Regulation of both structure and function by a *de novo* designed disulfide bond: A case study of heme protein in myoglobin

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Supporting Information

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, where the position of 123 is Asp (D), different from that of Asn (N) as reported by Springer and Sligar,[1] and purified using the procedure described previously.[2] V21C/V66C Mb, V21C/V66C/F46S Mb and F46S Mb genes were constructed by using the QuikChange Site Directed Mutagenesis Kit (Stratagene). The mutations were confirmed by DNA sequencing assay. All mutants were expressed in BL21(DE3) host cells and purified using the procedure described previously for WT Mb.[2]

1.2 UV-Vis spectroscopy

UV-Vis spectra of Mb and its mutants were recorded on a Hewlett-Packard 8453 diode array spectrometer. Deoxy Mbs were prepared by addition of a small amount of sodium dithionite and eluted from PD-10 column (GE Healthcare). Protein concentrations were determined with an extinction coefficient of ε_{409nm} = 157 $mM^{-1}\cdot cm^{-1}$ for WT Mb[1], and ε_{409nm} = 150 ± 5 $mM^{-1}\cdot cm^{-1}$ for the mutants, as calculated using the standard hemochromagen method. [3]

UV-Vis spectra (300-700 nm) of V21C/V66C Mb and WT Mb upon guanidine hydrochloride (Gdn·HCl)-induced unfolding were performed by titration of 10 μL protein solution to 2 mL Gdn·HCl stock solutions (0~4.0 M, pH 7.0) with a final protein concentration of ~10 μM. The samples were incubated at 25 ^{o}C for 1 h before collecting the UV-Vis spectra. The denaturation midpoint (C_m) was calculated by fitting the absorbance of Soret band *versus* the concentrations of Gdn·HCl to the two-state Boltzmann function (eq. 1):

$$A = A_2 + (A_1 - A_2)/(1+ e^{(C-Cm)/dC}) \qquad \text{(eq. 1)}$$

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.

For nitrite reductase activity assay, the reaction of V21C/V66C Mb and WT Mb

(5 μM in deoxygenated 100 mM potassium phosphate buffer, pH 7.4, 25 ^{o}C) was measured anaerobically by degassing the sealed cuvette and refilling with highly purified N_2 gas (>99.999 %) under pseudo-first order conditions, with varying sodium nitrite concentrations (0.1~1.0 mM) in presence of sodium dithionite (1 mM). The spectral changes upon nitrite reduction were monitored using an Agilent 8453 UV-Vis diode array spectrophotometer. The spectra were collected each 6 sec for 1 h. The observed rates (k_{obs}, s^{-1}) were calculated by fitting the absorbance of Soret band *versus* time to a single-exponential decay function. The k_{obs} values at each nitrite concentration were then plotted *versus* nitrite concentrations, and linearly fitted to obtain the observed bimolecular rate constants of nitrite reduction ($k_{nitrite}$, $M^{-1}s^{-1}$).

1.3 Mass spectrometry

Protein mass spectra were measured on G2-XS QToF mass spectrometer (Waters). The V21C/V66C Mb and V21C/V66C/F46S Mb samples were diluted with 0.1 M acetic acid (pH 3.0) to 1 μg/μL. 50 μL of protein was incubated without or in the presence of 10 mM tris-(2-carboxyethyl)-phosphine (TCEP) (Sigma-Aldrich) at 65 ^{o}C for 10 min, as the native form or reduced form, respectively. The protein solution was mixed with 1% formic acid, which was transferred into the mass spectrometer chamber for measurement under positive mode. For control study, the mass of V21C/V66C Mb reduced by addition of dithionite (50 μM) for 5 min was also carried out under the same conditions. The multiple m/z peaks were transformed to the protein molecular weight by using software MaxEnt1.

1.4 EPR spectroscopy

Electron paramagnetic resonance (EPR) spectra of V21C/V66C Mb and V21C/V66C/F46S Mb (0.3 mM) in the met form were collected at the high magnetic field laboratory of Chinese Academy of Science, Hefei, China. The sample was analyzed by X-band EPR on a Bruker EMX plus 10/12 spectrometer. A standard Bruker cavity (ER4119hs TE011) was used in conjunction with an Oxford Instrument EPR910 liquid helium continuous-flow cryostat for low-temperature analysis. The

spectrum was measured at a low temperature of 10 K, with frequency of 9.43 GHz, center field 2200 G and sweep width 3600 G, microwave power 2 mW and modulation amplitude 3.0 G. Control experiments of single mutant F46S Mb and WT Mb were carried out under the similar conditions.

1.5 Stopped-flow spectroscopy

The O_2 dissociation rate constants of oxy-WT Mb and oxy-V21C/V66C Mb were determined using a dual mixing stopped-flow spectrophotometer (SF-61DX2 Hi-Tech KinetAsyst^{TM}). The ferric protein was reduced by excess dithionite, which was removed from the solution by a PD-10 column (GE Healthcare). Oxy-Mbs were then obtained by exposure of dexoy-Mbs to the air, which were stable enough for kinetic measurements. Oxy-Mbs (10 μM, in 50 mM potassium phosphate buffer, pH 7.0) was mixed with an equal volume of 10 mM sodium dithionite in deoxygenated 50 mM potassium phosphate buffer, pH 7.0, at 25 ^{o}C. The changes of the Soret band for deoxy-Mbs were monitored and fitted to a single-exponential decay function.

The dehaloperoxidase activity assay of V21C/V66C/F46S Mb was performed on the same spectrophotometer at 25 ^{o}C. The absorbance at 272 nm (12 $mM^{-1}\cdot cm^{-1}$)[4] of the 2,4-dichloroquinone (DCQ) product was monitored *versus* time. The initial rate for each reaction was calculated from the linear initial portion of the trace. Control experiments were performed for V21C/V66C Mb, F46S Mb and WT Mb. All of the activity assays of Mbs were measured in 50 mM potassium phosphate buffer (pH 7.0). To determine the steady-state kinetic parameters (k_{cat} and K_m), varied concentrations of the substrate, 2,4,6-trichlorophenol (TCP), were mixed with the protein, and the reactions were then initiated by addition of H_2O_2. The final concentrations were 0~0.8 mM for TCP, 1 μM for protein and 10 mM for H_2O_2, respectively. The plots of initial rates as a function of TCP concentrations were fitted to the Michaelis-Menten equation (eq. 2) :

$$\upsilon/[\text{protein}] = k_{cat}[\text{TCP}]/(K_m + [\text{TCP}]) \qquad \text{(eq. 2)}$$

1.6 Substrate titration studies

UV-Vis spectra of substrates titration were collected on an Aglilent 8453 spectrometer at room temperature (25 °C). F46S Mb or V21C/V66C/F46S Mb was dissolved in 100 mM potassium phosphate buffer, pH 7.0 with a concentration of 10 μM. TCP was titrated dropwisely into the protein solution to a final concentration of 0.6 mM. The spectral changes of Soret band were plotted by the double reciprocal plot method [5], and fit to eq. 3.

$$1/\Delta A = (K_d/\Delta A_{inf})(1/[M]) + 1/\Delta A_{inf} \qquad \text{(eq. 3)}$$

where ΔF is the difference between the maximum and minimum absorption, [M] is the concentration of free substrate, which is assumed to be equal to the total concentration of added substrate, and ΔA_{inf} is the absorbance change for the complete formation of the adduct.

1.7 X-ray crystallography

V21C/V66C/F46S Mb with a high purity (A_{409nm}/A_{280nm} 3.5) was exchanged into 20 mM potassium phosphate buffer (pH 7.0) and concentrated to ~2.0 mM. The vapor diffusion hanging drop technique was used to crystallize the protein. 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.[6] The structure was solved by the molecular replacement method and the 1.6 Å structure of WT Mb (PDB entry 1JP6[7]) was used as the starting model. Manual adjustment of the model was carried out using the program COOT[8] and the models were refined by PHENIX[9] and Refmac5[10]. Stereochemical quality of the structures was checked by using PROCHECK.[11] All of residues locate in the favored and allowed region and none in the disallowed region (Table S1).

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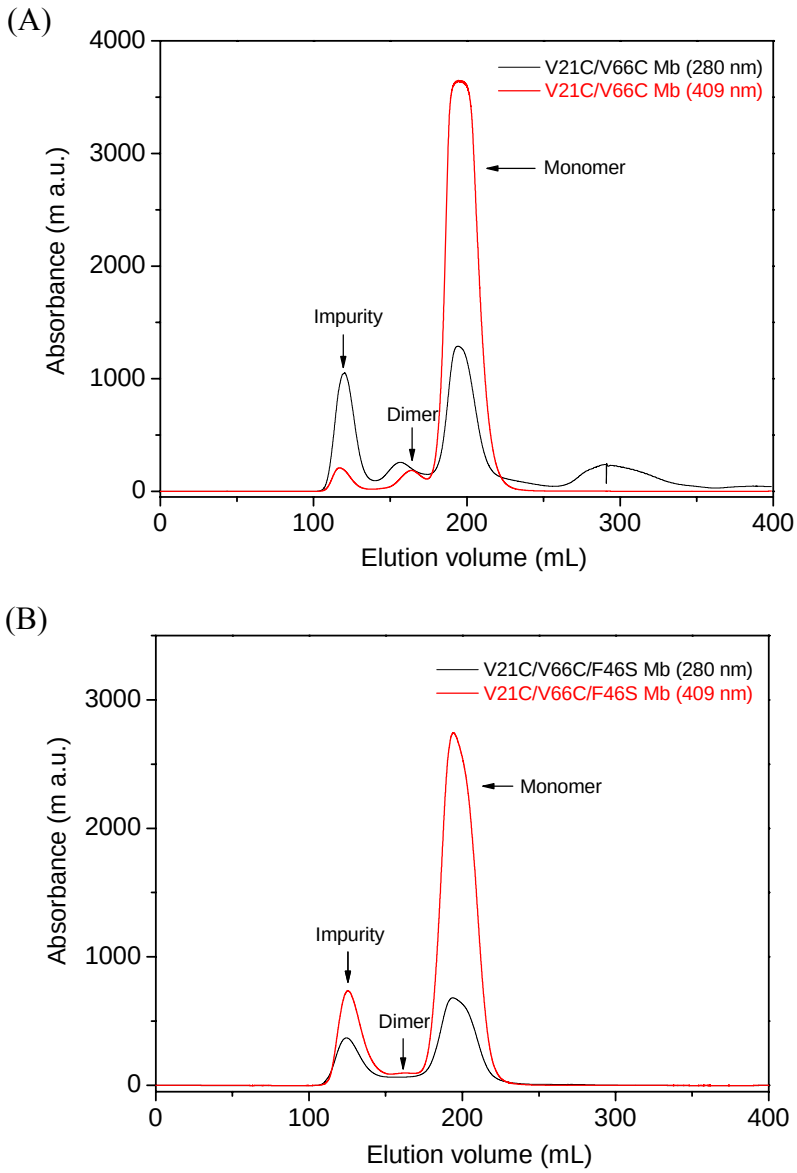


Fig. S1 Elution curves of (A) V21C/V66C Mb and (B) V21C/V66C/F46S Mb. Measurement conditions: Column, Superdex 75 10/300 GL; buffer, 100 mM potassium phosphate buffer, pH 7.0; monitoring wavelengths, 280 nm and 409 nm; temperature, 4 °C.

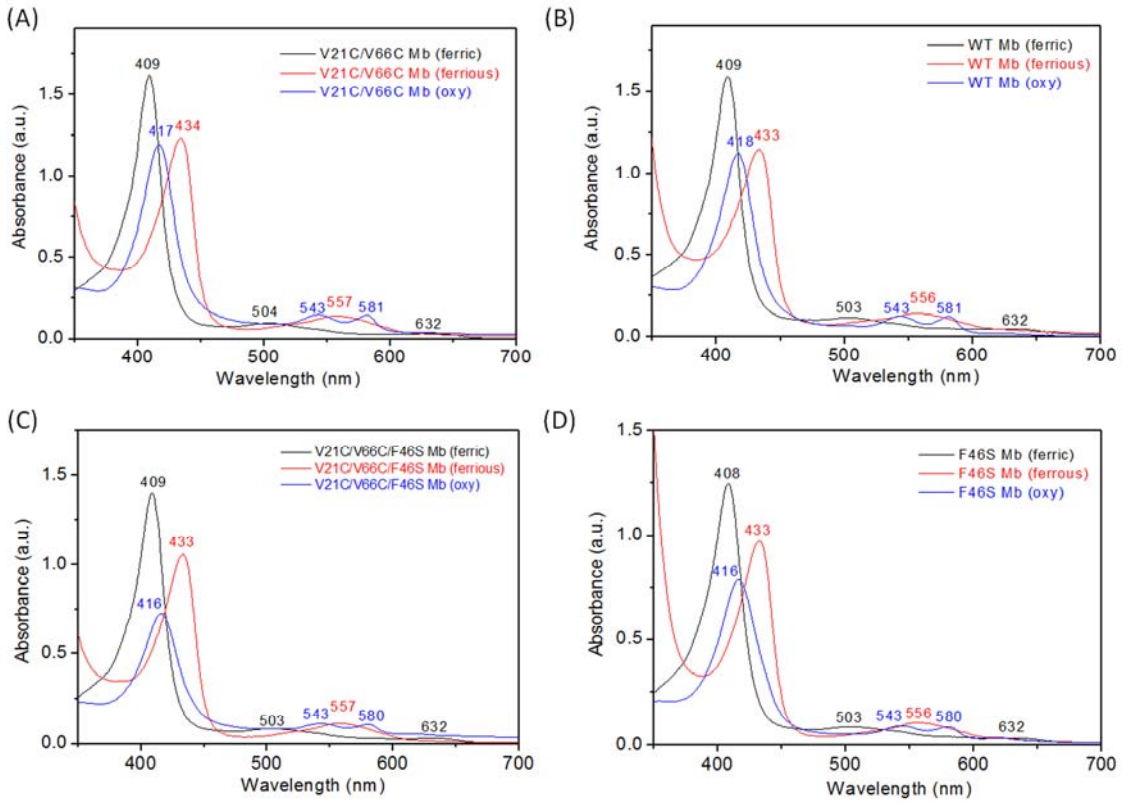


Fig. S2 UV-Vis spectra of (A) V21C/V66C Mb, (B) WT Mb, (C) V21C/V66C/F46S Mb, and (D) F46S Mb in the ferric (black), ferrous (red), and oxy (blue) forms.

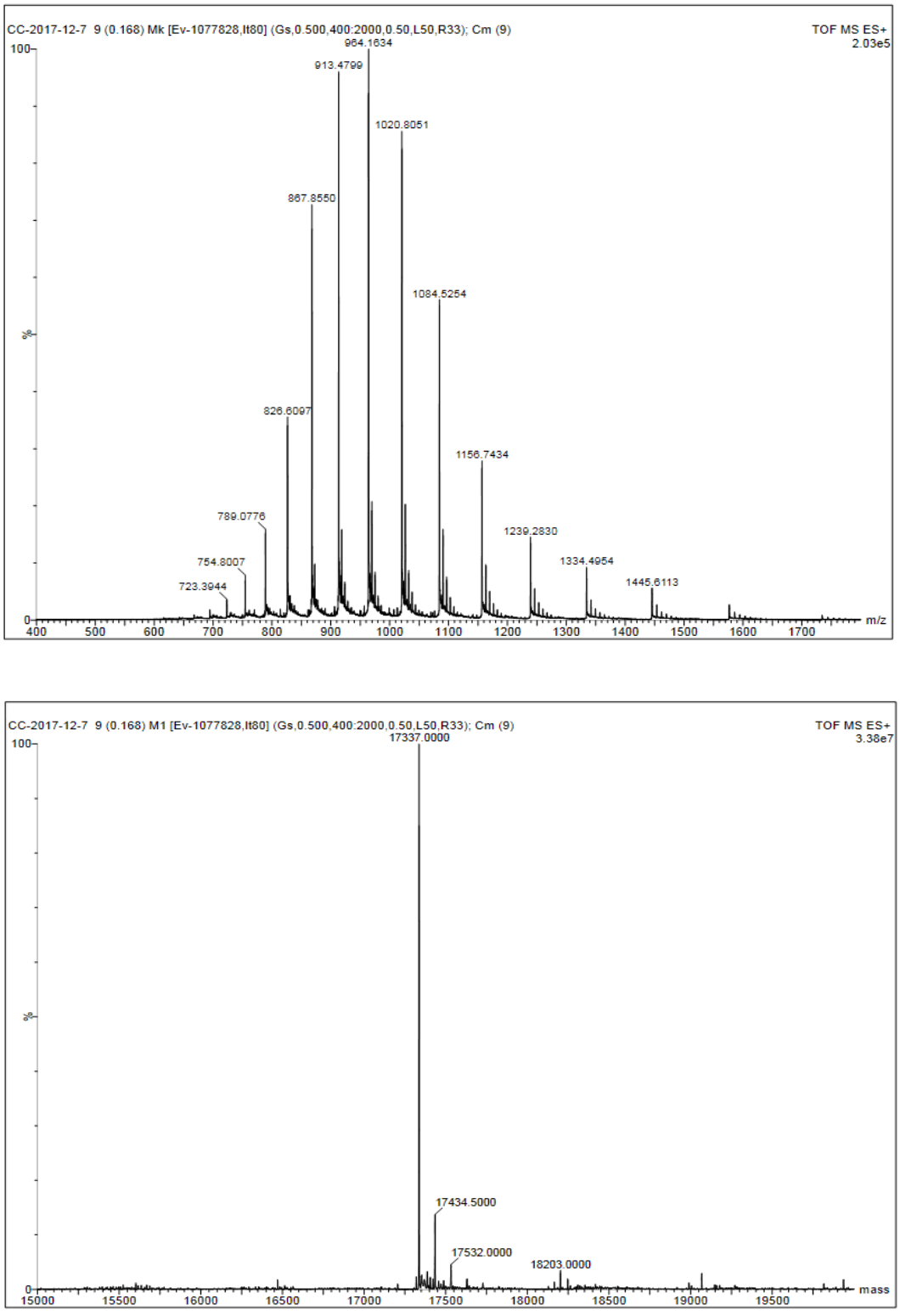
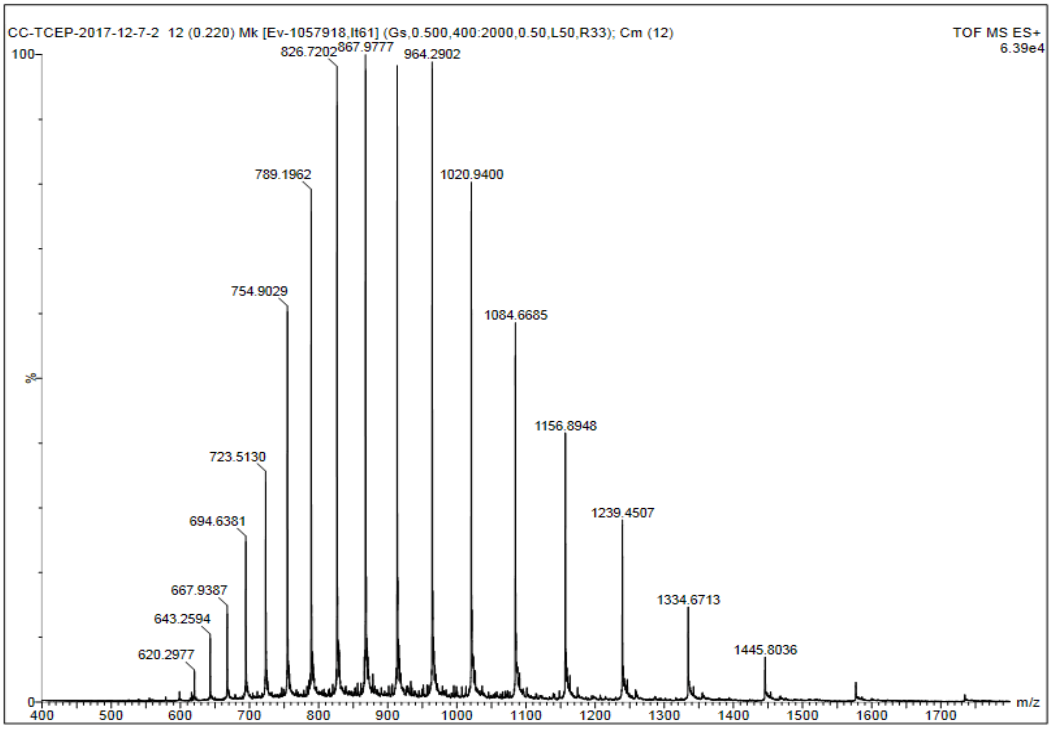


Fig. S3A MS spectrum of V21C/V66C Mb: Calculated molecular weight with a disulfide bond, 17337 Da, and the observed, 17337.0 ± 0.5 Da.



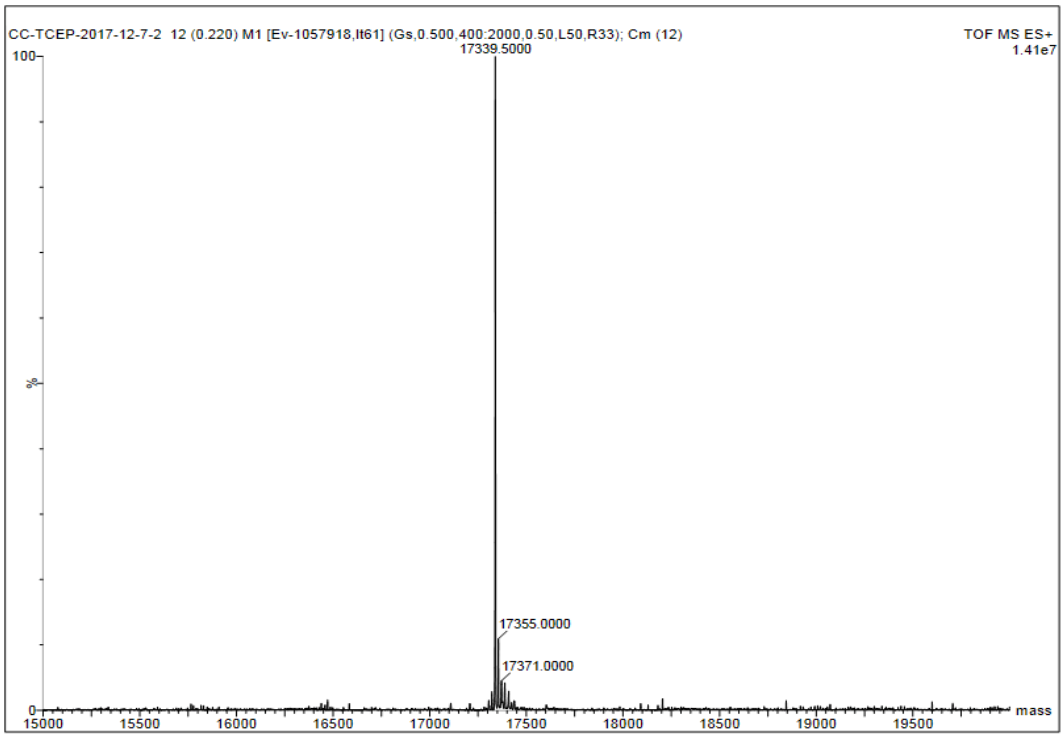


Fig. S3B MS spectrum of V21C/V66C Mb treated by TCEP: Calculated molecular weight without a disulfide bond, 17339 Da, and the observed, 17339.5 ± 0.5 Da.

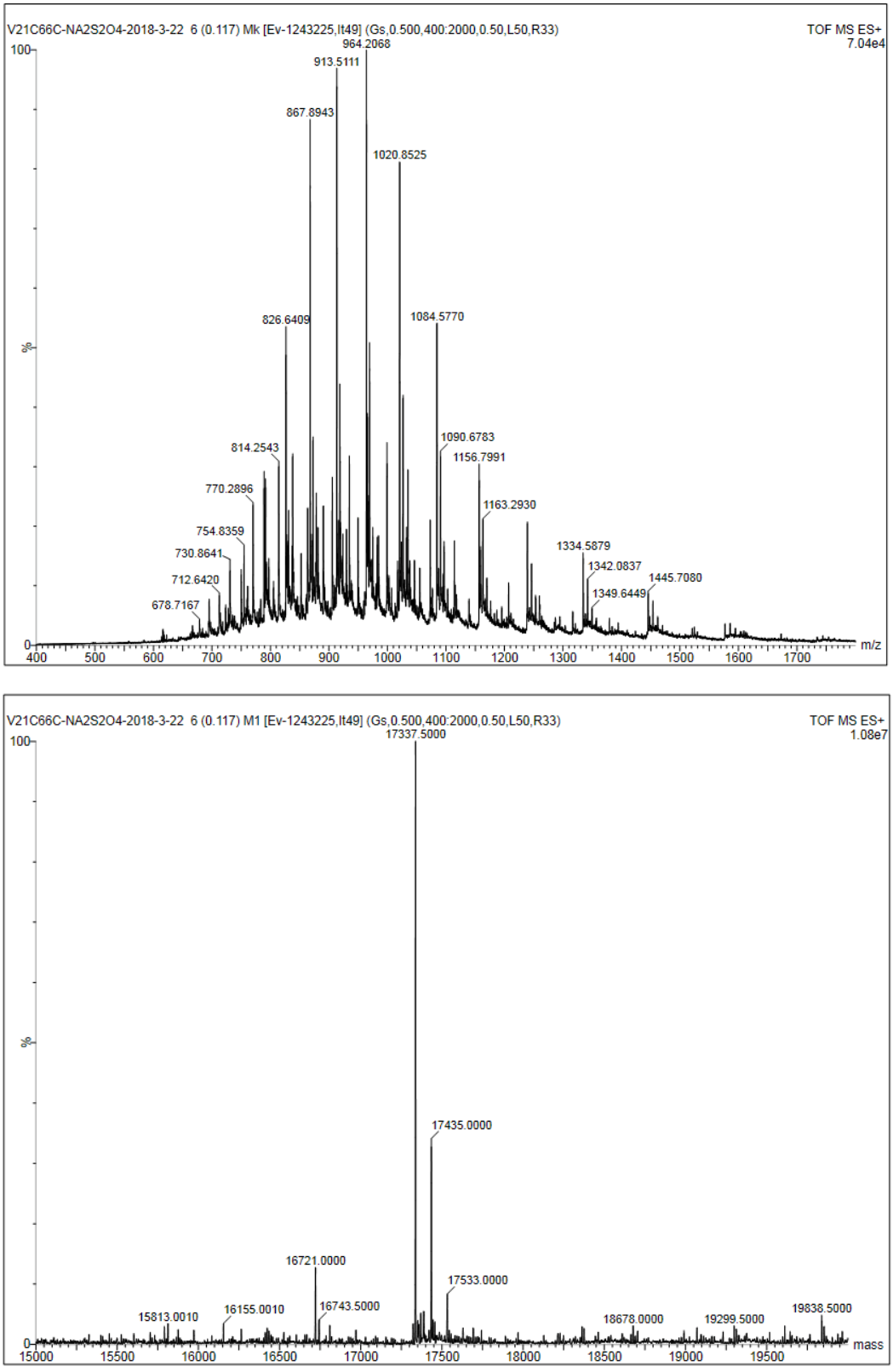
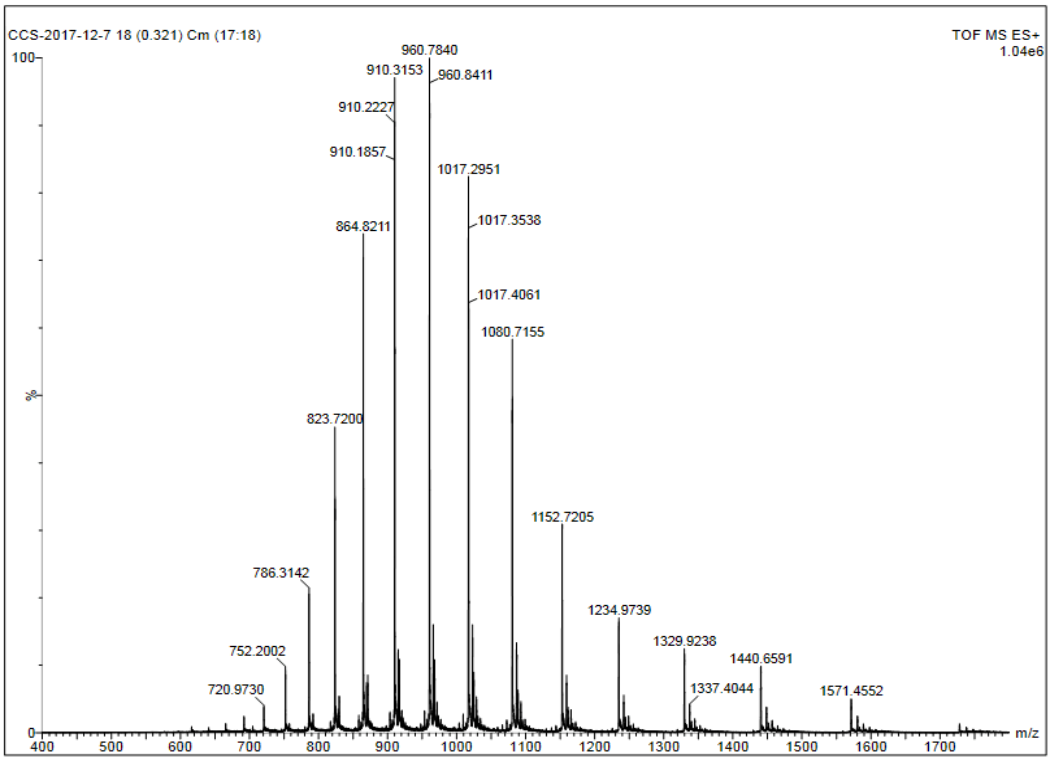


Fig. S3C MS spectrum of V21C/V66C Mb treated by dithionite: Calculated molecular weight with a disulfide bond, 17337 Da, and the observed, 17337.5 ± 0.5 Da.



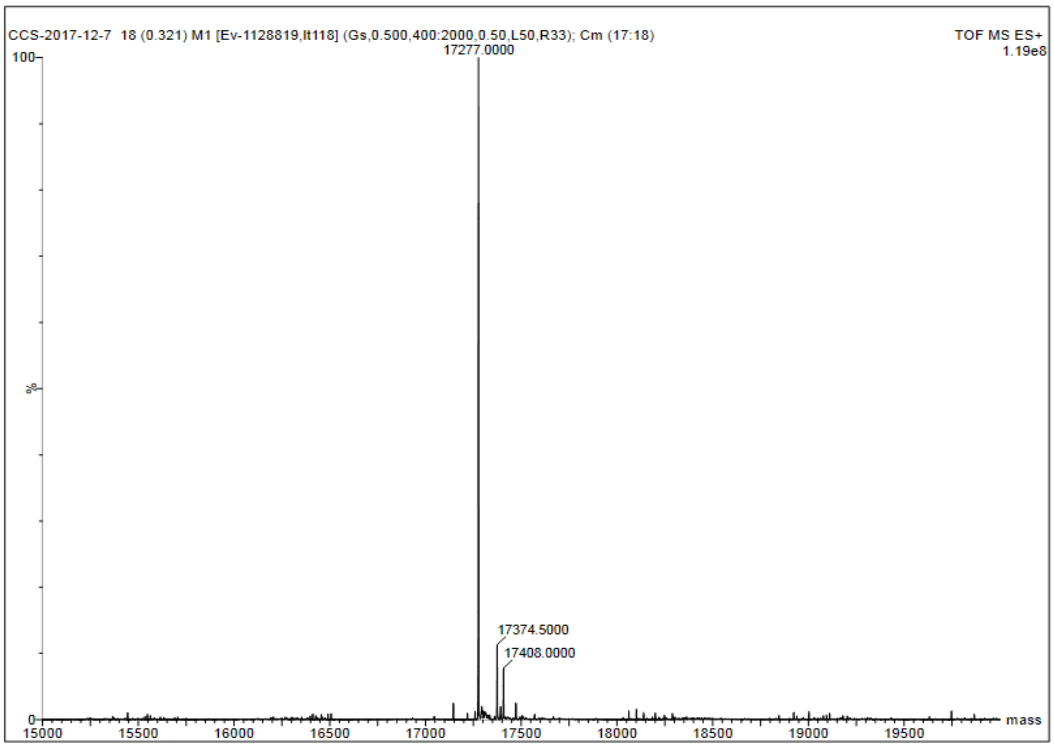
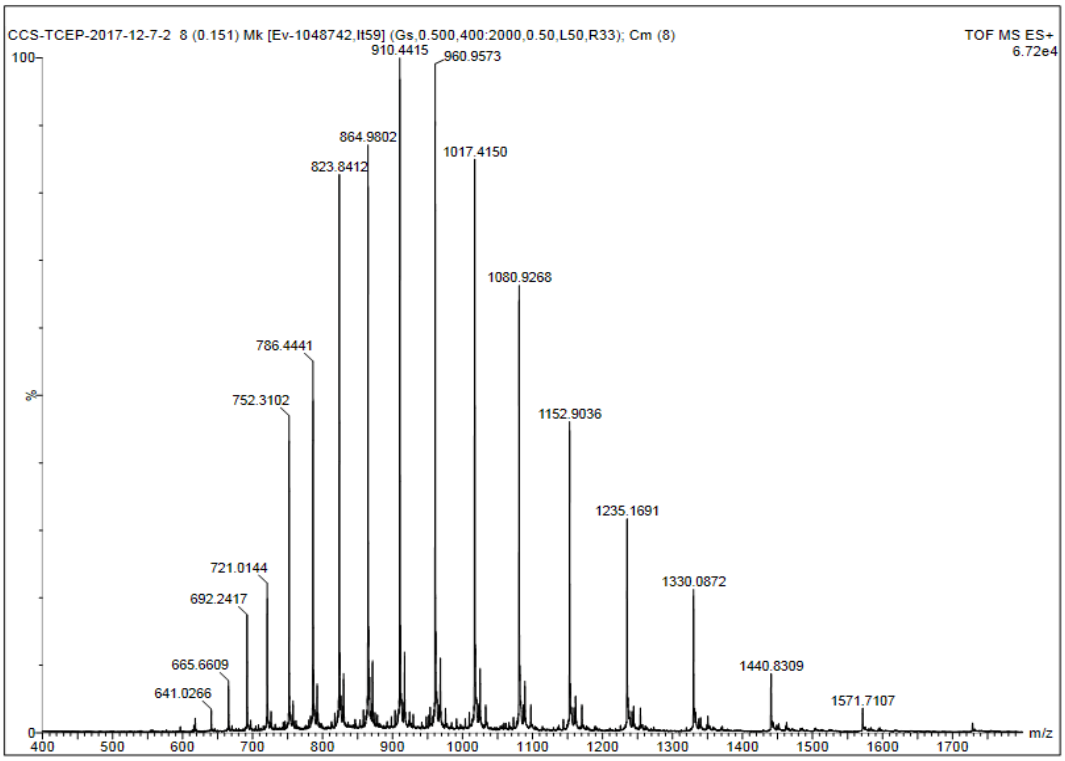


Fig. S3D MS spectrum of V21C/V66C/F46S Mb: Calculated molecular weight with a disulfide bond, 17277 Da, and the observed, 17277.0 ± 0.5 Da.



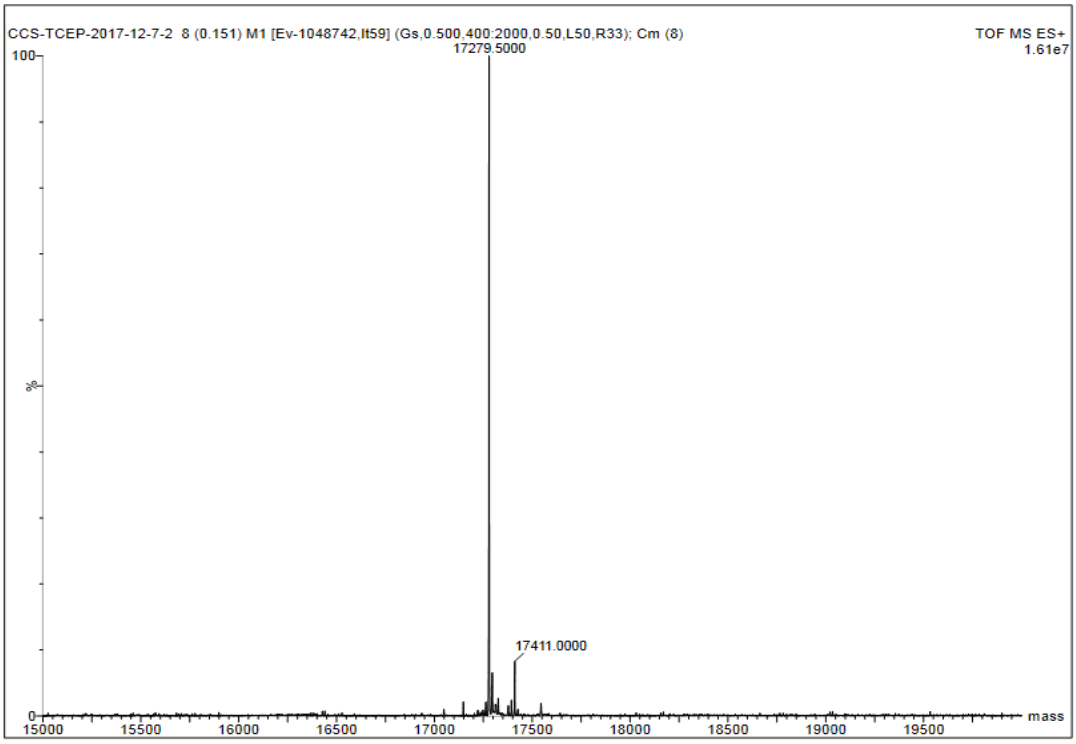


Fig. S3E MS spectrum of V21C/V66C/F46S Mb treated by TCEP: Calculated molecular weight without a disulfide bond, 17279 Da, and the observed, 17279.5 ± 0.5 Da.

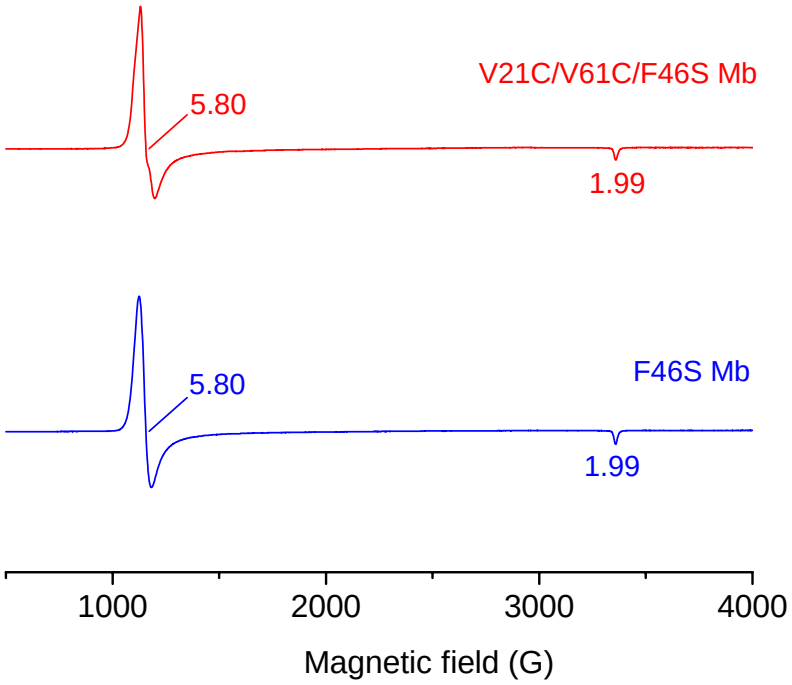


Fig. S4 EPR spectra of V21C/V66C/F46S Mb and F46S Mb (0.3 mM) measured at 10 K, 2 mW power and 9.43 GHz.

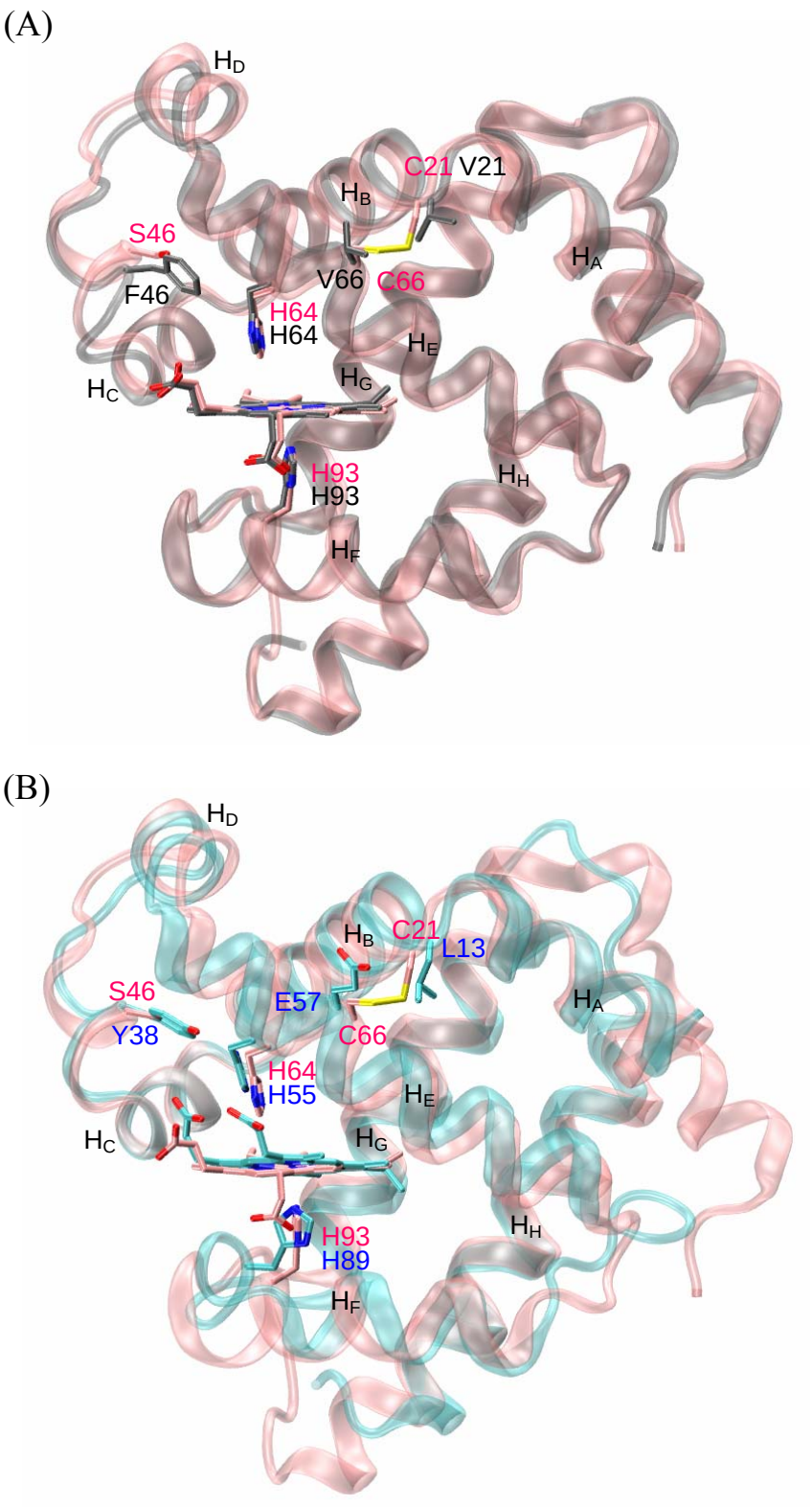


Fig. S5 Overlay of the X-ray structrue of V21C/V66C/F46S Mb (pink) and (A) WT Mb (gray, PDB code 1JP6), and (B) DHP from *Amphitrite ornata* (cyan, PDB code 1EW6), showing the heme active site and helices H_A-H_G.

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

	V21C/V66C/F46S Mb
Wavelength	0.9792
Space group	$P2_12_12_1$
Unit-cell dimensions (Å, °)	a =39.566, b = 47.351, c =79.111; $\alpha=\beta=\gamma$ = 90
Resolution (Å)	40.63-1.77 (1.80-1.77)
No. of observations	164550
No. of unique reflections[a]	15020 (725)[a]
Completeness (%)	99.4 (97.7)
<I>/ (I)	23.05 (5.33)
Redundancy	5.6 (5.5)
R_{sym}[b]	0.157 (0.468)
R_{cryst}[c] (%)/R_{free}[d] (%)	0.221 / 0.265
RMSD bonds (Å) / angles (°)	0.020 / 1.876
Ramachandran plot, residues in:	
Most favored regions (%)	96.1
Allowed regions (%)	3.9
Disallowed regions (%)	0.0
PDB code	5ZEO

[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%).

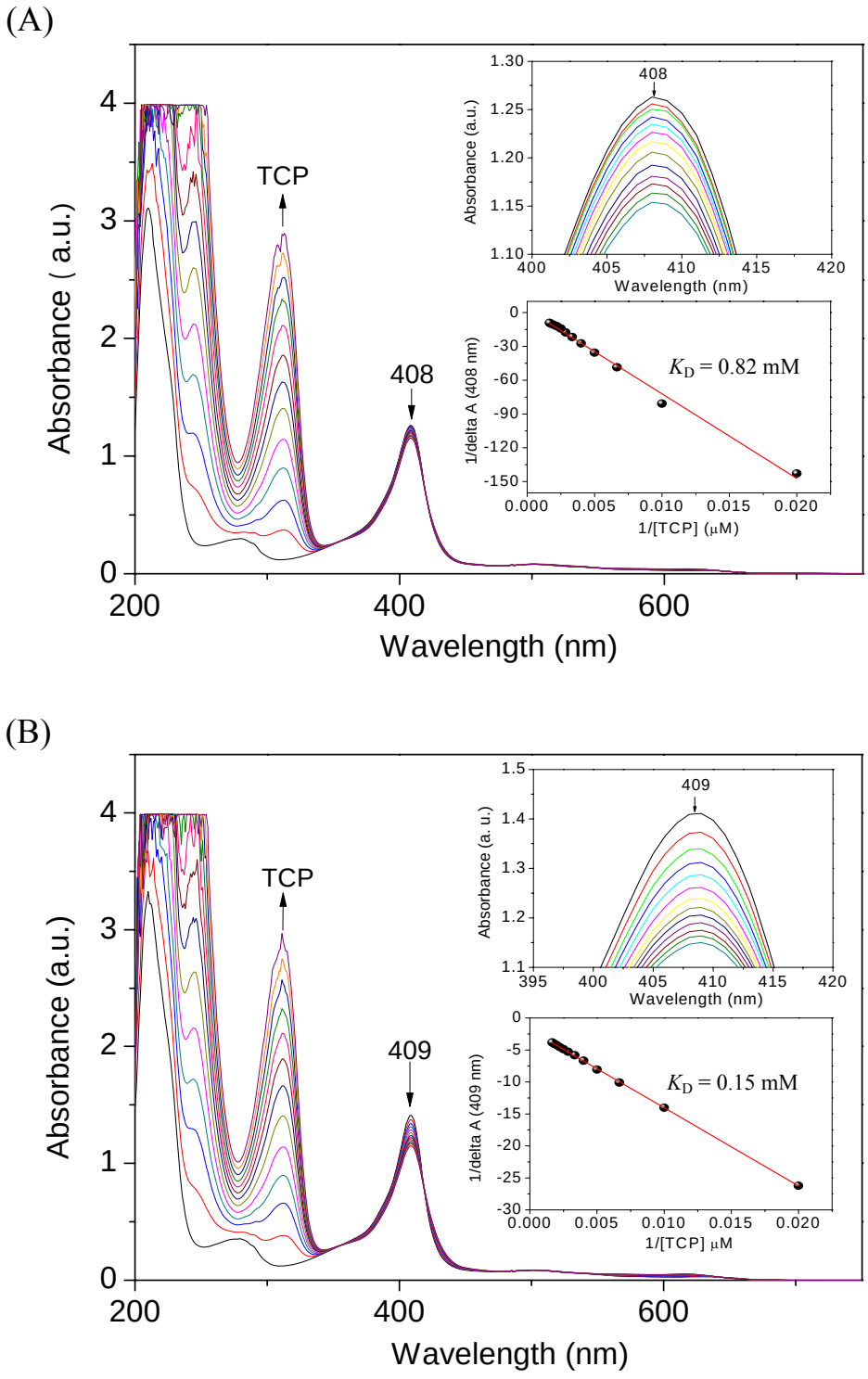


Fig. S6 UV-Vis spectra of titration of (A) F46S Mb and (B) V21C/V66C/F46S Mb (10 μM) in 100 mM potassium phosphate buffer (pH 7.0) with TCP to a final concentration of 0.6 mM. The changes of Sorect band, and the double reciprocal plot of the Soret absorbance change *versus* the substrate concentration are shown as insets.