Electronic Supplementary Material (ESI) for ChemComm.

Unique Tyr-heme double cross-links in F43Y/T67R myoglobin: an artificial enzyme with a peroxidase activity comparable to that of native peroxidases

Can Liu, ‡[a] Hong Yuan, ‡[b] Fei Liao,[a] Chuan-Wan Wei,[a] Ke-Jie Du,[a] Shu-Qin Gao,[c] Xiangshi Tan,[b] and Ying-Wu Lin [a,c]*

Supporting Information

Contents

1. Experimental Section

1.1 Protein preparation

Wild-type (WT) sperm whale Mb was expressed in BL21(DE3) cells using the Mb gene of pMbt7-7, and purified using the procedure described previously.[1] F43Y Mb was expressed and purified as reported in our previous study.[2] F43Y/T67R Mb gene was constructed using the QuikChange site directed mutagenesis kit (Stratagene), and the double mutations were confirmed by DNA sequencing assay. F43Y/T67R Mb was expressed and purified using a similar procedure as that for WT Mb and F43Y Mb.[1,2] Protein concentration was determined with an extinction coefficient of ε_{404} = 150 mM^{-1} cm^{-1} for F43Y/T67R Mb mutant, ε_{409} = 157 mM^{-1} cm^{-1} for WT Mb,[1] and ε_{403} = 146 mM^{-1} cm^{-1} for F43Y Mb mutant, respectively, as calculated using the standard hemochromagen method.[3] Note that due to the saturation of one vinyl group of heme in F43Y Mb and F43Y/T67R Mb by Tyr-heme cross-link, the α-band of pyridine spectrum shifted from 556 nm of WT Mb to 553 nm, the same as that of *c*-type heme with a single thioether bond,[4] which is between that of heme *b* (556 nm) and heme *c* (550 nm). Therefore, an average extinction coefficient of heme *b* and heme *c* was used to calculate that for the mutants.

1.2 UV-Vis spectroscopy

UV-Vis spectra were recorded on a Hewlett-Packard 8453 diode array spectrometer. Deoxy proteins were obtained by addition of a small amount of sodium dithionite. The pyridine hemochrome spectrum was obtained by using 8 μM protein in 19 % (vol/vol) pyridine and 0.15 M NaOH, and the protein was reduced by a small amount of sodium dithionite.

1.3 Mass spectrometry

Protein mass spectrum measurement was carried out on an G2-XS QTOF mass spectrometry (Waters). The F43Y/T67R 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 X-ray crystallography

Ferric F43Y/T67R Mb in met form with a high purity (A_{404nm}/A_{280nm}>4.0) was exchanged into 20 mM potassium phosphate buffer (pH 7.0) and concentrated to ~4.0 mM. The vapor diffusion hanging drop technique was used to crystallize the protein. The well buffer contained 0.2 M sodium acetate trihydrate, 0.1 M sodium cacodylate trihydrate pH 6.5, and 30% w/v polyethylene glycol 8,000 (Crystal screen, condition No. 28, Hampton Research). Crystal trays were set up by transferring 250 μL of well buffer into each well. Then, 2 μL of well buffer and 2 μL of protein were mixed and placed on a siliconized glass slide. Crystallization can be achieved at 10 °C after ~2 weeks. Diffractable crystals were soaked in a cryoprotectant solution of 30% PEG 400, mounted onto cryogenic loops, and frozen quickly in liquid nitrogen. Diffraction data was collected from a single crystal at Shanghai Synchrotron Radiation Facility (SSRF) BL17U beamline, China, using a MAR mosaic 225 CCD detector with a wavelength of 0.9793 Å at 100 K. The diffraction data were processed and scaled with HKL-2000[5]. The structure was solved by the molecular replacement method and the 1.6 Å structure of WT Mb (PDB entry 1JP6[6]) was used as the starting model. Manual adjustment of the model was carried out using the program COOT[7] and the models were refined by PHENIX[8] and Refmac5[9]. Stereochemical quality of the structures was checked by using PROCHECK[10]. All of residues locate in the favored and allowed region and none in the disallowed region.

1.5 Acid titration studies

Acid titration studies of F43Y/T67R Mb and F43Y Mb were 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 pK_a values were calculated by fitting the absorbance of Soret band vs. pH to the Boltzmann function.

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

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

1.6 Stopped-flow spectroscopy

Effects of pH on the peroxidase activity of F43Y/T67R Mb, F43Y Mb and WT Mb were investigated on a stopped-flow spectrophotometer (SF-61DX2 Hi-Tech KinetAsystTM) at 25 ^{o}C, by using 2,2'-azino-bis(3-ethylbenzothiazoline-6- sulfonic acid) diammonium salt (ABTS) as a substrate and H_2O_2 as the oxidant, respectively. Typically, one syringe contains 2 μM protein (in 100 mM potassium phosphate buffer, pH 4.5-7.0) in presence of 0.2 mM ABTS, and the second syringe contains 20 mM H_2O_2, as determined with $\varepsilon_{240\ nm}$ = 39.4 M^{-1} cm^{-1}. Upon mixing, the reaction was monitored by formation of the $ABTS^{+\cdot}$ cation radical at 660 nm. The initial rate was calculated based on the initial linear changes using an extinction coefficient of ε_{660nm} = 14.0 $mM^{-1}\cdot cm^{-1}$.[11]

The peroxidase activities were evaluated by using guaiacol or ABTS as a substrate. Typically, one syringe contains 2 μM protein (F43Y/T67R Mb in 100 mM potassium phosphate buffer, pH 5.5; F43Y Mb in the same buffer, pH 5.3; WT Mb in the same buffer, pH 5.0) in presence of guaiacol or ABTS, respectively, and the second syringe contains 20 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 (ε_{470nm} = 26.6 $mM^{-1}\cdot cm^{-1}$).[12] The initial rate was calculated based on the initial linear changes using an extinction coefficient, respectively. The curve of initial rates *versus* substrate concentrations was fitted to the Michaelis-Menten equation.

Kinetic studies of F43Y/T67R Mb and F43Y Mb in reaction with H_2O_2 were performed with the same stopped-flow spectrophotometer. Typically, one syringe contains 20 μM protein (F43Y/T67R Mb in 100 mM potassium phosphate buffer, pH 5.5, and F43Y Mb in the same buffer, pH 5.3), and the second syringe contains various concentrations of H_2O_2 (0.5-2 mM). The reaction was stated with mixing of equal volume of solutions from the both syringes. 50 time-dependent spectra were collected over 0.5 sec from 300 to 700 nm at 20 ^{o}C. The changes of the Soret band were plotted, and the observed rate constants (k_{obs}, s^{-1}) were calculated from single-exponential fits. The apparent rate constants (k_1, $mM^{-1}s^{-1}$) for compound II formation were obtained by linear regression fitting the plot of the observed rate constants, k_{obs}, versus the concentrations of H_2O_2.

$$k_{obs} = k_{off} + k_1[H_2O_2]$$

Here, k_{off} represents the dissociation rate constant of the Mb-H_2O_2 complex.[13]

1.7 ITC studies

Isothermal titration calorimetry (ITC) measurements were performed on a Microcal VP-ITC microcalorimeter (GE life sciences). The protein solution was thoroughly degassed in a ThermoVal apparatus (Microcal). For titration experiments, ~1.42 mL of F43Y/T67R Mb or F43Y Mb (10 μM) solution was placed in the reaction cell, and a solution of ABTS (350 μM) 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. The data were analyzed and the binding isotherm was fitted to a single-site model in the Origin 7.0 software.

References:

1 J. A. Sigman, B. C. Kwok, Y. Lu, *J Am Chem Soc* **2000**, *122*, 8192-8196.

2 D.-J. Yan, W. Li, Y. Xiang, G.-B. Wen, Y.-W. Lin, X. Tan, *Chembiochem* **2015**, *16*, 47-50

3 E. A. Berry, B. L. Trumpower, *Anal Biochem* **1987**, *161*, 1-15.

4 P. D. Barker, J. C. Ferrer, M. Mylrajan, T. M. Loehr, R. Feng, Y. Konishi, W. D. Funk, R. T. A. MacGillivray, A. G. Mauk, *Proc. Natl. Acad. Sci. USA* **1993**, 90, 6542-6546.

5 Z. Otwinowski and W. Minor, *Methods Enzymol.* **1997**, 276: 307-326.

6 P. Urayama, G. N. Jr Phillips and S. M. Gruner, *Structure*, **2002**, 10: 51–60.

7 P. Emsley and K. Cowtan, *ActaCrystallogr. Sect D*, **2004**, 60: 2126-2132.

8 P. D. Adams, R. W. Grosse-Kunstleve, L. W. Hung, T. R. Ioerger, A. J. McCoy, N. W. Moriarty, R. J. Read, J. C. Sacchettini, N. K. Sauter and T. C. Terwilliger, *Acta Crystallogr. Sect D*, **2002**, 58: 1948-1954.

9 G. N. Murshudov, A. A.Vagin and E. J. Dodson, *Acta Crystallogr. Sect D*, **1997**, 53: 240-255.

10 R. A. Laskowski, M. W. MacArthur, D. S. Moss and J. M. Thornton, *J. Appl. Cryst.* **1993**, 26: 283-291.

11 F. Nastri, L. Lista, P. Ringhieri, R. Vitale, M. Faiella, C. Andreozzi, P. Travascio, O. Maglio, A. Lombardi, V. Pavone, *Chemistry*, **2011**, *17*: 4444-4453.

12 D. A. Baldwin, H. M. Marques, J. M. Pratt, *J Inorg Biochem*, **1987**, *30*: 203-217.

13 K. K. Khan, M. S. Mondal, L. Padhy and S. Mitra, *Eur J Biochem*,**1998**, *257*: 547-555.

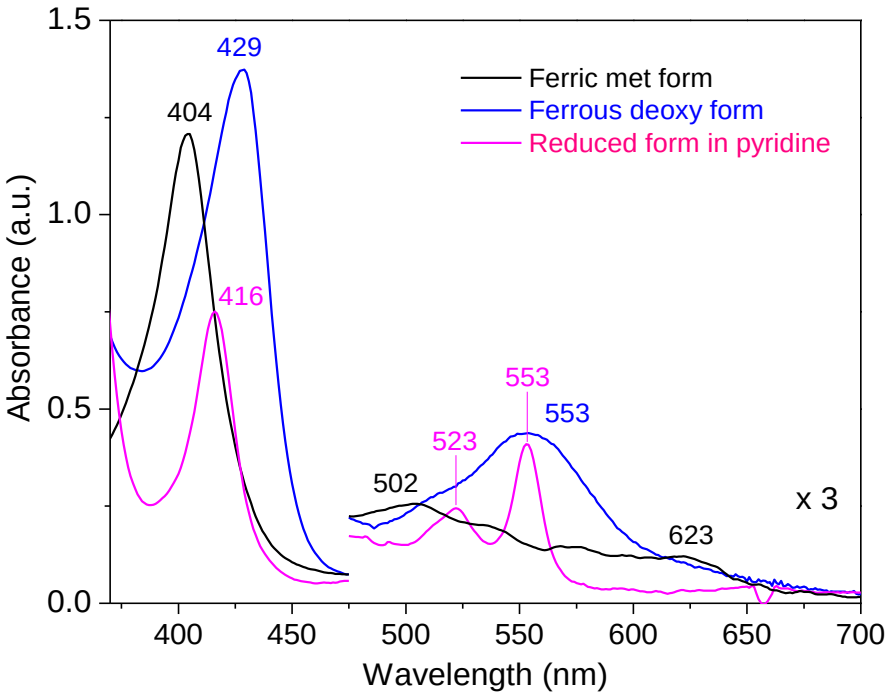

Fig. S1 UV-Vis spectra of F43Y/T67R Mb in the ferric met form, ferrous doxy form, and reduced form in pyridine.

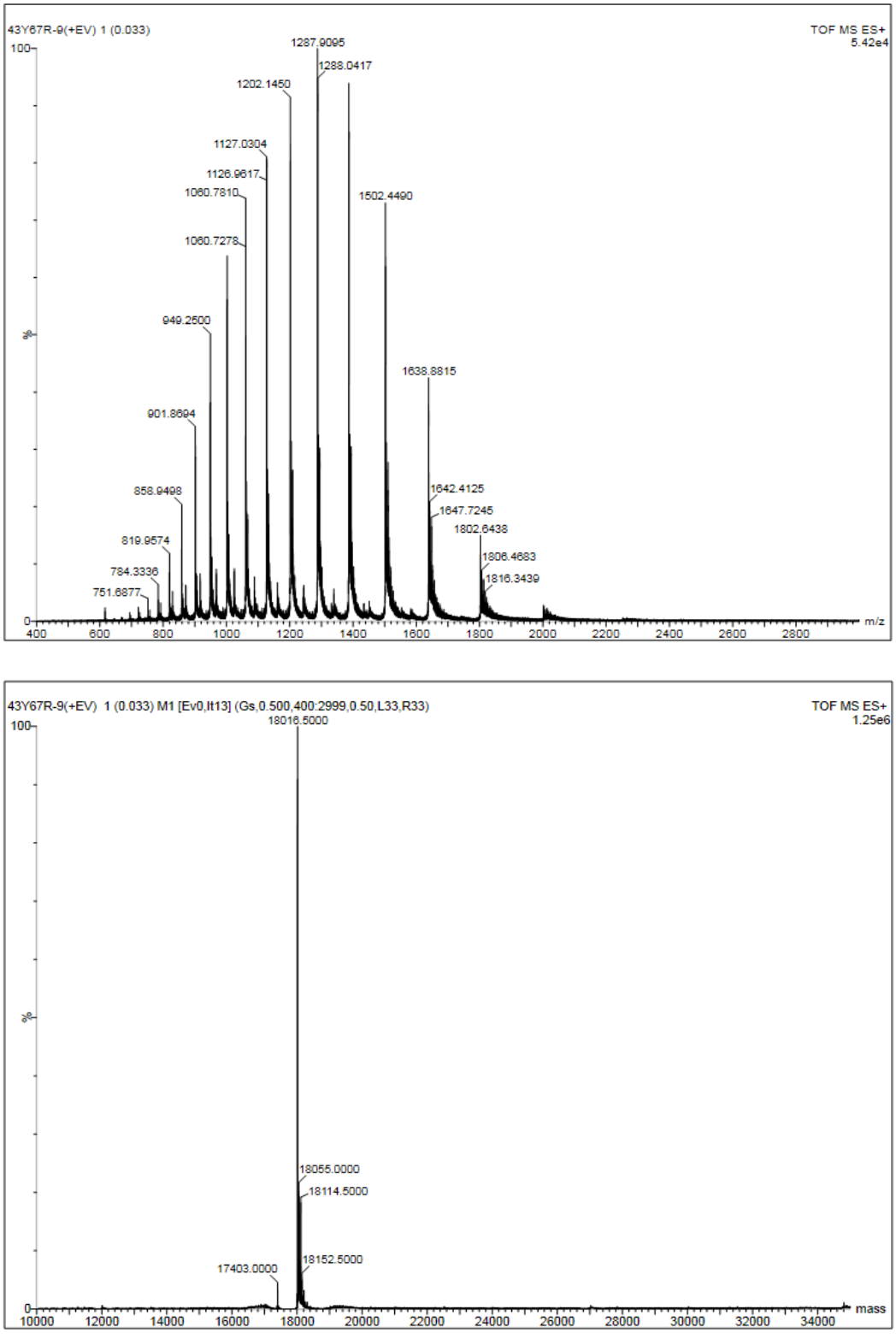

Fig. S2 MS spectrum of F43Y/T67R Mb: Calculated molecular weight for the holo-protein, 18018 Da, and the observed, 18016.5 ± 0.5 Da.

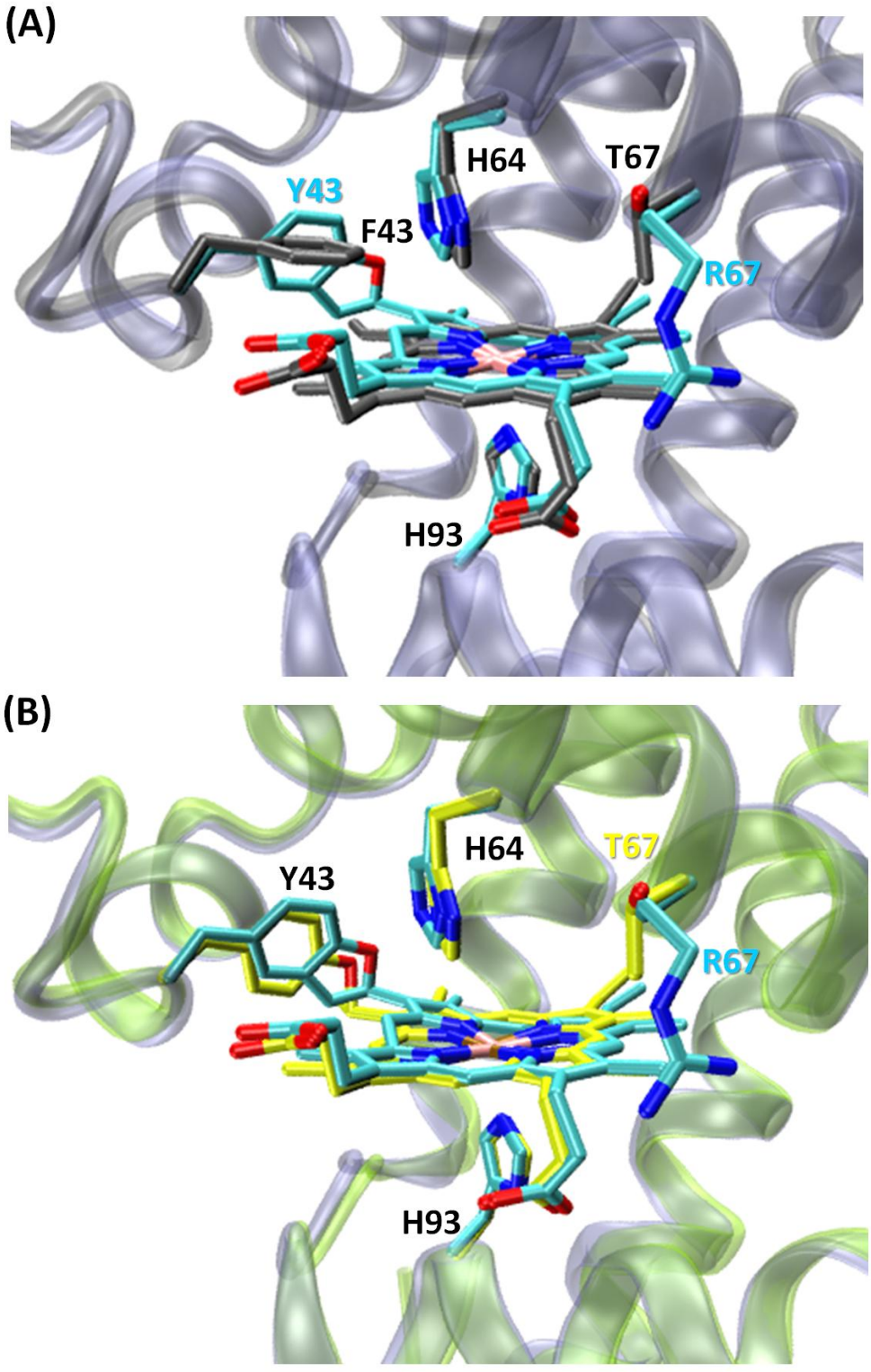

Fig. S3 Structural overlay of F43Y/T67R Mb (cyan) with (A) WT Mb (PDB code 1JP6, gray), and (B) F43Y Mb (PDB code 4QAU, yellow), respectively, showing the heme active site.

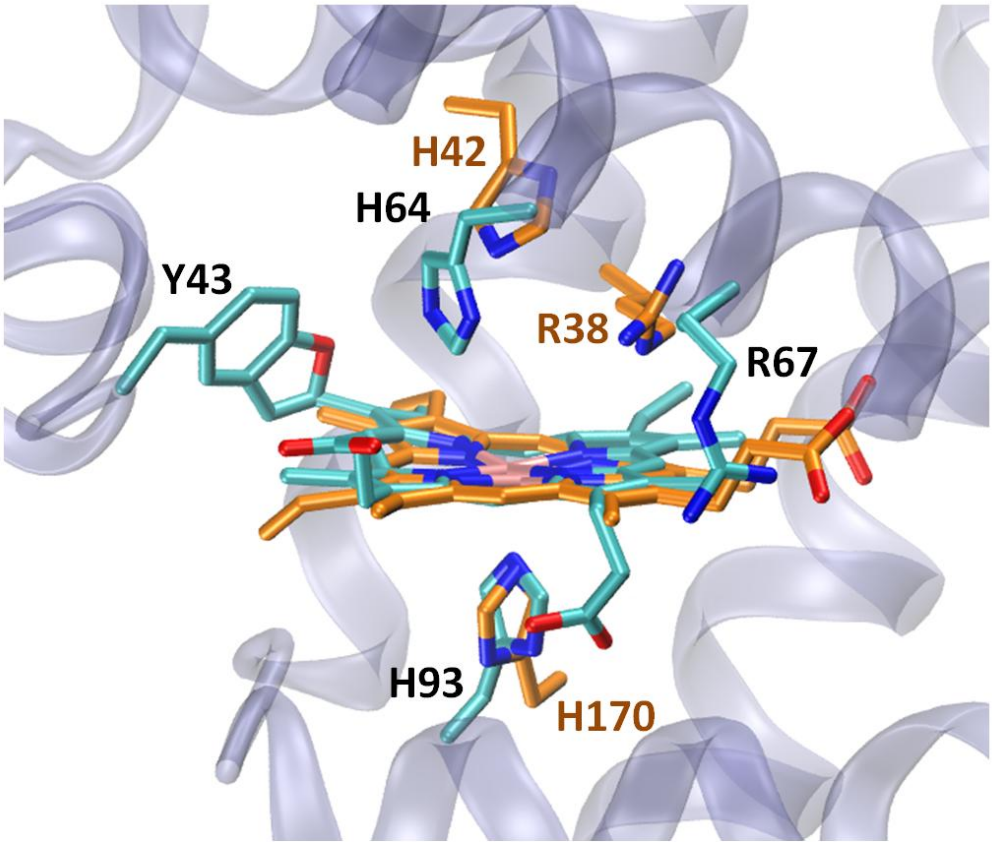

Fig. S4 Structural overlay of F43Y/T67R Mb (cyan) and HRP (PDB code 1H58, orange), showing the heme active site.

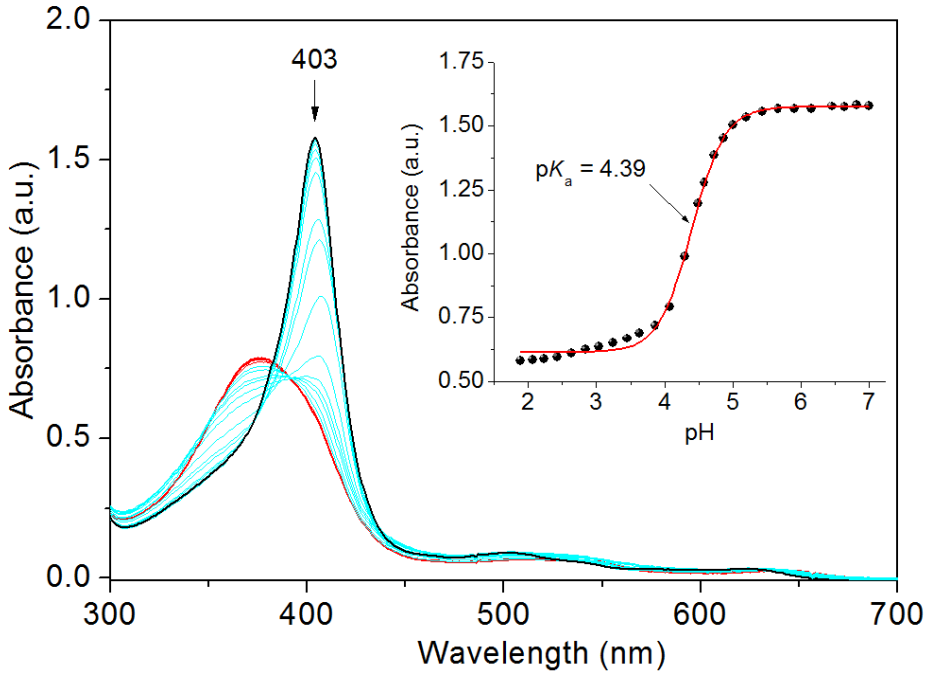

Fig. S5 UV-visible spectra of F43Y Mb upon pH titration. Plot of the Soret band at 403 nm against pH values was shown as an inset.

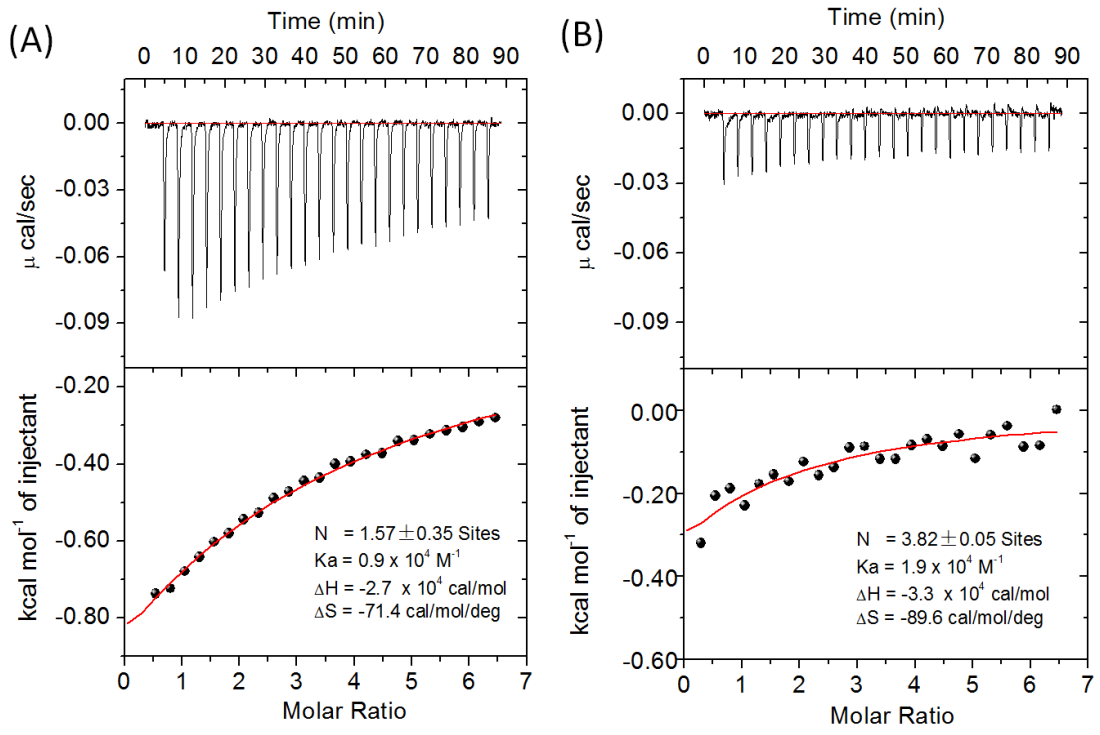

Fig. S6 ITC data for titration of F43Y/T67R Mb (A) and F43Y Mb (B) (10 μM) with RB19 (350 μM) at 25 ℃, pH 5.5. Top, raw data. Bottom, plot of integrated heats versus RB19/protein ratio.

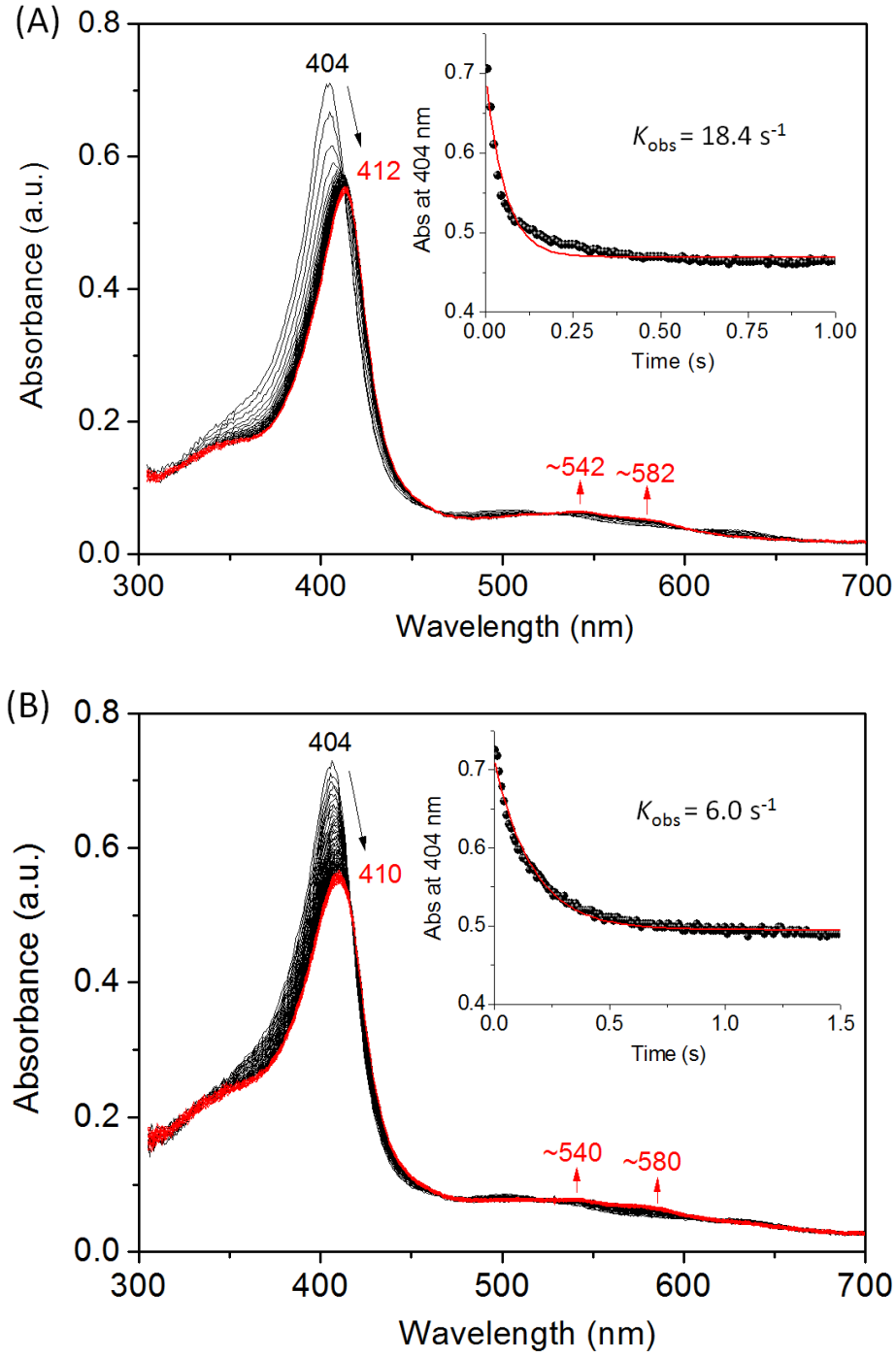

Fig. S7 Stopped-flow UV-Vis spectra of F43Y/T67R Mb (A) and F43Y Mb (B) in reaction with H_2O_2 (1 mM) for 0.5 s at pH 5.5 and pH 5.3, respectively, 20 ^{o}C. Inset, the single-exponential fit of the decay of Soret band at 404 nm.

Table S1. Summary of data collection and refinement statistics

	F43Y/T67R Mb
Wavelength	0.979
Space group	P 1 2_1 1
Unit-cell dimensions (Å, º)	a = 72.406, b = 33.604, c = 72.886 ;
	α = 90, β = 117.873, γ = 90
Resolution (Å)	50-1.99 (2.04-1.99)
No. of observations	156346
No. of unique reflections	21592 (2736)[a]
Completeness (%)	99.3 (94.3)
<I>/σ (I)	19.71 (2.59)
Redundancy	7.3 (6.4)
R_{sym}[b]	0.121 (0.892)
R_{cryst}[c] (%)/R_{free}[d] (%)	0.171 /0.220
RMSD bonds (Å) / angles (º)	0.010 /2.991
Ramachandran plot, residues in:	
Most favored regions (%)	95.96
Allowed regions (%)	4.04
Disallowed regions (%)	0.0

[a] Numbers in parentheses represent values in the highest resolution shell (Å).

[b] $R_{sym} = |I_j - \langle I \rangle| / I_j$, where I_j is the observed integrated intensity, <I> is the average integrated intensity obtained from multiple measurements, and the summation is over all observed reflections.

[c] $R_{cryst} = ||F_{obs}| - |F_{calc}|| / |F_{obs}|$, F_{obs} and F_{calc} are observed and calculated structure factor amplitudes, respectively.

[d] R_{free} calculated with randomly selected reflections (5%).