SUPPORTING INFORMATION

Systematic Tuning of Heme Redox Potentials and Its Effects on O_2 Reduction Rates in a Designed Oxidase in Myoglobin

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Materials and Methods:

All chemicals, unless otherwise specified, were obtained from Sigma (St. Louis, MO) or Fisher Scientific (Hampton, NH). Fe(III)-incorporated non-native heme cofactors were purchased from Frontier Scientific (Logan, UT).

Purification of proteins and incorporation of non-native heme cofactors

F33Y-Cu_BMb and S92A-F33Y-Cu_BMb were expressed and purified as reported previously.[1] Briefly, apo-protein was expressed and localized to the inclusion bodies of BL21(DE3) cells (New England Biolabs). The cells were then lysed by sonication and denatured with 6 M guanidine chloride. Heme *b* was added to the denatured cell solution, which was then dialyzed against 10 mM TRIS-H_2SO_4 pH 8 at least 4 times. The resulting protein solution was concentrated by Amicon with a 10,000 MWCO filter, and passed through a size exclusion column to achieve homogeneity. Final yield of the protein was ~20 mg/L. WTMb was expressed and purified as reported previously and its yield was typically ~100 mg/L.[2] Heme *b* was removed from F33Y-Cu_BMb and WTMb via acidified butanone extraction as per Teale's method.[3] Complete removal of heme *b* was ensured by taking a UV-vis spectra of the apo-protein, and only when the concentration of heme *b* (ε_{408nm} ~150 $mM^{-1}cm^{-1}$) was less than 1% of the concentration of protein (ε_{280nm} ~30 $mM^{-1}cm^{-1}$), non-native heme cofactors were incorporated.

Fe(III)-2,4-diacetyl deuteroporphyrin IX chloride (DA-heme), Fe(III)-2,4 (4,2) formyl vinyl deuteroporphyrin IX chloride (MF-heme) and Fe(III)-2,4-diformyl deuteroporphyrin IX chloride (DF-heme) were characterized by mass spectrometry before being incorporated into the apo-protein. The non-native heme cofactors were dissolved in dimethyl sulfoxide and titrated into the apo-WTMb/apo-F33Y-Cu_BMb until the ratio of the absorption of the heme band (408-430 nm depending on the cofactor) to that of the protein (280 nm) stopped increasing (usually 3 equivalent of heme was added to the apo-protein). Heme-protein mixture was then dialyzed against subsequently increasing concentration of pH 7 potassium phosphate buffers to ensure proper folding of the protein. The excess heme was then removed by a DEAE-Sepharose and size exclusion column. F33Y-Cu_BMb variants were finally chelexed to remove any metal ions at the Cu_B site as per a procedure reported previously.[2,4]

Characterization of F33Y-Cu_BMb and WTMb variants via UV-vis spectroscopy and Circular Dichroism

Table S1 enlists the spectral features of oxidized and reduced forms of F33Y-Cu_BMb and WTMb variants. The oxidized form of the proteins show a Soret band at 400 nm range followed by α and β bands in the visible region, typical of a well-folded oxidized myoglobin. Extinction coefficient of the proteins was determined by hemochromogen assay where the wavelength maximum (in nm) and extinction coefficient (in mM^{-1} cm^{-1}) used for the α peak of heme *b*, DA-, MF- and DF-heme were 556 (36), 576 (25), 583 (28) and 587 (20.5) respectively. Even though a racemic mixture of Fe(III)-2,4 (4,2)-formyl vinyl deuteroporphyrin IX chloride (MF-heme) was titrated into the apo-F33Y-Cu_BMb, comparison of the UV-vis spectroscopic properties of F33Y-Cu_BMb (MF-heme) (e.g. Soret wavelength maxima and the wavelength maxima of the hemochromogenic assay) with literature values reveals that only Fe(III)-2,4-formyl vinyl deuteroporphyrin IX got incorporated in the protein.[5,6] It should be noted that the wavelength maxima of MF-heme and DF-heme in the hemochromogenic assay exhibit a bathochromic shift as compared to heme *b* and match well with that of native heme *a* in the catalytic site of cytochrome *c* oxidase (585 nm).[7]

Circular dichroism (CD) measurements as a function of wavelength were obtained using a JASCO J-715 spectrometer at 20°C in a standard 1 cm path length cuvette with protein samples in 100 mM phosphate buffer pH 6.0 at a protein concentration of 2-3 μM. The Mean Residue Ellipticities $[\Theta]_{222nm}$ was calculated with the following equation :

$$[\Theta]_{222nm} = \Theta_{222nm} / L\ (cm) \times C\ (decimole) \times n\ (deg.cm^2.dmol^{-1})$$

Helical content of the protein was determined as described by Chen *et al,* and compared to the literature record of WTMb.[8,9]

L: length of light path of cuvette; C: protein sample concentration; n: total number of the amino acid residues in the protein.

Spectroelectrochemical Measurements The reduction potential (E°) was measured by a spectroelectrochemical method using an optically transparent thin-layer cell (OTTLE). The working electrode was made from a piece of 52 mesh platinum gauze. A 1 mm diameter platinum wire was used as the auxiliary electrode, while a piece of Pasteur pipet filled with agar gel containing 0.93 M KCl was used a salt bridge to connect the Ag/AgCl (3 M KCl) reference electrode to the bulk solution containing the working and auxiliary electrodes. Generally, the redox titration was performed using ~ 0.6 mL of working solution containing 0.2 mM protein, 45 μM phenazine methosulfate, 45 μM anthraquinone-2-sulfonate, and 20 μM N,N,N',N'-tetramethyl-p-phenylenediamine (TMPD) as redox mediators in 100 mM phosphate buffer, pH6. The working solution was purged gently with Argon before transferring to the OTTLE cell. A model 362 potentiostat from Princeton Applied Research was used to control the potential of working electrode. After the potential was applied (typically with 25 mV increments), the UV-vis spectra were recorded using a Cary 3E spectrophotometer until no further spectral changes occurred. The Ag/AgCl (3M KCl) reference electrode was calibrated using ferrocene carboxylic acid as the standard and was found to be 220 mV versus SHE. All reduction potentials mentioned in this work are reported against SHE. The system was calibrated using WTMb (Fig. S4); the measured E^o of 61 ± 2 mV matched closely to reported literature values.[10,11]

Data Analysis for spectroelectrochemical measurements

The spectroelectrochemical titration data over the entire spectra were analyzed by global analysis using singular value decomposition (SVD) and nonlinear regression modeling with SpecFit/32 (Spectrum Software Associates, Inc.) using a method followed previously.[12-14] All of the data were fit with a model of A ↔ B as the Nernst plot at a single wavelength gave a straight line fit with n (no. of electrons transferred) value close to 1.

Oxygen consumption assay and Turnover studies using an Oxygen Electrode.

The rate of water and ROS production was measured and calculated as reported previously with slight modifications.[1] 6 μM of protein was incubated in air saturated 100 mM phosphate buffer at pH 6. The oxygen consumption assay was initiated by the addition of 10 mM ascorbate and 1 mM of TMPD.

To calculate the percentage of water with respect to O_2^- and H_2O_2 (ROS) formation, we repeated the measurement of O_2 reduction in the presence of superoxide dismutase (SOD) (250 units) and catalase (2.2 μM), which selectively react with O_2^- and H_2O_2, respectively. By comparing the rates of reduction in the absence of and in the presence of SOD and catalase, the portion of O_2 reduction that is due to H_2O formation can be calculated, using a protocol reported previously.[1] To investigate if TMPD is required for O_2 reduction experiments, we measured O_2 reduction rates of F33Y-Cu_BMb and its variants by ascorbate in the absence of TMPD (Fig. S6). The O_2 reduction rates of F33Y-Cu_BMb, F33Y-Cu_BMb (MF-heme) and F33Y-Cu_BMb (DF-heme) are much lower than those in the presence of TMPD. Interestingly, even in the absence of TMPD, the rate of O_2 reduction increases in direct correlation with heme E^o. Therefore, our results are consistent with the hypothesis that TMPD is a redox mediator to help ascorbate reduce the proteins more efficiently.[15]

The turnover experiment in Fig. 4B was performed similarly except that after the O_2 in the reaction chamber was exhausted, the chamber was vented with pure O_2 to boost O_2 concentration to 500-600 μM. The concentration of ascorbate was kept at 10 mM.

Xray Crystallography Crystals were grown at 4ºC on hanging drops containing 0.1M MES pH6.57, 0.2M NaOAc.3H_2O, 30% PEG 6K as well buffer. Drops contained an equal volume of 1 mM S92A-F33Y-Cu_BMb in 20 mM TRIS.H_2SO_4 pH 8 and the well buffer. Prior to mounting, the S92A-F33Y-Cu_BMb crystals were soaked in buffer containing 0.1M MESpH 6.0, 0.2M NaOAc.3H_2O, 30% PEG 6K for ~ 30 min and then frozen in a cryoprotectant solution of 50 mM MES pH6.0, and 30% PEG 400. Diffraction data were collected at the Brookhaven National Lab Synchrotron Light Source x29 beamline. The crystal structure was solved by molecular replacement using F33Y-Cu_BMb (4FWX).

Supplementary Figures and Tables:

Table S1: UV-vis spectral features of F33Y-Cu_BMb variants

	ferric*			deoxy*	
	Soret	vis		Soret	vis
F33Y-Cu_BMb	407	501	628	431	553
S92A- F33Y-Cu_BMb	407	502	628	429	556
F33Y-Cu_BMb (DA-heme)	416	507	635	444	562
F33Y-Cu_BMb (MF-heme)	422	542	656	443	585
F33Y-Cu_BMb (DF-heme)	430	547	662	460	575
WTMb	408	504	626	431	556
WTMb (DA-heme)	416	507	634	442	566
WTMb (DF-heme)	432	548	663	460	575

*wavelength maxima reported (in nm)

Table S2: Circular Dichroism results for F33Y-Cu_BMb variants

Protein	**Conc (μM)**	**CD_{222nm}**	**[Θ] * 10^3 Deg. cm^2 $dmol^{-1}$/residue**	**α helical content (%)**
Sw WTMb	-	-	24-25	80-85[9]
F33Y-Cu_BMb	2.15	-84.79	25.82	86
S92A-F33Y-Cu_BMb	1.97	-85.33	28.28	94
F33Y-Cu_BMb (DA-heme)	1.95	-84.35	28.27	94
F33Y-Cu_BMb (MF-heme)	1.95	-82.55	27.56	91
F33Y-Cu_BMb (DF-heme)	1.93	-82.55	27.90	91

Table S3: Diffraction and refinement data for S92A-F33Y-Cu_BMb

Data Collection Statistics	
X-ray source	x29 NSLS
X-ray detector	ADSC Q315r
X-ray wavelength (Å)	1.075
Space group	$P2_12_12_1$
Unit Cell (Å)	a=39.467, b=47.910, c=76.854
Molecules in asymmetric unit	1
Resolution range (Å)	50-1.64 (1.73-1.64)
Total reflections	278363
Unique Reflections	18486 (1682)
Overall B-factor, ML ($Å^2$)	20.70
Completeness (%)	99.3 (92.9)
Redundancy	15.1 (13.3)
<I/σI>	31.8 (6.1)
Linear R-merge	0.097 (0.462)
Refinement Statistics	
Program	Phenix
Resolution (Å)	40.66-1.64 (1.66-1.64)
Reflections used in refinement	34403
Test set	3447
Completeness (%)	99.76
R_{all}	0.160
R_{work}	0.156 (0.182)
R_{free}	0.198 (0.260)
Coordinate error (ML)	0.17
Wilson B-factor	21.06
TLS groups (6)	1-35|36-51|52-58|59-100 101-124|125-153
Atoms	
Protein	1240
Water	178
Heme	42
RMSD	
Bonds (Å)	0.017
Angles (º)	1.784
Ramachandran outliers (%)	0.00

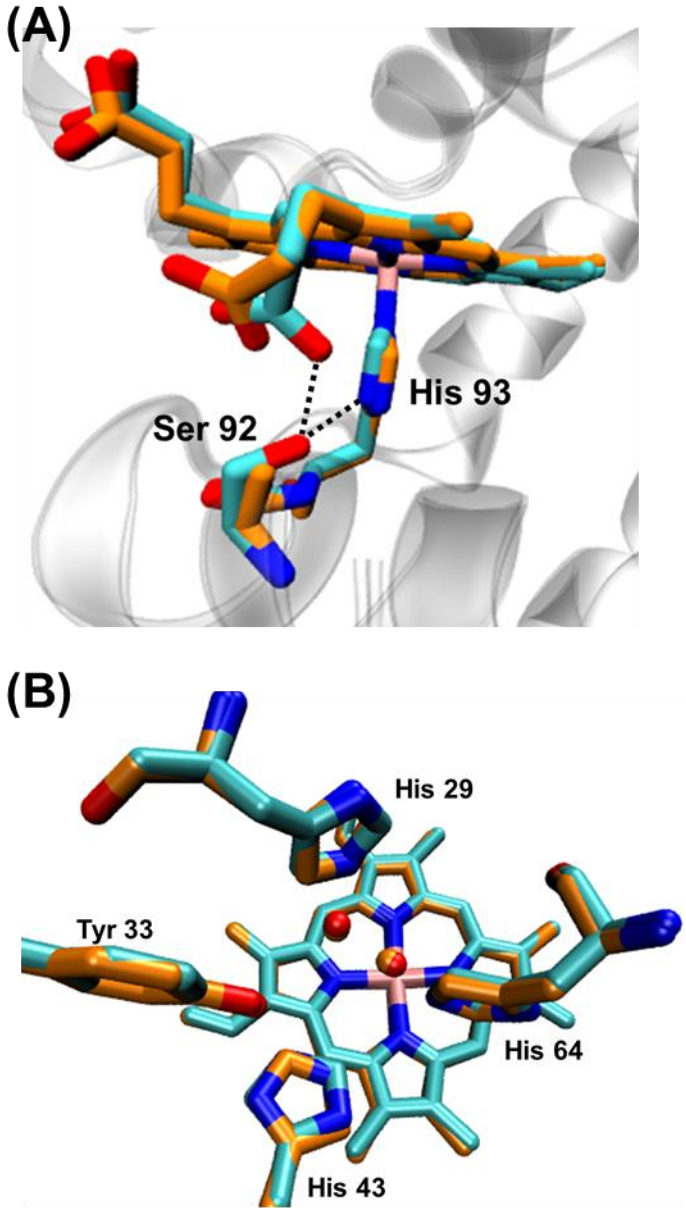

Figure S1: Overlay of the X-ray crystal structure of F33Y-Cu_BMb (4FWX, cyan) and S92A-F33Y-Cu_BMb (4TYX, orange) (A) Proximal side of the heme. Ser92 is hydrogen bonded to His93 and heme propionate in F33Y-Cu_BMb (represented by dotted lines) and its mutation to Ala in S92A-F33Y-Cu_BMb causes the heme propionate to move out. Ser92, Ala92, His93 and heme is shown in licorice while the protein backbone is shown in the cartoon representation. (B) Distal side of the heme. The key residues and the water molecules of F33Y-Cu_BMb (responsible for its oxidase activity) stay unperturbed on mutating Ser92 to Ala. His43, His64, His29, Tyr 33 and heme is shown in licorice. For purpose of clarity propionate side chains of heme and protein backbone are not shown.

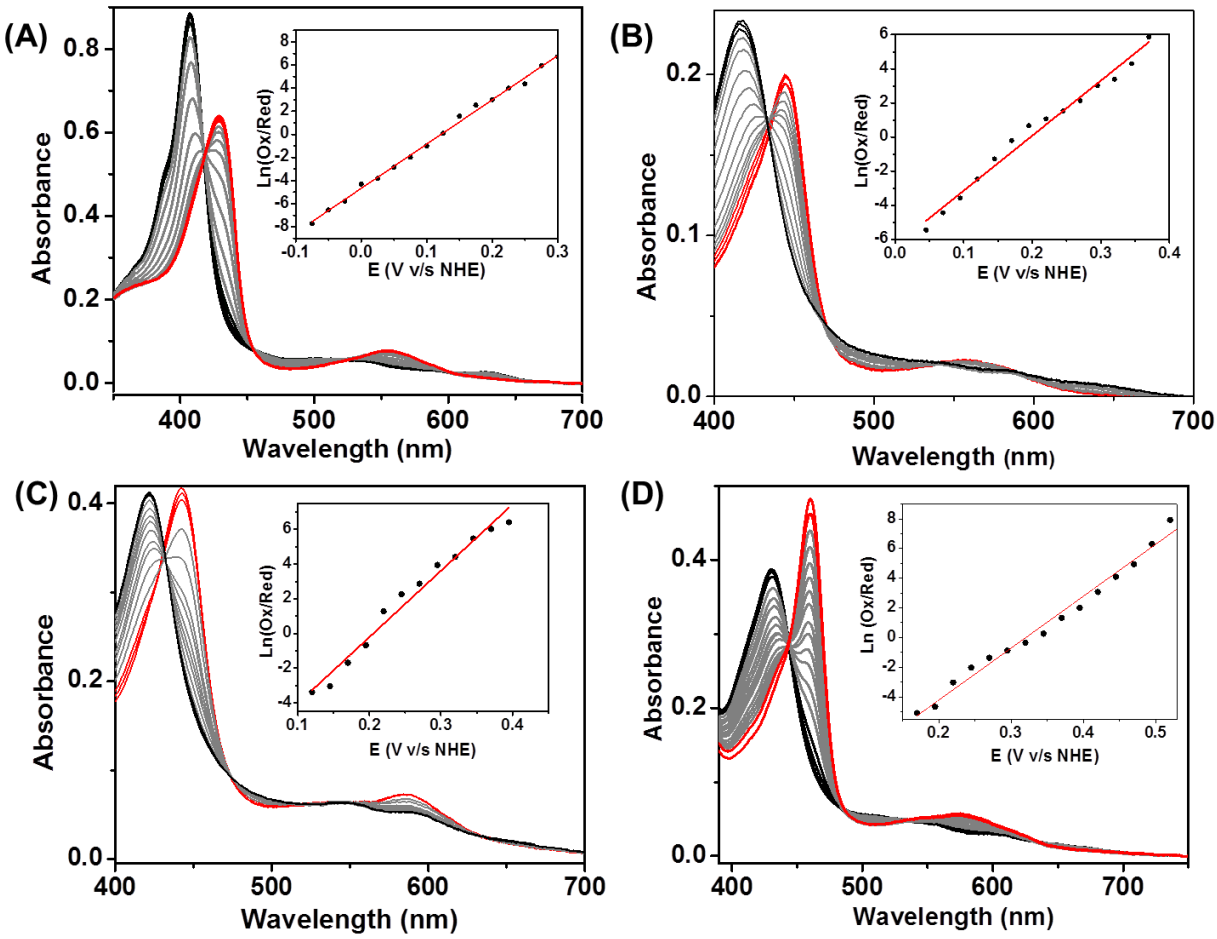

Figure S2: Spectroelectrochemical reduction of F33Y-Cu$_B$Mb variants (A) S92A- F33Y-Cu$_B$Mb (B) F33Y-Cu$_B$Mb (DA-heme) (C) F33Y-Cu$_B$Mb (MF-heme) (D) F33Y-Cu$_B$Mb (DF-heme). The inset shows the Nernst plot from the dependence of the absorbance at 429 nm (A), 444 nm (B), 443 nm (C) and 460 nm (D) on the applied potential.

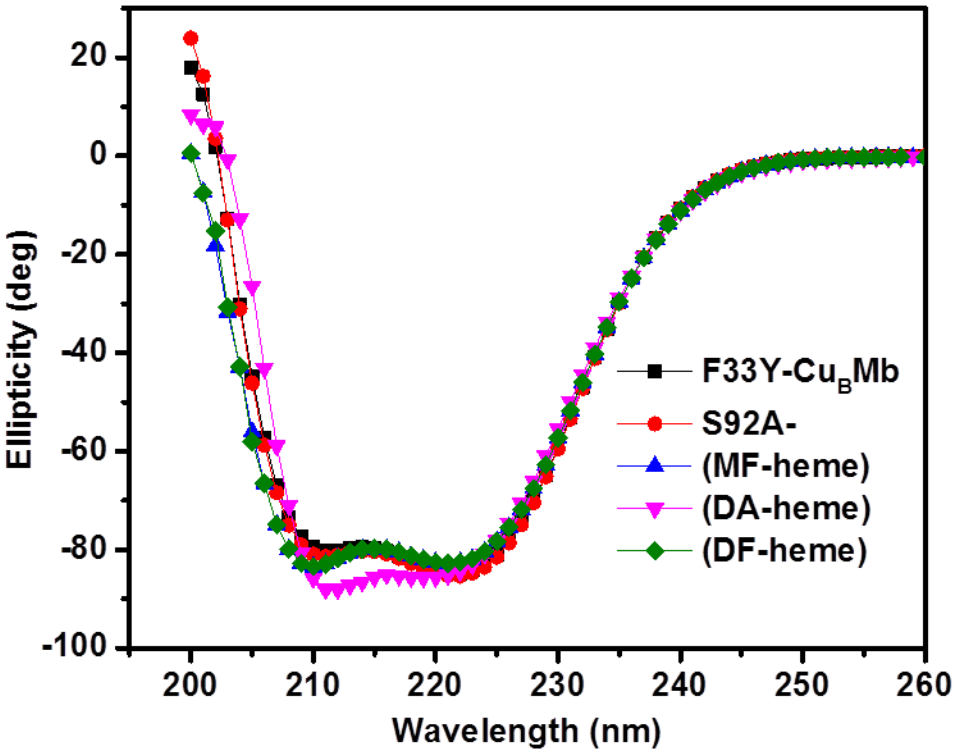

Figure S3: Circular Dichroism spectra of F33Y-Cu$_B$Mb variants

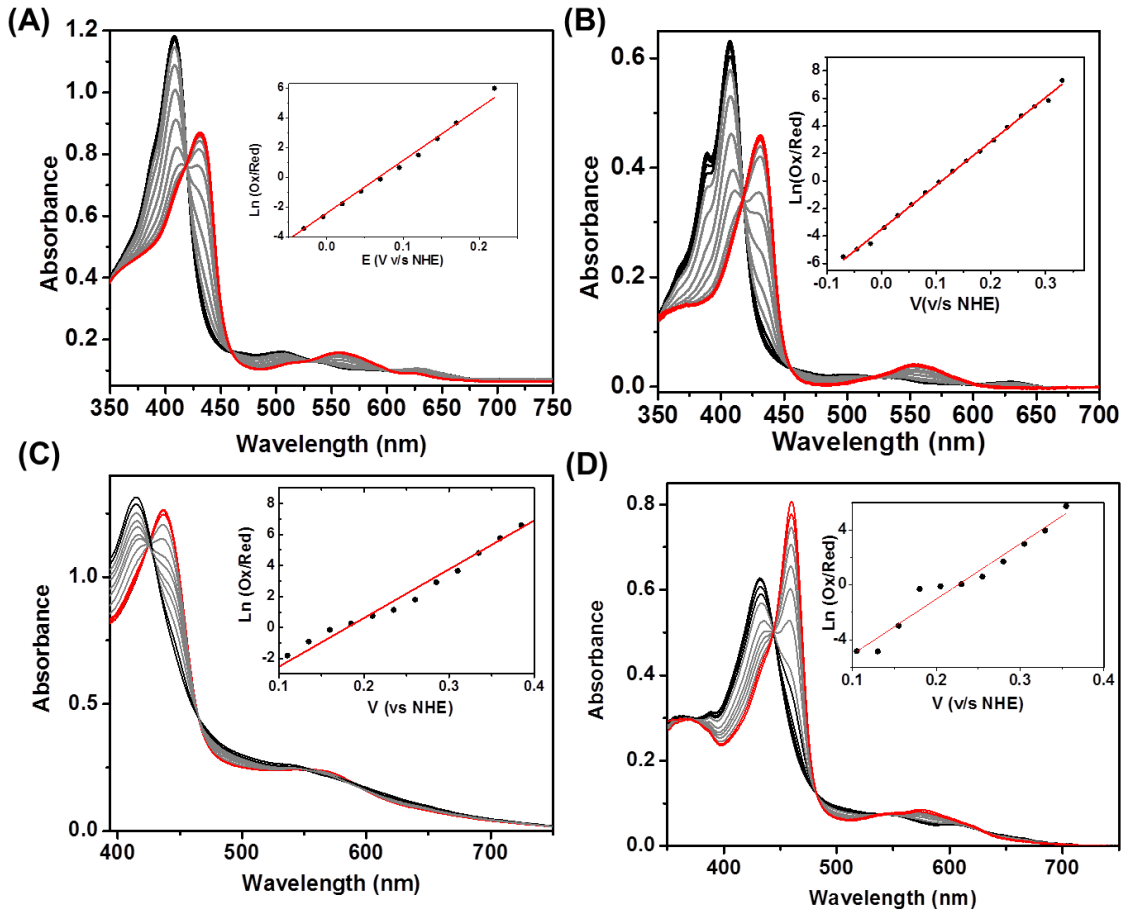

Figure S4: Spectroelectrochemical reduction of (A) WTMb (B) F33Y-Cu_BMb (C) WTMb (DA-heme) (D) WTMb (DF-heme). The inset shows the Nernst plot from the dependence of the absorbance at 431 nm (for A and B), 442 nm (C) and 460 nm (D) on the applied potential.

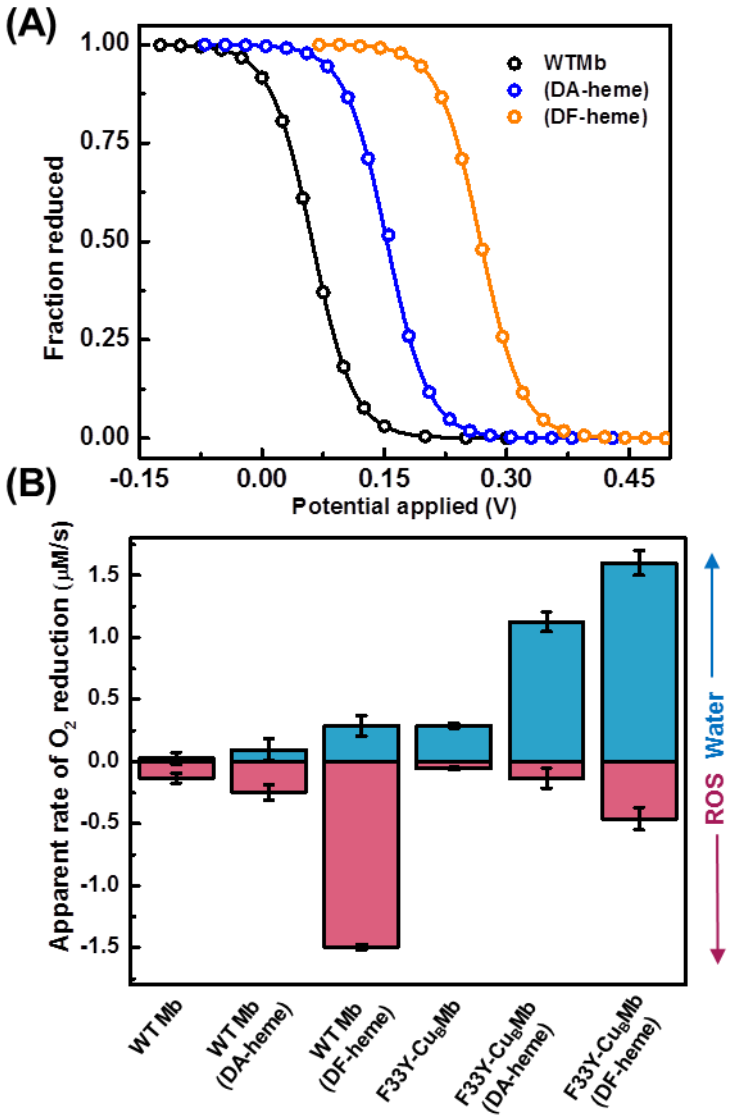

Figure S5. (A) Nernst fit of the spectral plot for WTMb and its DA-heme and DF-heme variants. (B) Rates of O_2 reduction to form either water (blue) or ROS (red) catalyzed by 6 μM F33Y-Cu$_B$Mb, WTMb and their DA-heme and DF-heme variants in 100 mM phosphate buffer (pH6) containing 258 μM O_2, 1 mM TMPD and 10 mM ascorbate.

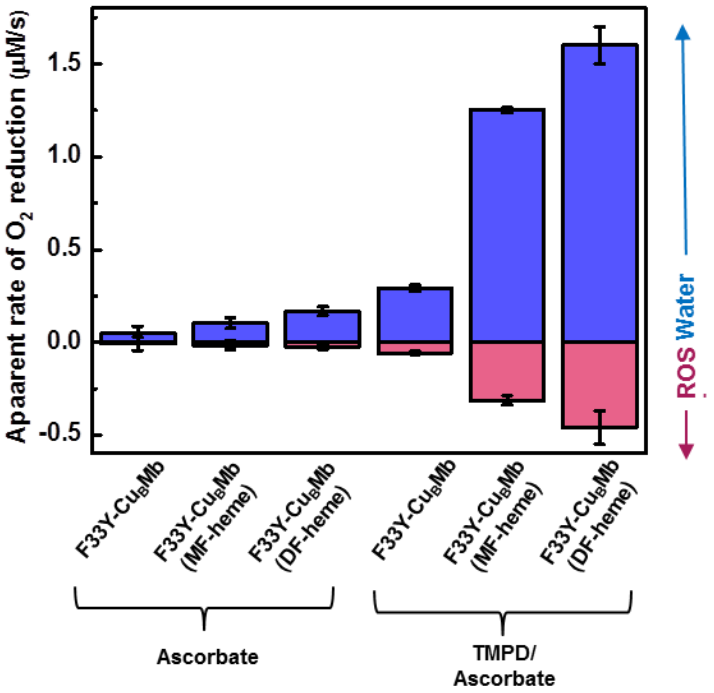

Figure S6. Rates of O_2 reduction to form either water (blue) or ROS (red) catalyzed by 6 μM F33Y-Cu_BMb and its MF-heme and DF-heme variants in 100 mM phosphate buffer (pH6) containing 258 μM O_2 and 10 mM ascorbate. The rates were measured in the presence (left) and absence (right) of 1 mM TMPD.

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