43Y/T67R/F138W Mb was recorded on a Hewlett-Packard 8453 diode array spectrometer at room temperature. The pH value of the protein solution (10 μM) was adjusted by addition of a small amount of highly concentrated HCl (10 M), and measured directly in the cuvette using a microelectrode (type LE422) connected to a Mettler Toledo pH meter (type FE20). The plot of normalized changes at 404 nm against pH, which was divided into two curves and fitted to the Boltzmann function, and pK_{a1} and pK_{a2} values were calculated, respectively.

$$A = A_2 + (A_1 - A_2)/(1+ e^{(pH-pKa)/dpH})$$

Here, A is the absorbance of Soret band; A_1 and A_2 are the initial and final absorbance of Soret band, respectively.

1.5 Stopped-flow spectroscopy

The effect of pH (3.2-9.2) on the peroxidase activity of F43Y/T67R/F138W Mb was investigated on a stopped-flow spectrophotometer (SF-61DX2 Hi-Tech KinetAsystTM) at 25 ^{o}C, by using guaiacol as a substrate and H_2O_2 (75 mM) as the oxidant, respectively. The steady-state kinetic parameters for the peroxidase activity determined by using both guaiacol and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) as the representative substrates and H_2O_2 as the oxidant. One syringe contains 2 μM protein (in 100 mM KH_2PO_4 buffer, pH 7.0) in presence of guaiacol with various concentrations (0.1-10 mM), and the second syringe contains 150 mM H_2O_2. The reaction using guaiacol as a substrate was followed by monitoring the change in absorbance of the product at 470 nm, as proposed to be a dimeric form of guaiacol (3, 3'-dimethoxy-4, 4'-biphenylquinone) [4]. The reaction using ABTS as a substrate was followed by monitoring the formation of the $ABTS^{+\cdot}$ cation radical at 660 nm [5]. The initial rate was calculated based on the initial linear changes using an extinction coefficient of ε_{470} = 26.6 $mM^{-1}\cdot cm^{-1}$ [6], and ε_{660nm} = 14.0 $mM^{-1}\cdot cm^{-1}$ [5], respectively. The curve of initial rates *versus* substrate concentrations was fitted to the Michaelis-Menten equation:

$$\upsilon/[\text{protein}] = k_{cat}[\text{substrate}]/(K_m + [\text{substrate}])$$

Control experiments were performed for F43Y/T67R Mb, F43Y Mb and WT Mb under the same conditions.

1.6 ITC studies

Isothermal titration calorimetry (ITC) measurements were performed on a Microcal VP-ITC microcalorimeter (GE life sciences). Both protein and ABTS solution were thoroughly degassed in a ThermoVal apparatus (Microcal). For titration experiment, ~1.5 mL of F43Y/T67R/F138W Mb (10 μM) in potassium phosphate buffer (50 mM, pH 7.0) was placed in the reaction cell, and a solution of ABTS (0.2 mM) was injected over 20 sec with a total of 25 injections (2 μL for the first injection and 10 μL for later injections), with a 150 sec interval between each injection. The reaction cell was continuously stirred at 502 rpm, and heat changes were recorded at

25 ˚C. Control experiments were performed for F43Y/T67R Mb, F43Y Mb and WT Mb under the same conditions. The data were analyzed and the binding isotherm was fitted to a single-site model in the Origin 7.0 software (GE life sciences).

References:

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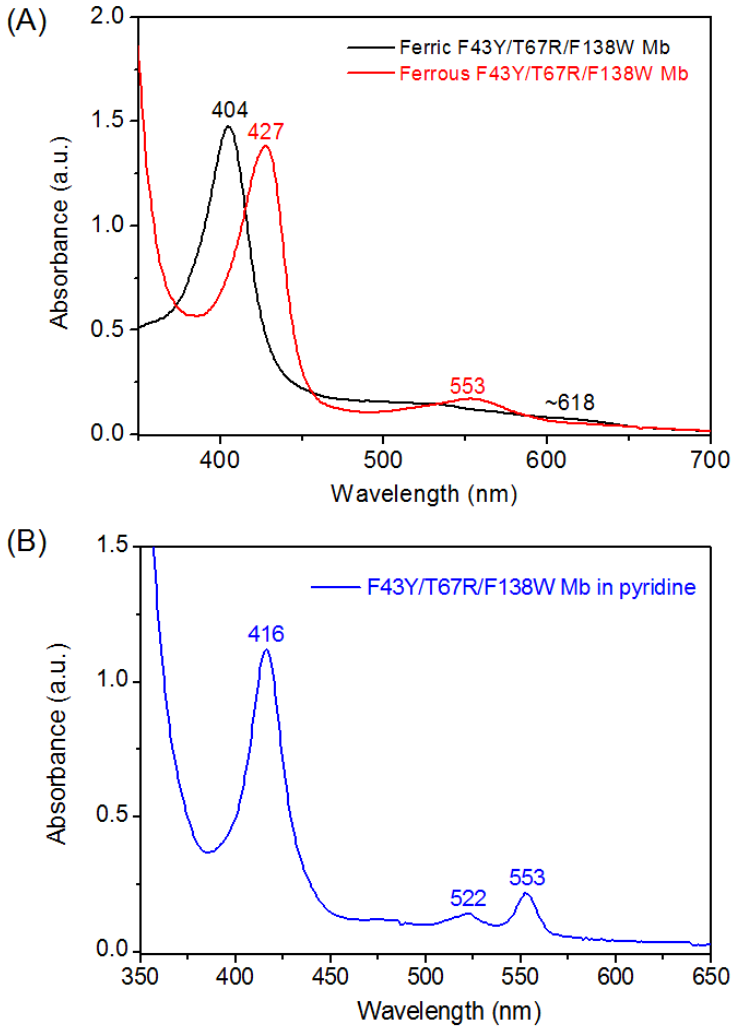

Figure S1. UV-Vis spectra of F43Y/T67R/F138W Mb in the ferric met form and ferrous doxy form (A), and the reduced form in pyridine (B).

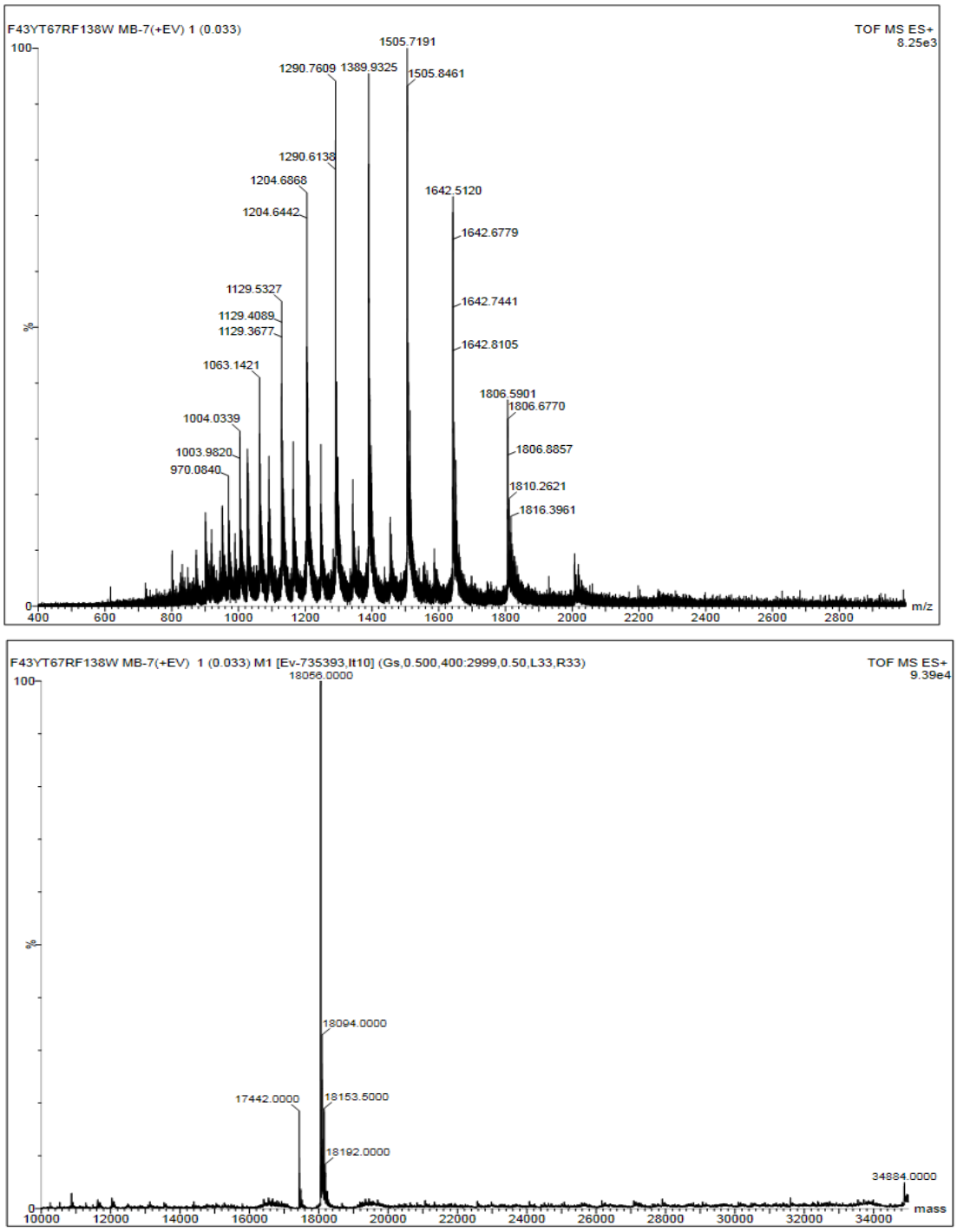

Figure S2. MS spectrum of F43Y/T67R/F138W Mb: Molecular weight for the apo-protein, calculated 17441 Da, observed 17442 Da; the holo-protein, calculated 18057.5 Da, observed 18056.0 Da, respectively.

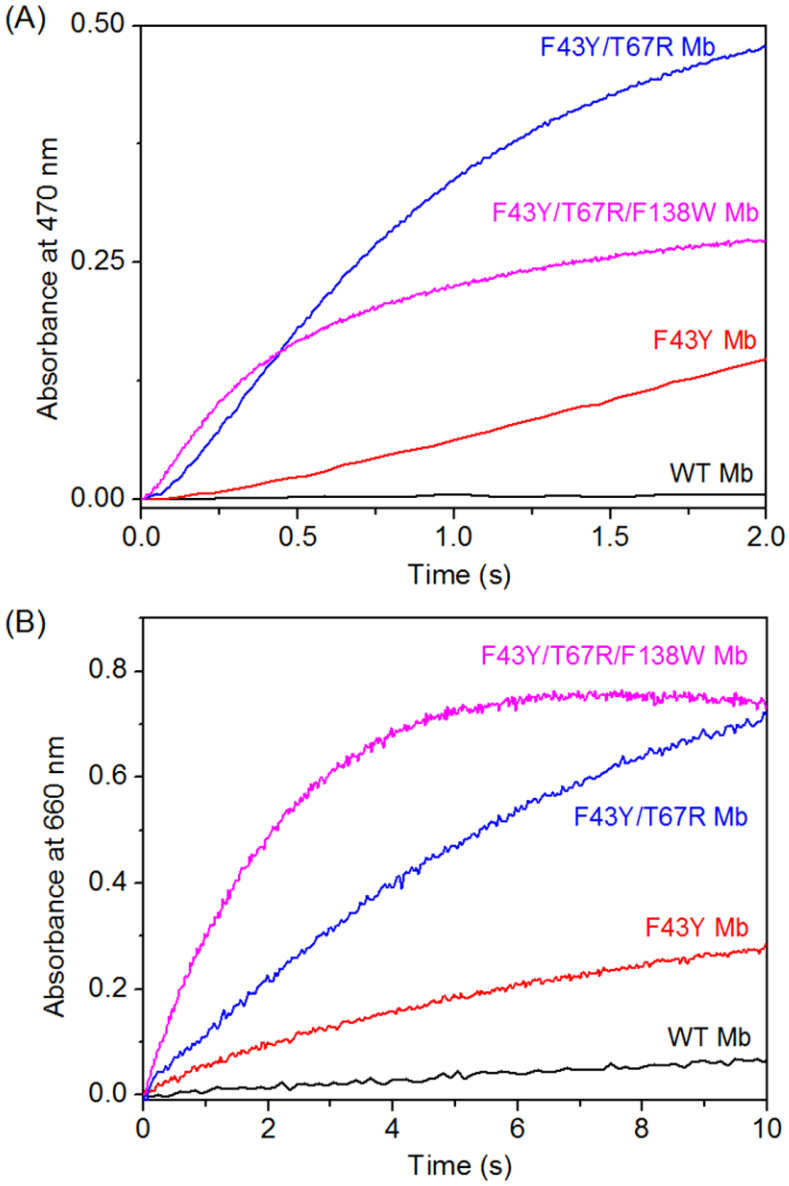

Figure S3. Time-dependent oxidation of (A) Guaiacol (2 mM) and (B) ABTS (250 μM) catalyzed by F43Y/T67R/F138W Mb, F43Y/T67R Mb, F43Y Mb and WT Mb, and monitored at 470 nm and 660 nm, respectively. Reaction conditions: 1 μM protein, 75 mM H_2O_2, 50 mM potassium phosphate buffer at pH 7.0, 25 ℃.

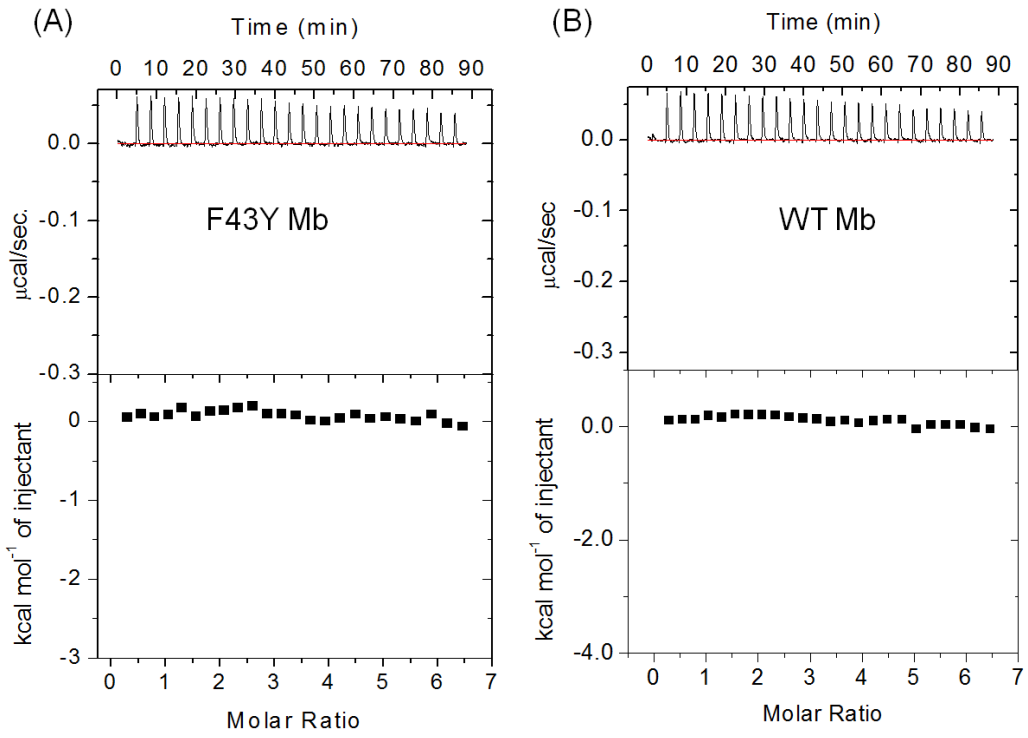

Figure S4. ITC data for titration of (A) F43Y Mb and (B) WT Mb with ABTS in potassium phosphate buffer (50 mM, pH 7.0) at 25 ^{o}C. Top, raw data. Bottom, plot of integrated heats versus ABTS/protein ratio.