How a novel tyrosine-heme cross-link fine-tunes the structure and function of heme protein: A direct comparison study in myoglobin

Dao-Jing Yan[a†], Hong Yuan[b†], Wei Li[b†], Yu Xiang[c], Bo He,[a] Chang-Ming Nie,[a]

Ge-Bo Wen[d], Ying-Wu Lin[a, d]*, and Xiangshi Tan[b]*

Contents

1. Experimental section

1.1 Protein preparation

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]. L29H Mb and F43Y Mb single mutants were expressed and purified as described in previous studies [2, 3]. L29H/F43Y Mb gene was constructed by using the QuickChange Site Directed Mutagenesis Kit (Stratagene), and the double mutations were confirmed by DNA sequencing assay. L29H/F43Y Mb was expressed using a similar procedure as that for WT Mb, L29H Mb and F43Y Mb. Two different forms of L29H/F43Y Mb were obtained in protein purification when the condition was kept in oxidative or reductive state by adding 1 mM potassium ferricyanide ($K_3[Fe(CN)_6]$) or dithiothreitol (DTT), respectively. The form purified in oxidative condition was found to have no Tyr-heme cross-link, termed L29H/F43Y Mb, whereas the other form purified in reductive condition was found to have a novel Tyr-heme cross-link similar to that in F43Y Mb, termed L29H/F43Y Mb-X for clarification. Protein concentration was determined with an extinction coefficient of ε_{404} = 157 ± 3 $mM^{-1}\cdot cm^{-1}$ for L29H/F43Y Mb and ε_{406} = 155 ± 3 $mM^{-1}\cdot cm^{-1}$ for L29H/F43Y Mb-X, respectively, as calculated using the standard hemochromagen method [4].

1.2 MALDI-TOF mass spectrometry

Protein mass spectrum measurement was carried out on an AXIMA Performance MALDI-TOF/TOF mass spectrometer (Shimadzu Corp., Kyoto, Japan).

The matrix used for sample treatment in the MALDI-TOF MS measurement was 10 mg/mL sinapinic acid in 50:50 water/acetonitrile with 0.1% TFA. A mixture of 0.5 μL L29H/F43Y Mb and L29H/F43Y Mb-X solution (0.1 mM) and 2 μL matrix solution was dropped on a standard sample plate. After dried under ambient condition, the plate was transferred into the mass spectrometer chamber for measurement under positive mode.

1.3 X-ray crystallography

L29H/F43Y Mb and L29H/F43Y Mb-X, both with a high purity (A_{soret}/A_{280nm}>4.0), were exchanged into 20 mM KH_2PO_4 (pH 7.0) and concentrated to ~1.5 mM. The vapor diffusion hanging drop technique was used to crystallize the protein under the similar conditions to that for F43Y Mb in previous study [3]. 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.

1.4 UV-vis spectroscopy

UV-vis spectra of L29H/F43Y Mb and L29H/F43Y Mb-X were recorded in 100 mM KH_2PO_4 (pH 7.0) on a Hewlett-Packard 8453 diode array spectrometer. Deoxy proteins were prepared by addition of a small amount of sodium dithionite in anaerobic conditions. 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. The reduction of ferric L29H/F43Y Mb (10 μM) by 1 mM DTT in air-saturated 100 mM KH_2PO_4 (pH 7.0) was performed by monitoring the UV-vis spectra changes for 1 hour, and the protein was oxidized back to its ferric state by addition of a small amount of $K_3[Fe(CN)_6]$. Deoxy L29H/F43Y Mb was prepared by addition of a small amount of sodium dithionite and eluted from PD-10 column (GE Healthcare). The protein was then diluted into O_2-saturated 100 mM KH_2PO_4 (pH 7.0).

1.5 Gdn·HCl-induced unfolding study

Guanidine hydrochloride (Gdn·HCl)-induced unfolding of L29H/F43Y Mb and L29H/F43Y Mb-X were performed by addition of 10 μL protein solution to 2 mL Gdn·HCl stock solutions (0-5.0 M, pH 7.0) to a final concentration of 10 μM. The samples were incubated 25 ^{o}C for 30 min before collecting the UV-vis spectra. Control experiment was also performed for single mutant L29H Mb. Gdn·HCl-induced unfolding studies of single mutant F43Y Mb and WT Mb were reported in previous study [3]. The denaturation midpoints (C_m values) were

calculated by fitting the absorbance of Soret band versus the concentrations of Gdn·HCl to the two-state Boltzmann function (eq. 1).

$$A = A_2 + (A_1 - A_2)/(1+ e^{(C- Cm)/dC}) \quad (1)$$

Here, A is the absorbance of Soret band; A_1 and A_2 are the initial and final absorbance of Soret band, respectively; C is the concentration of Gdn·HCl.

1.6 Imidazole binding study

L29H/F43Y Mb and L29H/F43Y Mb-X (10 μM) were dissolved in 20 mM KH_2PO_4 (pH 7.0) and titrated with imidazole at 25 ˚C. The UV-Vis spectra were recorded in a range of 300-700 nm with dropwise addition of imidazole. Control experiments were performed for single mutants F43Y Mb and L29H Mb, as well as WT Mb under the same conditions. The dependency of the visible band change (ΔA) due to the formation of His/Imidazole coordination on concentrations of imidazole was analyzed by the following equation (eq. 2):

$$\Delta A = \Delta A_{max}[[C_P + C_L + K_D - [(C_P + C_L + K_D)^2 - 4C_PC_L]^{1/2}]]/2C_p \quad (2)$$

Here, ΔA is the absorbance difference for the visible band; ΔA_{max} is the maximum absorbance difference with ferric heme fully occupied by imidazole; C_P and C_L are the total protein and total imidazole concentration, respectively; and K_D is the equilibrium dissociation constant.

1.7 Peroxidase reaction kinetics

The reactions of L29H/F43Y Mb and L29H/F43Y Mb-X with hydrogen

peroxide (H_2O_2) were determined using a dual mixing stopped-flow spectrophotometer (SF-61DX2 Hi-Tech KinetAsystTM). Typically, one syringe contains 10 μM of protein (in 100 mM KH_2PO_4 buffer, pH 7.0), and the second syringe contains H_2O_2 with concentration ranging from 0.125 to 1 mM. The reaction was stated with mixing of equal volume of solutions from the both syringes. 100 time-dependent spectra were collected over 20-50 sec from 350 to 700 nm at 25 ^oC. The time traces of Soret band absorbance were biphasic for the double and single mutants. The corresponding peseudo-first-order rate constants, k_{obs1} and k_{obs2}, were calculated by fitting to double-exponential decay function (eq. 3), and k_{obs1} was used for comparison. For WT Mb, the observed rate constants (k_{obs}, s^{-1}) were calculated from single-exponential fits.

$$y = y_0 + ae^{-k1t} + ae^{-k2t} \quad (3)$$

The apparent rate constant, k_1 ($mM^{-1}s^{-1}$), was obtained by linear regression fitting the plot of the observed rate constants, k_{obs}, versus the concentrations of H_2O_2 (eq. 4).

$$k_{obs} = k_{off} + k_1[H_2O_2] \quad (4)$$

where k_{off} represents the dissociation rate constant of the Mb-H_2O_2 complex.

1.8 EPR spectroscopy

Electron Paramagnetic Resonance (EPR) spectra of L29H/F43Y Mb was recorded on a Bruker A300 spectrometer (X-band) equipped with Bruker ER4141VTM liquid nitrogen system. The protein sample (0.5 mM in 100 mM

KH_2PO_4, pH 7.0) was transferred into an EPR tube with a volume of 300 μL. The spectra were measured at 90 K, with frequency of 9.43 GHz, center field 2200 G and sweep width 3600 G, microwave power 0.595 mW and modulation amplitude 3.0 G. EPR spectra of L29H/F43Y Mb (0.5 mM) after addition of DTT (50 mM) were recorded under the similar condition for 15, 30 and 60 min, respectively.

References:

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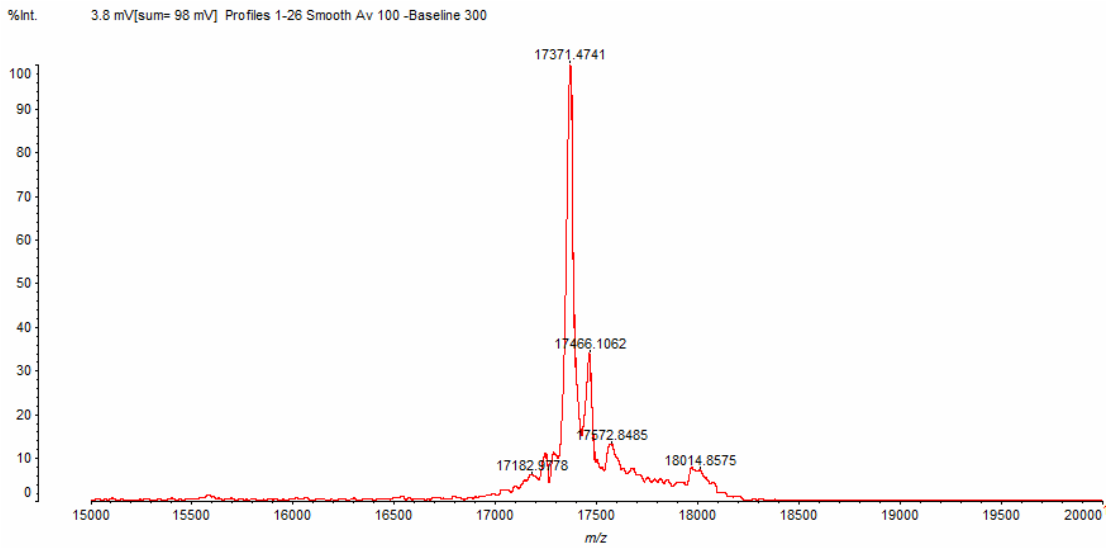
(A)



(B)

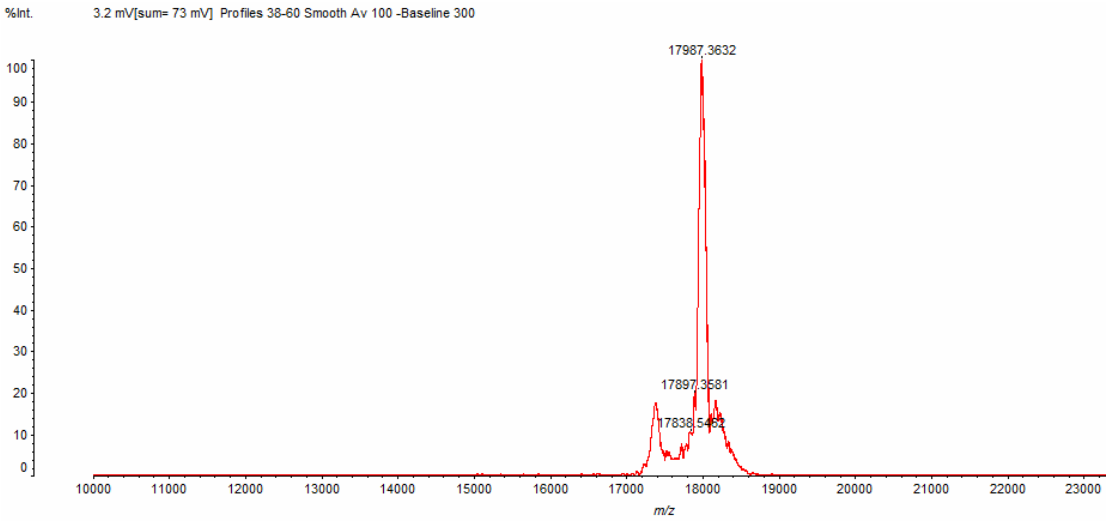


Figure S1. MALDI-TOF mass spectra of L29H/F43Y Mb (A, Calculated apo-protein, 17371 Da; Observed: 17371.5 Da), and L29H/F43Y Mb-X (B, Calculated holo-protein: 17987 Da; Observed: 17987.4 Da).

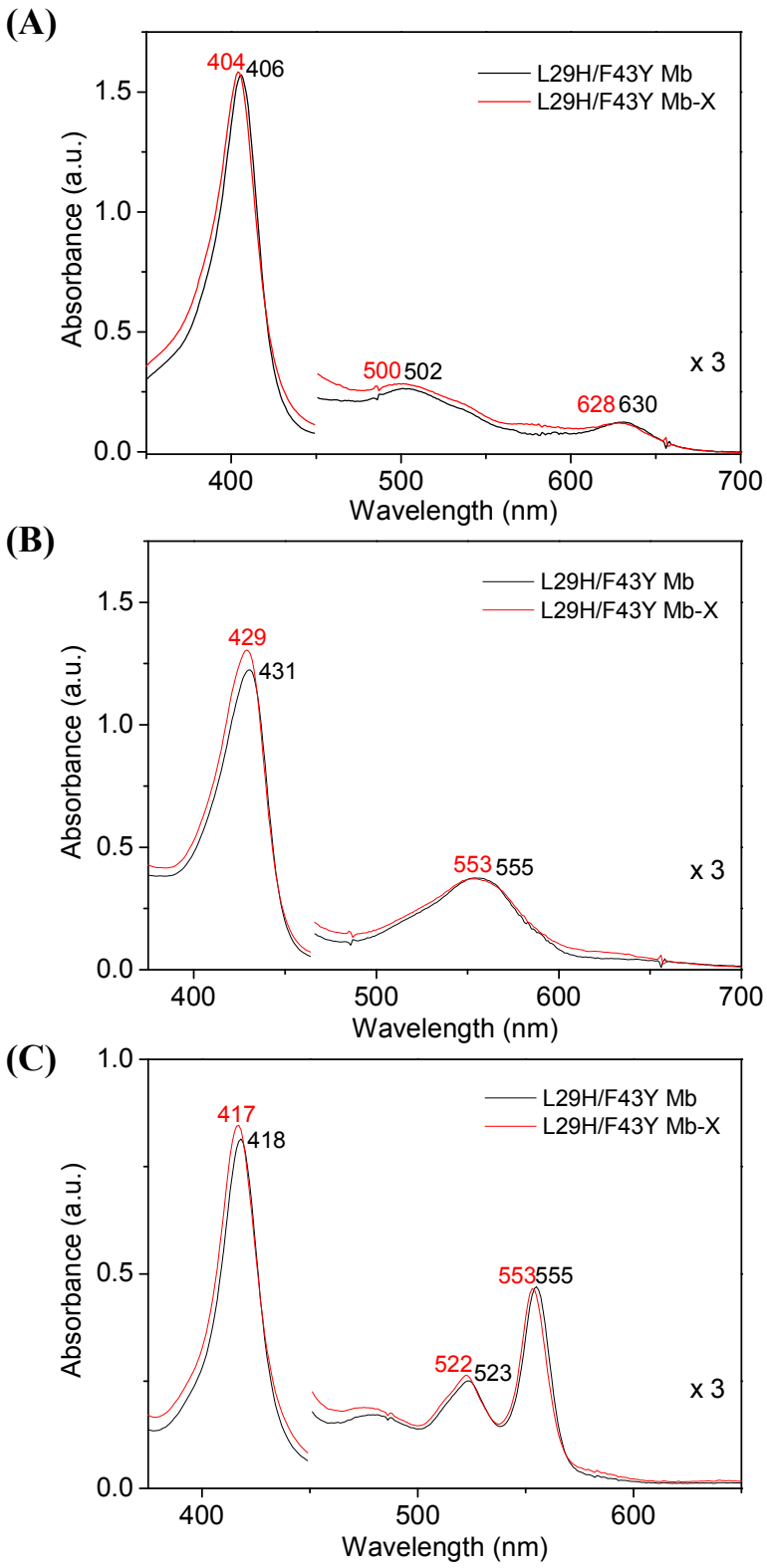


Figure S2. UV-vis spectra comparison of L29H/F43Y Mb (black) and L29H/F43Y Mb-X (red) in met form (A), deoxy form (B), and in the reduced pyridine hemochromagen complexes (C).

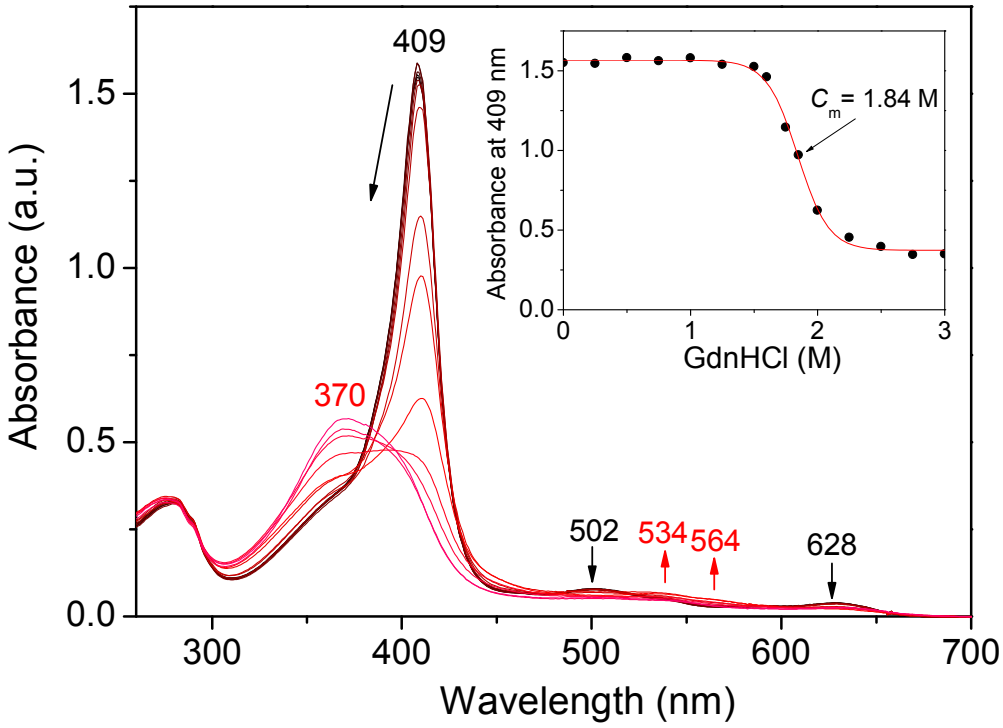
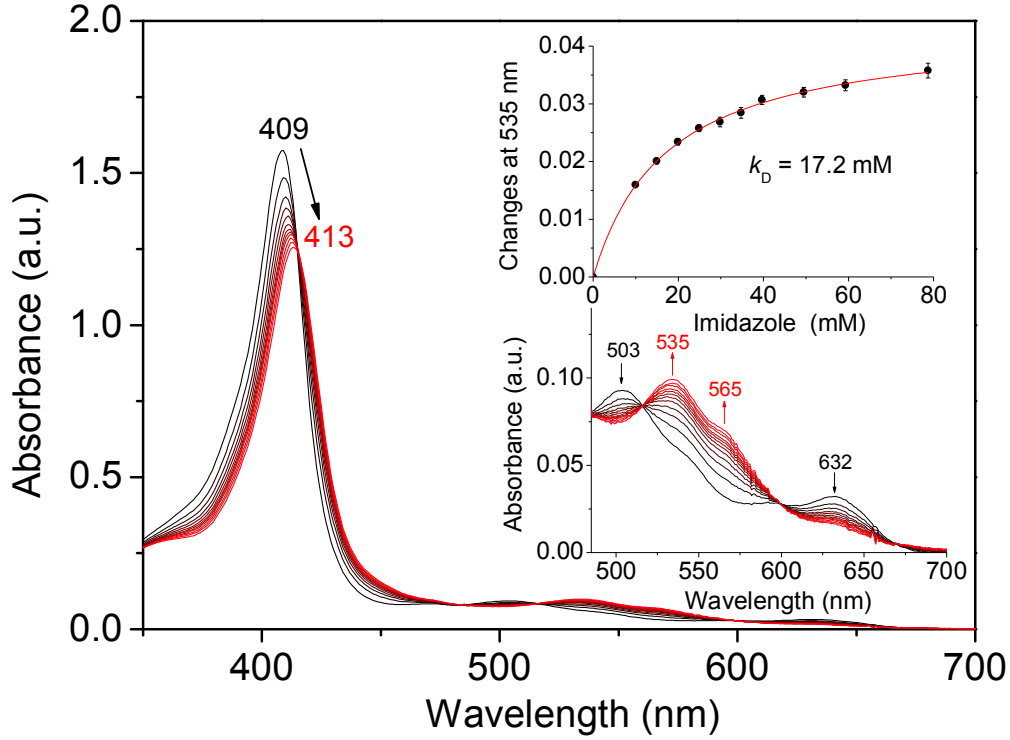
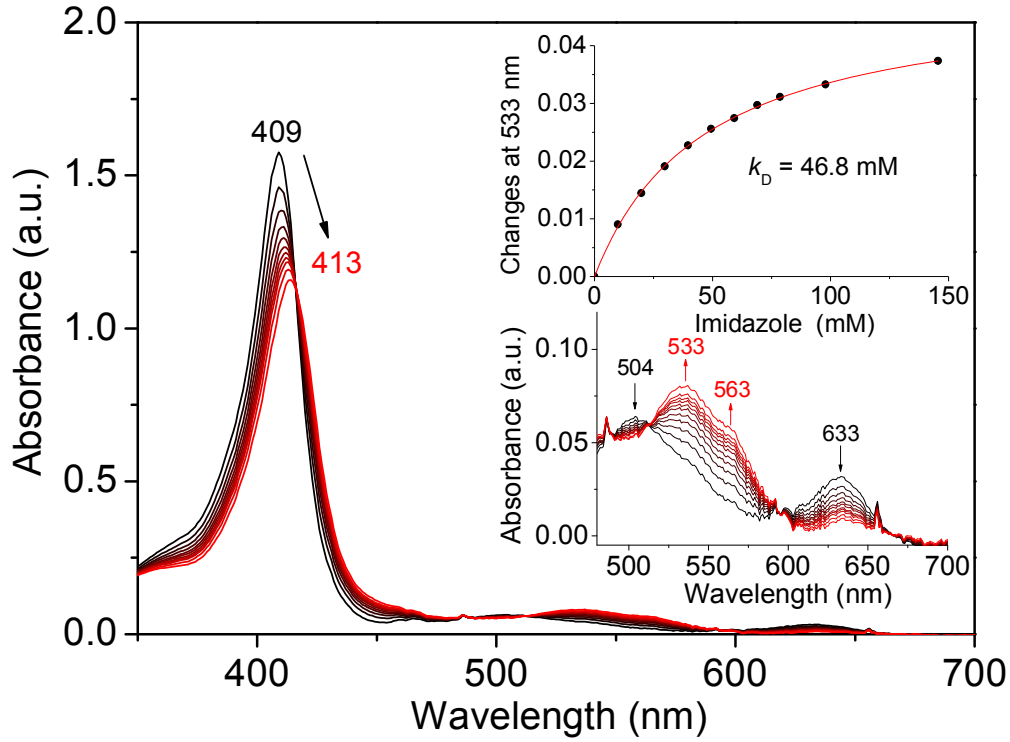


Figure S3. UV-visible spectra of Gdn·HCl-induced unfolding of L29H Mb. The changes of Soret band versus Gdn·HCl concentrations were shown as insets.

(A)


Absorbance (a.u.)
Wavelength (nm)
409
413
Changes at 535 nm
k_D = 17.2 mM
Imidazole (mM)
503
535
565
632
Absorbance (a.u.)
Wavelength (nm)


Absorbance (a.u.)
Wavelength (nm)
409
413
Changes at 533 nm
k_D = 46.8 mM
Imidazole (mM)
504
533
563
633
Absorbance (a.u.)
Wavelength (nm)

(C)

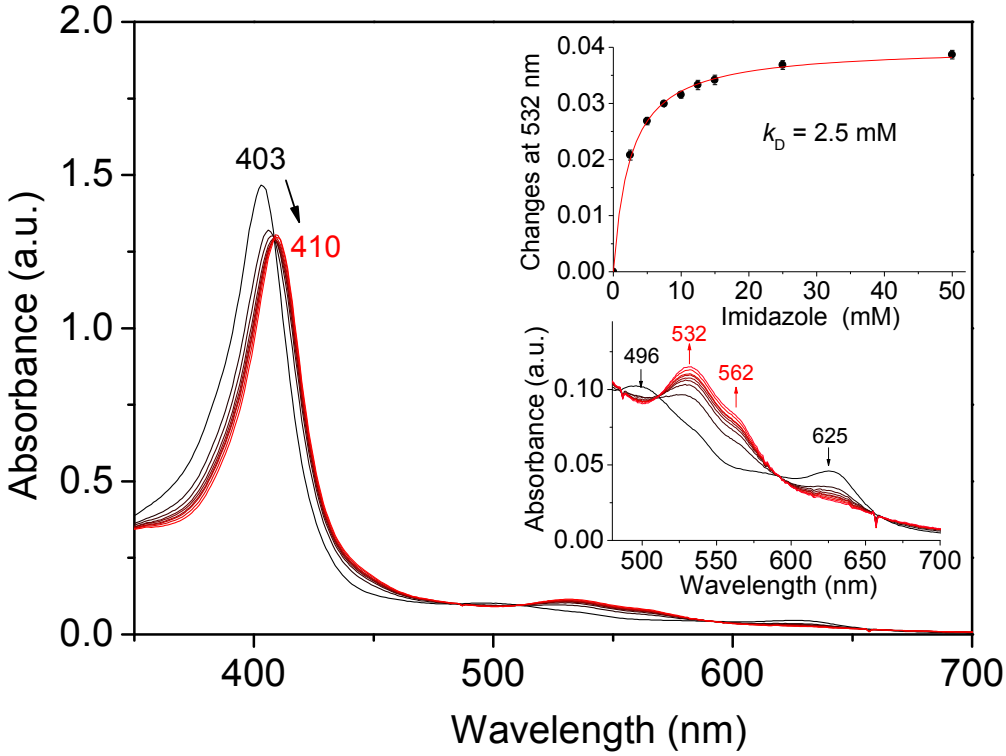
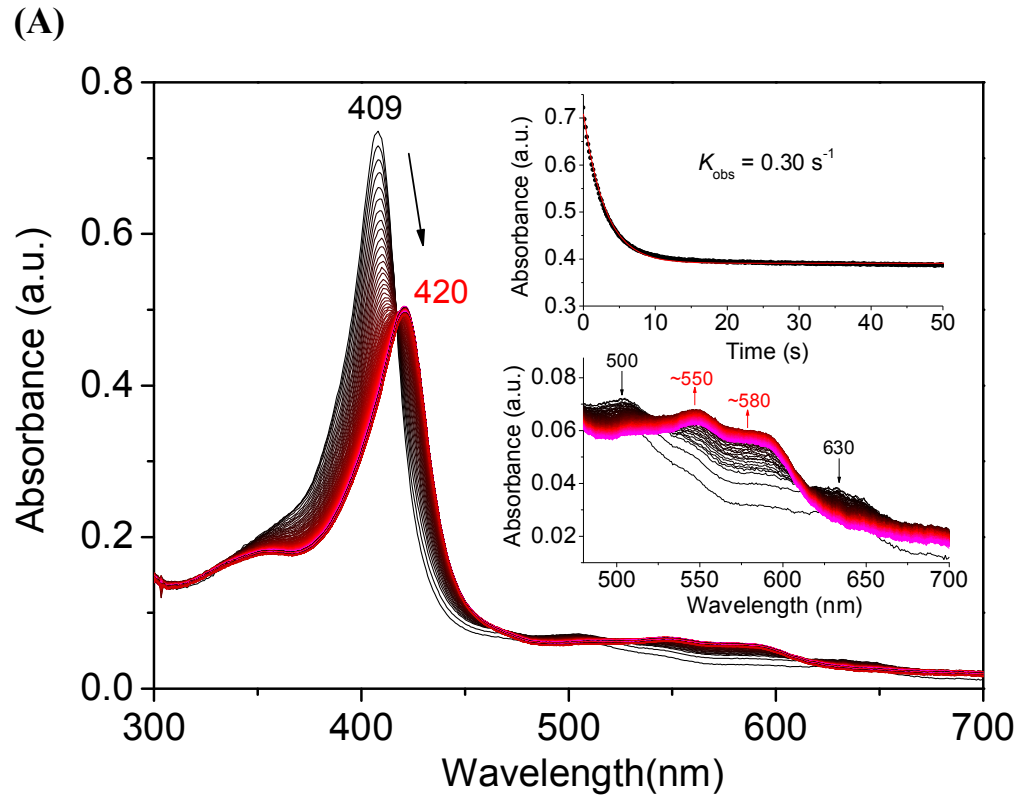
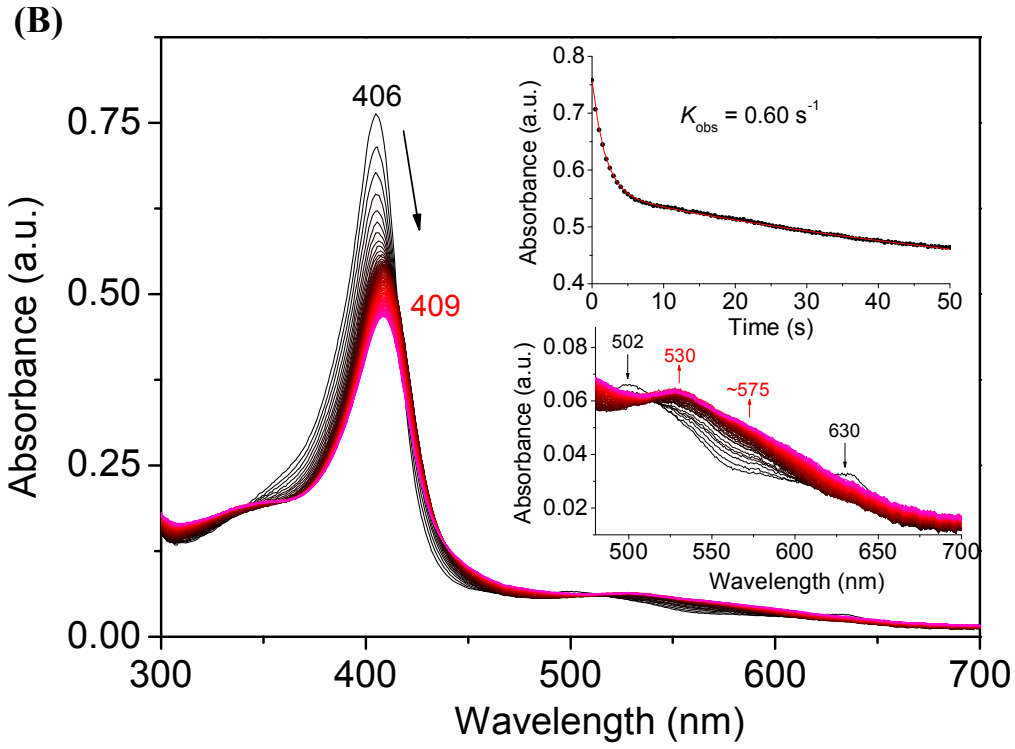


Figure S4. UV-vis spectra of WT Mb (A), L29H Mb (B), and F43Y Mb (C) upon titration with imidazole. The fitting of the intensity changes of Soret band versus imidazole concentrations is shown as an inset.


(A)
409
420
Absorbance (a.u.)
Wavelength(nm)
K_{obs} = 0.30 s^{-1}
Time (s)
500
~550
~580
630
Wavelength (nm)


(B)
406
409
Absorbance (a.u.)
Wavelength (nm)
K_{obs} = 0.60 s^{-1}
Time (s)
502
530
~575
630
Wavelength (nm)

(C)

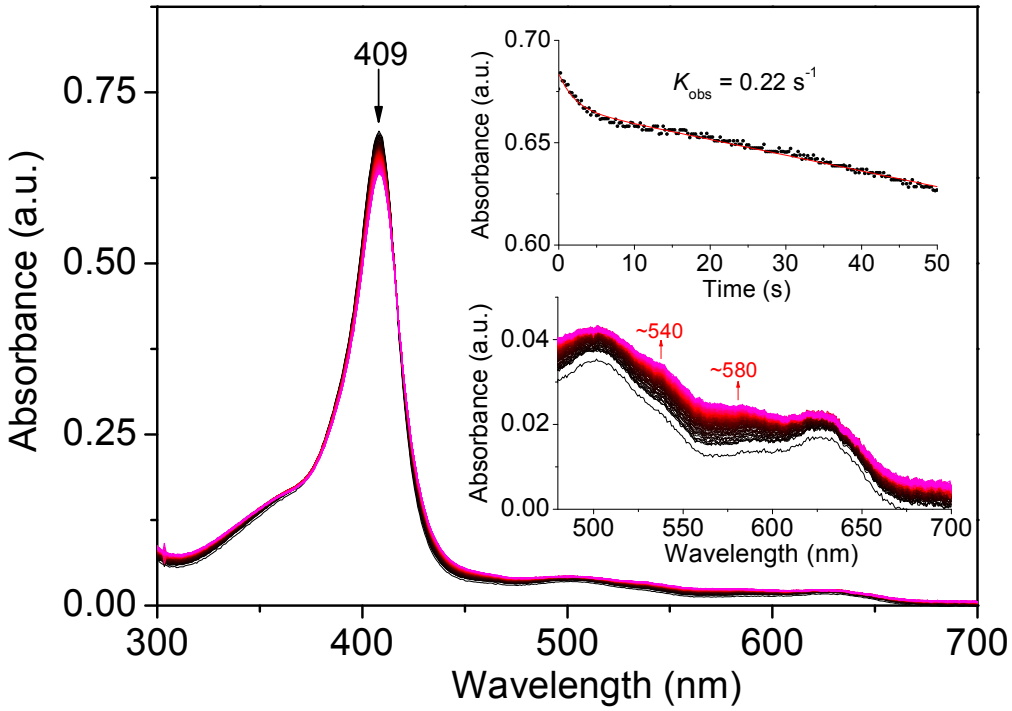


Figure S5. Stopped-flow spectra upon mixing 10 μM WT Mb (A), L29H/F43Y Mb-X (B) and L29H Mb (C) and 0.5 mM H_2O_2 in 100 mM KH_2PO_4 buffer, pH 7.0, 25 ^oC, for 20 sec. Insets, the single-exponential fits (A) and double-exponential fit (B and C) of the decay of Soret band.

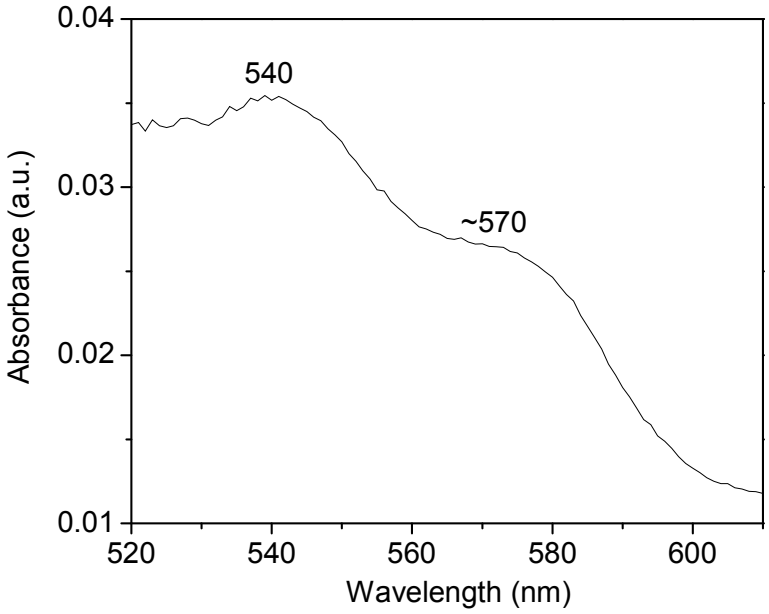


Figure S6. UV-vis spectrum of the visible bands for L29H/F43Y Mb in oxy form.

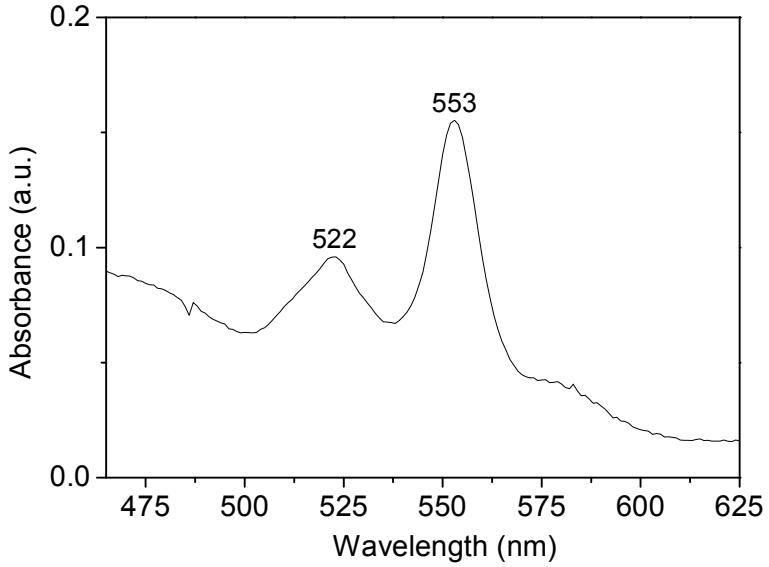


Figure S7. UV-vis spectra of the reduced pyridine hemochromagen complex of L29H/F43Y Mb after reaction. The protein was obtained by reaction with DTT in presence of O_2 and re-oxidized by $K_3[Fe(CN)_6]$.

Table S1. X-ray crystallography data collection and refinement statistics.

	L29H/F43Y Mb	**L29H/F43Y Mb-X**
Wavelength	0.9792	0.9792
Space group	$P2_12_12_1$	$P2_12_12_1$
Unit-cell dimensions (Å, °)	a = 39.871, b = 48.400, c = 78.787; $\alpha = \beta = \gamma = 90$	a =40.062, b = 48.348, c =79.141; $\alpha = \beta = \gamma = 90$
Resolution (Å)	1.36	1.79
No. of observations	427643	541169
No. of unique reflections[a]	33291 (1638)	14678 (738)
Completeness (%)	99.9 (100.0)	99.7 (100.0)
<I>/ (I)	16.1 (2.0)	19.8 (10.1)
Redundancy	12.8 (12.8)	13.0 (12.9)
R_{sym}[b]	0.068 (0.466)	0.163 (0.524)
R_{cryst}[c] (%)/R_{free}[d] (%)	0.1801 / 0.1991	0.1568 / 0.1808
RMSD bonds (Å)/angles (°)	0.007 / 1.132	0.0262 /2.2276
Ramachandran plot, residues in:		
Most favored regions (%)	96.7	98.0
Additional allowed regions (%)	2.3	2.0
Generously allowed regions (%)	1.0	0
Disallowed regions (%)	0.0	0
PDB code	4LPI	5C6Y

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

[b] $R_{sym} = \Sigma|I_j - \langle I \rangle| / \Sigma I_j$, 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] $R_{cryst} = \Sigma||F_{obs}| - |F_{calc}||/\Sigma|F_{obs}|$, F_{obs} and F_{calc} are observed and calculated structure factor amplitudes, respectively.

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