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Distinct roles of tyrosine-water hydrogen-bond network in fine-tuning the structure and function of heme proteins: Two cases designed for myoglobin

Fei Liao,[a‡] Hong Yuan,[b‡] Ke-Jie Du,[a] Yong You,[c] Shu-Qin Gao,[c] Ge-Bo Wen,[c]

Ying-Wu Lin,[a, c*] and Xiangshi Tan[b*]

[a] *School of Chemistry and Chemical Engineering, University of South China, Hengyang 421001, China; E-mail: linlinying@hotmail.com; ywlin@usc.edu.cn*

[b] *Department of Chemistry/Shanghai Key Lab of Chemical Biology for Protein Research & Institute of Biomedical Science, Fudan University, Shanghai 200433, China; E-mail: xstan@fudan.edu.cn*

[c] *Laboratory of Protein Structure and Function, University of South China, Hengyang 421001, China.*

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]. I107Y Mb and F138Y Mb gene was constructed by using the QuickChange Site Directed Mutagenesis Kit (Stratagene), and the mutations were confirmed by DNA sequencing assay. I107Y Mb and F138Y Mb single mutants were expressed and purified as described in previous studies [2, 3]. Protein concentration was determined with an extinction coefficient of ε_{408} = 157 ± 3 $mM^{-1}\cdot cm^{-1}$ for I107Y Mb and ε_{408} = 155 ± 3 $mM^{-1}\cdot cm^{-1}$ for F138Y Mb, respectively, as calculated using the standard hemochromagen method [4].

1.2 X-ray crystallography

I107Y Mb and F138Y Mb, both with a high purity (A_{soret}/A_{280nm}>4.0), were exchanged into 20 mM potassium phosphate (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.

1.3 UV-vis spectroscopy

1.3.1 General spectroscopic studies

UV-Vis spectra of I107Y Mb and F138Y Mb were recorded in 100 mM potassium phosphate (pH 7.0) on a Hewlett-Packard 8453 diode array spectrometer.

1.3.2 Gdn·HCl-induced unfolding study

Guanidine hydrochloride (Gdn·HCl)-induced unfolding of I107Y Mb and F138Y Mb 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. Gdn·HCl-induced unfolding study of WT Mb was reported in previous study [3].

The denaturation midpoint (C_m) of WT Mb was calculated by fitting the absorbance of Soret band *versus* the concentrations of Gdn·HCl to the two-state Boltzmann function (eq. 1):

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

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.

The plots of I107Y Mb and F138Y Mb were fitted to a three-state model using

following equation (eq. 2) [11]:

$$A = f_1/(1+ e^{(C- Cm1)/dC}) + f_2/(1+ e^{(C- Cm2)/dC}) \qquad (2)$$

Here, A is the absorbance of Soret band; C_{m1} and C_{m2} are the denaturation midpoints for transition from initial folded state (F) to intermediate state (I), and from I to unfolded state (U), respectively; C is the concentration of Gdn·HCl. f_1 and f_2 are the contribution to A from two unfolding transitions, and $f_1 + f_2 = 1$.

1.3.3 pH titration study

I107Y Mb and F138Y Mb (10 μM) were dissolved in 10 mM Tris·HCl and titrated with increasing amounts of 10 M NaOH at 25 ˚C. The pH values were measured directly in the cuvette using a microelectrode (type LE422) connected to a Mettler Toledo pH meter (type FE20). The acid-alkaline equilibrium was determined with an Agilent 8453 diode array spectrophotometer. The pK_a values were calculated by fitting the absorbance of Soret band vs. pH to the Boltzmann function (eq. 3).

$$A = A_2 + (A_1 - A_2)/(1+ e^{(pH-pKa)/dpH}) \qquad (3)$$

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

1.4 Stopped-flow studies

1.4.1 Fluoride binding kinetics

Fluoride binding to the heme center of WT Mb, I107Y Mb and F138Y Mb were carried out with a SF-61DX2 Hi-Tech KinetAsyst™ dual mixing stopped-flow spectrophotometer. The binding kinetics was measured by mixing the protein (10 μM

in 100 mM potassium phosphate buffer, pH 7.0) in one syringe with increasing concentrations of NaF (0.1-0.4 M) in the second syringe, with an equal volume of solutions. The observed rate constant (k_{obs}) was obtained by fitting the change of protein Soret band to mono-exponential decay equation. The association rate constant, k_a (k_{on}), and the dissociation rate constant, k_d (k_{off}), were determined from a plot of k_{obs} *versus* the fluoride concentration (eq. 4) [12]:

$$k_{obs} = k_{on}[F^-] + k_{off} \quad (4)$$

where the slope and intercept correspond to k_{on} and k_{off}, respectively.

1.4.2 Peroxide reaction kinetics

The reactions of I107Y Mb and F138Y Mb 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 potassium phosphate buffer, pH 7.0), and the second syringe contains H_2O_2 with concentration ranging from 0.25 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 mutants. The corresponding peseudo-first-order rate constant, k_{obs}, was calculated by fitting to single-exponential decay function (eq. 5):

$$y = y_0 + ae^{-kt} \quad (5)$$

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.

1.5 EPR spectroscopy

Electron Paramagnetic Resonance (EPR) spectra of WT Mb, I107Y Mb and F138Y Mb were recorded on a Bruker A300 spectrometer (X-band) equipped with Bruker ER4141VTM liquid nitrogen system. The protein samples (0.5 mM in 100 mM potassium phosphate, pH 7.0) was transferred into an EPR tube with a volume of 200 μL. The spectra were measured at 100 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 WT Mb, I107Y Mb and F138Y Mb (0.5 mM) after addition of two equivalents of H_2O_2 (1.0 mM) for 1 min, and I107Y Mb (0.5 mM) after addition of one, two and five equivalents of H_2O_2 for 1 min, were recorded under the same conditions.

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

	I107Y Mb	**F138Y Mb**
Wavelength	0.9795	0.9795
Space group	$P2_12_12_1$	$P2_12_12_1$
Unit-cell dimensions (Å, °)	a = 39.911, b = 48.560, c = 78.184; $\alpha = \beta = \gamma = 90$	a =39.881, b = 48.679, c =77.821; $\alpha = \beta = \gamma = 90$
Resolution (Å)	50-1.60 (1.63-1.60)	50-1.85 (1.88-1.85)
No. of observations	125054	184084
No. of unique reflections[a]	19658 (940)	13363 (632)
Completeness (%)	96.2 (94.8)	99.6 (99.7)
<I>/ (I)	45.4 (5.6)	23.9 (8.2)
Redundancy	6.4 (6.8)	13.8 (14.0)
R_{sym}[b]	0.079 (0.305)	0.111 (0.442)
R_{cryst}[c] (%)/R_{free}[d] (%)	0.166 / 0.194	0.176 / 0.225
Ramachandran plot, residues in:		
Most favored regions (%)	96.6	95.4
Additional allowed regions (%)	3.4	4.6
Generously allowed regions (%)	1.0	0.0
Disallowed regions (%)	0.0	0
PDB code	5B84	5B85

[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%).