An intramolecular disulfide bond designed in myoglobin fine-tunes both protein structure and peroxidase activity

Lei-Bin Wu [a], Hong Yuan [b], Hu Zhou [c], Shu-Qin Gao [d], Chang-Ming Nie [a], Xiangshi Tan [b], Ge-Bo Wen [d], Ying-Wu Lin [a, d, *]

[a] School of Chemistry and Chemical Engineering, University of South China, Hengyang 421001, China
[b] Department of Chemistry, Shanghai Key Lab of Chemical Biology for Protein Research, Institute of Biomedical Science, Fudan University, Shanghai 200433, China
[c] Department of Analytical Chemistry, CAS Key Laboratory of Receptor Research, Shanghai Institute of Materia Medica, Chinese Academy of Sciences, Shanghai, 201203, China
[d] Laboratory of Protein Structure and Function, University of South China, Hengyang 421001, China

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ABSTRACT

Disulfide bond plays crucial roles in stabilization of protein structure and in fine-tuning protein functions. To explore an approach for rational heme protein design, we herein rationally introduced a pair of cysteines (F46C/M55C) into the scaffold of myoglobin (Mb), mimicking those in native neuroglobin. Molecular modeling suggested that it is possible for Cys46 and Cys55 to form an intramolecular disulfide bond, which was confirmed experimentally by ESI-MS analysis, DTNB reaction and CD spectrum. Moreover, it was shown that the spontaneously formed disulfide bond of Cys46-Cys55 fine-tunes not only the heme active site structure, but also the protein functions. The substitution of Phe46 with Ser46 in F46S Mb destabilizes the protein while facilitates H_2O_2 activation. Remarkably, the formation of an intramolecular disulfide bond of Cys46-Cys55 in F46C/M55C Mb improves the protein stability and regulates the heme site to be more favorable for substrate binding, resulting in enhanced peroxidase activity. This study provides valuable information of structure-function relationship for heme proteins regulated by an intramolecular disulfide bond, and also suggests that construction of such a covalent bond is useful for design of functional heme proteins.

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1. Introduction

Protein design has been shown to be powerful, not only in revealing the structure-function relationship of native proteins, but also in creating artificial proteins with advanced functions [1–6]. Rational heme protein design has received much attention during the last two decades, and various approaches have been successfully developed, including using non-heme metal ions, unnatural amino acids, and heme mimics to modify the heme active site for functional fine-tuning [7–14]. Moreover, computer modeling plays key roles in guiding the protein design by providing clues for experiments [15–18]. For example, computer modeling was successfully applied to design of a non-heme iron binding site in the heme pocket of myoglobin (Mb), which converted the oxygen

carrier into a functional nitric oxide reductase [19].

Disulfide bond, as formed by the oxidation of two thiol groups within cysteine residues, plays a crucial role in stabilization of the tertiary structure of proteins [20]. For example, manganese peroxidase contains five disulfide bonds, and introduction of one more disulfide bond can further increase its tolerance for heat inactivation [21,22]. Moreover, disulfide bond has been shown to regulate ligand binding and migration in the native heme proteins, as observed for neuroglobin (Ngb) [23,24] and cytoglobin (Cgb) [25–27]. Both Ngb and Cgb have an intramolecular disulfide bond, Cys46-Cys55 and Cys38-Cys83, and a six-coordinated heme with bis-His ligations, His64/His96 and His81/His113, respectively [24,25]. This is distinct from Mb, although these proteins belong to the same globin family. Based on the structural information, we envisaged that it might be able to artificially construct an intramolecular disulfide bond to fine-tune the structure and function of heme proteins that are lacking of such a covalent bond such as Mb. This method could thus be explored for rational heme protein

design.

The C-D region (two short helices and a long loop) of the globin family have been shown to play a crucial role in regulating the heme coordination state and ligand binding to the heme iron [16,28]. Recently, Nadra, Estrin and co-workers [29] engineered a chimeric protein of Mb and Ngb by replacing the entire C-D region of Mb with that of Ngb where the disulfide bond of Cys46-Cys55 locates, and revealed the regulatory role of the entire C-D region in Ngb for bis-His coordination. Meanwhile, the individual role of the disulfide bond in fine-tuning the structure and function of heme proteins deserves to be fully addressed. In this study, we showed that an intramolecular disulfide bond can be designed in Mb with its own C-D region by merely introducing a pair of cysteines, F46C/M55C, which was also found to regulate both the structure and peroxidase activity of Mb, making it possible for exploring a disulfide bond for rational heme protein design.

2. Materials and methods

2.1. Protein preparation

WT sperm whale Mb was expressed using the Mb gene of pMbt7-7 and purified using the procedure described previously [30]. F46C/M55C Mb, F46S/M55C Mb and F46S Mb gene were constructed using the QuickChange Site Directed Mutagenesis Kit (Stratagene). The mutations were confirmed by DNA sequencing assay. F46C/M55C Mb and F46S Mb mutants were expressed in BL21(DE3) host cells and purified using the procedure described previously for WT Mb [30]. F46S/M55C Mb was expressed in inclusion bodies and purified using the procedure described previously for L29E/F43H Mb double mutant [31], but with a very low yield.

2.2. Molecular modeling

The initial structure of F46C/M55C Mb and F46S Mb were constructed based on the X-ray crystal structure of WT Mb (PDB code 1JP6 [32]) using program VMD 1.9. The heme axial water molecule in the X-ray structure was retained in simulation. A patch of disulfide bond was applied to Cys46 and Cys55 for simulation F46C/M55C Mb. The protein was then solvated in a cubic box of TIP3 water, which extended 10 Å away from any given protein atom. Counter ions (Na^+ and Cl) were further added to obtain the physiological ionic strength of 0.15 M by using the autoionize plug-in of VMD 1.9 [33]. The resulting system was minimized with NAMD2.9 (Nanoscale Molecular Dynamics) [34], using 50,000 minimization steps with conjugate gradient method at 0 K, and equilibrated for 10,000,000 molecular dynamics steps (1 fs per step, 10 ns in total) at 300 K, then further minimized for 50,000 steps at 0 K. The last 5 ns were used for analysis. Control experiment of WT Mb was performed under the same conditions. The trajectory data was saved every 5000 steps. Visualization and data analysis were done with VMD 1.9.

2.3. ESI-MS analysis

Electrospray ionization mass spectrometry (ESI-MS) was used to confirm whether an intramolecular disulfide was formed or not. The F46C/M55C Mb protein sample was 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 °C for 10 min, as the native form or reduced form, respectively. 2 μg of protein samples was analyzed on the EASY-nLC1000 HPLC system (Thermo Fisher Scientific) using a self-packed column (75 μm × 70 mm; 3 μm ReproSil-Pur C4 beads,

300 Å, Dr. Maisch GmbH, Ammerbuch, Germany) at a flow rate of 300 nL/min using 25 min gradients. MS data were acquired on an Orbitrap Elite (Thermo Fisher Scientific) platform. The full mass (400–1800 m/z) was scanned in the Orbitrap analyzer with R = 60,000 (defined at m/z 200). The calibration standards for the mass spectrometer were purchased from Thermo Fisher Scientific (ESI Positive Ion Calibration Solution, Cat. No. 88323).

2.4. UV–vis spectroscopy

UV–vis spectra of ferric F46C/M55C Mb was recorded in 100 mM KH_2PO_4 (pH 7.0) on a Hewlett-Packard 8453 diode array spectrometer. Deoxy protein was prepared by addition of a small amount of sodium dithionite in anaerobic condition. Protein concentration was determined with an extinction coefficient of $\varepsilon_{411} = 130 \pm 5$ mM^{-1} cm^{-1} for F46C/M55C Mb, and $\varepsilon_{408} = 150 \pm 5$ mM^{-1} cm^{-1} for F46S Mb, respectively, as calculated using the standard hemochromagen method [35]. The free thiols in F46S/M55C Mb and F46C/M55C Mb (10 μM) were estimated by addition of 1 equivalent of Ellman's reagent [36], 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB), in 6 M guanidinium hydrochloride (Gdn·HCl) at pH 7.5. Control experiment was performed for WT Mb under the same conditions. Gdn·HCl-induced unfolding studies of F46S Mb and F46C/M55C Mb were performed using the procedure as described in previous study for WT Mb [37].

2.5. CD spectroscopy

Circular Dichroism (CD) spectra of F46C/M55C Mb was collected the near UV–visible region from 240 to 350 nm (0.1 cm path length) at 25 °C, with a Jasco model J720 spectropolarimeter. The protein concentration was 16 μM, dissolved in 100 mM KH_2PO_4 (pH 7.0) buffer. Control experiments of single mutant F46S Mb and WT Mb were carried out under the same conditions.

2.6. EPR spectroscopy

Electron paramagnetic resonance (EPR) spectrum of F46C/M55C Mb (0.3 mM) in met form was 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.

2.7. Stopped-flow spectroscopy

The reaction of F46S Mb or F46C/M55C Mb with H_2O_2 was determined using a dual mixing stopped-flow spectrophotometer (SF-61DX2 Hi-Tech KinetAsystTM). Typically, one syringe contains 10 μM protein (in 100 mM KH_2PO_4 buffer, pH 7.0), and the second syringe contains H_2O_2 with concentration ranging from 0.25 to 1 mM, as determined with $\varepsilon_{240} = 39.4$ M^{-1} cm^{-1}. The reaction was stated with mixing of equal volume of solutions from the both syringes. 100 time-dependent spectra were collected over 10 s from 350 to 700 nm at 25 °C. The observed rate constant, k_{obs} (s^{-1}), was calculated by fitting the Soret band changes over time to a single-exponential decay function. The apparent rate constant, k_1 (mM^{-1} s^{-1}), was further obtained by linear regression fitting the plot of the k_{obs} value versus the concentration of H_2O_2.