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

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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 H₂O₂ 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.

Keywords: Heme proteins; Protein design; Disulfide bond; Coordination state; Peroxidase activity

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, or 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 10000,000 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 5,000 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 ThermoFisher 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 $\epsilon_{411} = 130 \pm 5 \text{ mM}^{-1}\cdot\text{cm}^{-1}$ for F46C/M55C Mb, and $\epsilon_{408} = 150 \pm 5 \text{ mM}^{-1}\cdot\text{cm}^{-1}$ 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 $\epsilon_{240} = 39.4 \text{ M}^{-1}\cdot\text{cm}^{-1}$. The reaction was started with mixing of equal volume of solutions from the both syringes. 100 time-dependent spectra were collected over 10 sec 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 ($\text{mM}^{-1}\cdot\text{s}^{-1}$), was further obtained by linear regression fitting the plot of the k_{obs} value versus the concentration of H_2O_2 .

The peroxidase activity of F46S Mb or F46C/M55C Mb was determined using

guaiacol as a representative substrate and H₂O₂ as the oxidant. One syringe contains 2 μ M protein (in 100 mM KH₂PO₄ buffer, pH 7.0) in presence of 2 mM guaiacol, and the second syringe contains 200 mM H₂O₂. The reaction was followed by monitoring the change in absorbance of the product at 470 nm, as proposed to be a dimeric form of guaiacol (3, 3'-dimethoxy-4, 4'-biphenylquinone).[38] The initial rate was calculated based on the initial linear changes using an extinction coefficient of $\epsilon_{470} = 26.6 \text{ mM}^{-1}\cdot\text{cm}^{-1}$. [39] To determine the steady-state kinetic parameters, one syringe contains 2 μ M protein mixed with various concentrations (0.1-6 mM) of guaiacol, and the second syringe contains 200 mM H₂O₂. The curve of initial rates *versus* substrate concentrations was fitted to the Michaelis-Menten equation: $v/[\text{protein}] = k_{\text{cat}}[\text{guaiacol}]/(K_{\text{m}} + [\text{guaiacol}])$. Control experiment of WT Mb was performed under the same condition.

3. Results and Discussion

3.1 Design of an intramolecular disulfide bond in Mb

As one member in the globin family, Mb has a similar tertiary structure to the other members such as Ngb[24]. Differently, Ngb has a six-coordinated heme with bis-His (His64/His96) ligations. Moreover, it has a unique intramolecular disulfide bond formed between Cys46 and Cys55 in the C-D region, which was found to regulate ligand binding and protein function.[23, 24] Inspired by this information, we attempted to introduce an intramolecular disulfide bond into Mb (Fig. 1A), aimed to explore this approach for rational heme protein design. Sequence alignment shows that Mb has two residues of Phe46 and Met55 in the C-D region corresponding to the two cysteine residues in Ngb (Fig. 1B). Although sequence alignment shows that Mb has more charged residues in the C-D region compared to that of Ngb, the spatial

position of Phe46 and Met55 with a close distance of 7.8 Å between the C_B atoms of the two residues (Fig. 1A, dashed line [32]) suggests that it is possible to form an intramolecular disulfide bond if two cysteine residues are introduced at these two positions. Moreover, Phe46 locates in the flexible loop linking helices C and D, which would allow Cys46 at this position to form a disulfide bond with neighboring Cys55 by local conformational changes. Therefore, we replaced both Phe46 and Met55 with a cysteine residue by mutagenesis study, and purified the double mutant of F46C/M55C Mb with the same procedure as that for WT Mb.[30]

(Fig. 1 should be here)

3.2 Modeling of F46C/M55C Mb with an intramolecular disulfide bond

In addition to sequence alignment, we performed molecular dynamics (MD) simulation of the double mutant F46C/M55C Mb, to reveal the possibility of formation of Cys46-Cys55 disulfide bond. Control experiments were also performed for single mutant F46S Mb and WT Mb. With minimization and equilibration, the simulated structure of F46C/M55C Mb is shown in Fig. 2A (orange), and overlapped with that of simulated F46S Mb (pink) and WT Mb (gray). Generally, the three structures overlapped well with one another excepting for the C-D loop in F46C/M55C Mb where Cys46 locates. Cys55 retained a similar position to Met55 in WT Mb, whereas Cys46 underwent a large conformational change upon formation of the Cys46-Cys55 disulfide bond, due to the flexibility of the long loop linking helices C and D. Control simulation of F46S Mb showed that Ser46 has a similar position to that of Phe46 in WT Mb. This observation also agrees with the previously reported X-ray structure of F46L Mb.[40]

(Fig. 2 should be here)

Detailed modeling structure showed that when the disulfide bond was formed, the conformation of adjacent Arg45 was changed simultaneously, with the nitrogen atom of guanidinium group shifted closer to the heme iron (~ 8.5 Å) compared to that in WT Mb (~ 9.5 Å). Meanwhile, the double hydrogen-bonds between Arg45 and Asp60 in WT Mb were still formed in the modeling structure, playing a crucial role in stabilization of the local conformation of the C-D region (Fig. 2B). The modeling structure further revealed that the micro-environment of the heme active site was altered subtly (Fig. 2B). For example, the conformation of distal His64 was slightly altered, and the distance between the N_ε atom of His64 and the heme iron was increased from ~ 4.7 Å in WT Mb to ~ 4.9 Å in the double mutant. The simulated structure of F46S Mb also showed a conformational change for the distal His64 (pink in Fig. 2C), with a similar conformation and distance (~ 4.9 Å) to that in F46C/M55C Mb (orange in Fig. 2D). Arg45 in F46S Mb was found to undergo a slight conformational change and form a hydrogen bonding network with Asp60 and both heme 6-propionate (6-P) and 7-P groups, with a distance of ~ 8.8 Å to the heme iron. Overlapping view showed that a larger conformational change occurred for the 7-P group in F46S Mb with respect to that in F46C/M55C Mb, leading to a change in the planarity of the heme group (Fig. 2D).

These observations indicate that Phe46 is a crucial hydrophobic residue in the C-D region of Mb, and Phe to Ser substitution will induce a conformational rearrangement for the heme site. More importantly, the modeling study shows that, theoretically, an intramolecular disulfide bond of Cys46-Cys55 can be formed in the double mutant F46C/M55C Mb, and it is also capable of fine-tuning the heme active structure, although it is remote from the heme site.

3.3 Confirming the formation of an intramolecular disulfide bond in F46C/M55C Mb

Since molecular modeling suggested that Cys46 and Cys55 can form an intramolecular disulfide bond in F46C/M55C Mb, we needed to confirm it experimentally. ESI-MS analysis has been well established for determining the number of disulfide bonds present in a given protein, simply through measurement of the intact mass of the protein with and without the formation of the disulfide bonds.[41] In formation of a disulfide bond, one hydrogen atom is lost per cysteine, resulting in a mass difference of 2 Da. Therefore, the mass of a protein can be measured in both unreduced and reduced forms, and a mass increase of 2 Da should be measured upon reduction of one disulfide bond. Based on this theory, we performed ESI-MS analysis for F46C/M55C Mb as purified, and that treated with TCEP to reduce the disulfide bond if it was formed, as shown in Fig. 3A and B, respectively. The results showed that the purified F46C/M55C Mb has an observed mass of 17256.1 Da. Upon reduction, a mass increase of 2 Da was observed for F46C/M55C Mb (17258.1 Da), in agreement with the calculated mass for the double mutant without a disulfide bond (17259 Da; WT Mb: calculated, 17331 Da; observed, 17329.5 Da[37]). These observations demonstrate that a single disulfide bond was formed in F46C/M55C Mb, and the formation was spontaneous *in vivo* or *in vitro* during protein purification.

(Fig. 3 should be here)

To further confirm the formation of a disulfide bond between Cys46 and Cys55 in F46C/M55C Mb, we tested whether there are free thiols in the protein using Ellman's reagent, DTNB [36]. In chemical reaction, DTNB will react with the free thiol of Cys

and release a product of 5-thio-2-nitrobenzoate, the latter has a characteristic UV-vis absorption with a peak of 412 nm [36]. After adding 1 equivalent of DTNB to the protein solution of denatured F46C/M55C Mb, the UV-vis spectrum was similar to that of denatured WT Mb (Fig. 4A), suggesting no free Cys residues in the protein. To provide a positive control for this experiment, we constructed a double mutant of F46S/M55C Mb with a single Cys residue, and did DTNB reaction under the same conditions. The resultant UV-vis spectrum exhibited a shoulder peak at ~412 nm, which is more obvious in the difference spectrum between F46S/M55C Mb and F46C/M55C Mb (Fig. 4A), suggesting that the single Cys was reacted with DTNB. These results thus indicate that the two Cys residues in F46C/M55C Mb are formed an intramolecular disulfide bond.

(Fig. 4 should be here)

Moreover, to find additional information for the disulfide bond, we collected the CD spectrum of F46C/M55C Mb in the near UV-Vis region, and compared it to that of F46S Mb and WT Mb (Fig. 4B). Since the double mutant F46S/M55C Mb has a very low expression level, we used a single mutant F46S Mb for the control, and in following sections as well. The CD spectrum showed that F46S Mb has a less intensity in region of 250-310 nm compared to that of WT Mb, which is due to the removal of Phe46 as phenylalanine exhibits absorption in this region.[42] Differently, F46C/M55C Mb showed an altered spectrum in region of 250-290 nm with respect to that of F46S Mb. The difference spectrum between F46C/M55C Mb and F46S Mb exhibits a negative Cotton effect at ~275 nm and a positive Cotton effect with a shoulder at ~260 nm. Since the near UV absorption of the disulfide bond occurs near 260 nm, although is generally weak,[43] this observation provides further information for the presence of Cys46-Cys55 disulfide bond. It should be noted that the

conformational changes for other aromatic residues such as Phe33 and Phe43 in the C-D region, as induced by the formation of the disulfide bond, may also contribute to the alteration of CD spectrum of F46C/M55C Mb.

3.4 Spectroscopic properties of F46C/M55C Mb with an intramolecular disulfide bond

After confirming that Cys46 and Cys55 indeed formed an intramolecular disulfide bond, we recorded the UV-vis spectra of F46C/M55C Mb in both ferric and ferrous states (Fig. 5A). It was found that ferric F46C/M55C Mb exhibited 2-nm red shift in the Soret band (411 nm) relative to that of WT Mb (409 nm). In the visible region, although a charge transfer band was observed at ~635 nm, the band appeared at 525 nm suggested a partial formation of the bis-His coordination species. Meanwhile, in ferrous state F46C/M55C Mb exhibited a spectrum (432, 556 nm) similar to that of WT Mb (433, 556 nm). Control study of single mutant F46S Mb showed that it has similar UV-vis spectra in both ferric (408, 502 and 635 nm) and ferrous (556 nm) states to that of WT Mb, suggesting that removal of the hydrophobic side chain of Phe46 only causes slight structural change for the heme center. These observations are different from the introduction of the entire C-D region of Ngb into Mb, which showed that the modified Mb adopted a bis-His coordination state at both ferric (413, 535, 565 nm) and ferrous (424, 528, 556 nm) states.[29] These findings indicate that not only the intramolecular disulfide bond, but also the other residues in the C-D region control the heme coordination state of Mb. This is also true for Ngb, since X-ray crystal structure shows that both WT Ngb and a mutant C46G/C55S/C120S Ngb, in which the disulfide bond was removed, are in bis-His heme coordination state.[24, 44]

(Fig. 5 should be here)

Moreover, to provide structural information for the heme site, we collected the EPR spectrum of F46C/M55C Mb in ferric state, since ferrous heme is EPR silent. As shown in Fig. 5B, the EPR spectrum exhibits the dominate high-spin signals ($g \sim 5.92, 1.99$), similar to that of ferric WT Mb ($g \sim 5.94, 1.99$).^[45] Control study showed that the single mutant F46S Mb also has similar EPR signals ($g \sim 5.97, 1.99$). Meanwhile, it is different from F46S Mb and WT Mb that small signals ($g \sim 2.97, 2.22$) were observed for F46C/M55C Mb, which are typical of a low-spin ferric heme with a bis-His coordination. This is similar to that for L29E/F43H Mb ($g \sim 2.97, 2.20$),^[31] which was confirmed by X-ray crystallography to have a bis-His coordination with native ligands (His64/His93).^[46] These observations suggest that the intramolecular disulfide bond plays a certain role in fine-tuning the heme coordination state, although other residues in the C-D region are also important. It should be noted that due to the technique limitation of MD simulation, an artificial force of bis-His coordination has to be applied for the heme iron if we attempt to model the low-spin species of F46C/M55C Mb, as performed in previous study for simulation of L29E/F43H Mb.^[16] Since F46C/M55C Mb has a low amount of low-spin species that is not responsible for the peroxidase activity (see following sections), we did not model the corresponding structure in this study.

To evaluate the stability of F46C/M55C Mb with an intramolecular disulfide bond, we performed Gdn·HCl-induced unfolding studies and compared with that of F46S Mb and WT Mb. The results showed that the Soret band of F46C/M55C Mb decreased with increasing the concentration of Gdn·HCl, and a shoulder peak appeared at ~ 370 nm of the free heme (Fig. 6A), similar to the observation for WT Mb,^[37] suggesting the release of heme upon unfolding. The half denaturation

concentration (C_m) of F46C/M55C Mb was found to be 1.60 ± 0.03 M, which is ~ 0.28 M lower than that of WT Mb (1.88 ± 0.02 M) (Fig. 6B). Meanwhile, control unfolding study showed that F46S Mb exhibited a C_m value of 1.43 ± 0.03 M, which is ~ 0.17 and ~ 0.45 M lower than that of F46C/M55C Mb and WT Mb, respectively (Fig. 6B). These observations indicate that substitution of bulky Phe46 with Ser46 destabilizes the protein, presumably due to alteration of the hydrophobic interactions in the C-D region of Mb, whereas the formation of an intramolecular disulfide bond improves the protein stability with respect to that in the absence of the disulfide bond.

(Fig. 6 should be here)

3.5 H₂O₂ activation by F46C/M55C Mb with an intramolecular disulfide bond

To probe the functional effect of an intramolecular disulfide bond in Mb, we examined the ability of H₂O₂ activation by F46C/M55C Mb. Stopped-flow kinetic studies showed that F46C/M55C Mb readily reacted with H₂O₂, and the Soret band (411 nm) decreased and shifted to 418 nm, with visible bands increased at 545 and 585 nm, suggesting the formation of an oxoferryl heme (Compound II) (Fig. 7A). The decay curve of Soret band was found to obey the pseudo first-order kinetics (Fig. 7A, inset). The rate constant (k_1) of Compound II formation, as determined by plot of observed rate constant (k_{obs}) versus H₂O₂ concentrations, is 7.20 ± 0.02 mM⁻¹·s⁻¹ at 25 °C (Fig. 7B). Control experiments showed that WT Mb has a k_1 value 0.57 ± 0.01 mM⁻¹·s⁻¹, in agreement with previously reported by Watanabe and co-workers (0.51 mM⁻¹·s⁻¹ at pH 7.0, 20 °C).[47]

(Fig. 7 should be here)

On the other hand, the single mutant of F46S Mb was found to have a k_1 value of 9.47 ± 0.02 mM⁻¹·s⁻¹, which is ~ 16.6 -fold that of WT Mb (Fig. 7B). This observation

agree with previous study, in which Smith, Mauk and co-workers[48] showed that horse F46L Mb mutant exhibits a k_1 value ($1.32 \text{ mM}^{-1}\cdot\text{s}^{-1}$) 2.4-fold that of horse WT Mb ($0.54 \text{ mM}^{-1}\cdot\text{s}^{-1}$ at pH 6.0, 25 °C). Olson and co-workers[40] revealed that Phe46 orients the distal His64 for hydrogen-bonding to bound ligands in Mb, and F46L or F46V mutation increases the conformational freedom of distal His64. Watanabe and co-workers [47] showed that a distal histidine with a suitable position is crucial for H_2O_2 activation, ideally with a distance of 5.5-6.0 Å to the heme iron, as observed in native peroxidases.[49] Moreover, a cooperative distal His-Arg pair is crucial for the peroxidase activity [49]. For example, horseradish peroxidase (HRP) (PDB code 1HCH[50]) and cytochrome *c* peroxidase (CcP) (PDB code 2CYP[51]) has a distal Arg with a distance of ~5.0 and 6.9 Å to the heme iron. The simulated structure of both F46S Mb and F46C/M55C Mb showed that the distal His64 has a slightly longer distance (~4.9 Å) to the heme iron compared to that for simulated WT Mb (~4.7 Å), whereas the distal Arg45 has a slightly shorter distance (~8.8 and 8.5 Å, respectively) to the heme iron compared to that for simulated WT Mb (~9.5 Å). These differences presumably contribute to the enhanced activity for these mutants. With an intramolecular disulfide bond of Cys46-Cys55, the H_2O_2 activation ability of F46C/M55C Mb was found to be ~12.6-fold that of WT Mb, slightly lower than that of F46S Mb (~16.6-fold). These results indicate that Phe46 to Ser46 substitution enhances the ability of H_2O_2 activation, whereas formation of Cys46-Cys55 disulfide bond slightly inhibits it, presumably due to the formation of a small amount of low-spin species with bis-His coordination.

3.6 Peroxidase activity of F46C/M55C Mb with an intramolecular disulfide bond

Since both F46S Mb and F46C/M55C Mb exhibited an enhanced ability to

activate H₂O₂ compared with WT Mb, we were interested in evaluating their peroxidase activity in presence of H₂O₂ using guaiacol as a typical substrate. The oxidation product was monitored by an absorption increase at 470 nm (Fig. 8A), and the calculated initial rate was $1.63 \pm 0.05 \mu\text{M}\cdot\text{s}^{-1}$ and $2.56 \pm 0.08 \mu\text{M}\cdot\text{s}^{-1}$ for F46S Mb and F46C/M55C Mb, respectively, which is ~20-fold and ~32-fold higher than that of WT Mb ($0.08 \pm 0.01 \mu\text{M}\cdot\text{s}^{-1}$) under the same conditions. These results indicate that an enhancement of peroxidase activity of Mb was achieved by replacement of Phe46 with Ser46, and introduction of an intramolecular disulfide bond of Cys46-Cys55 in Mb further fine-tunes the peroxidase activity.

(Fig. 8 should be here)

To further probe the substitution effect of Phe to Ser and the fine-tuning effect of an intramolecular disulfide bond on peroxidase activity, we determined the steady-state kinetic parameters for F46S Mb and F46C/M55C Mb, respectively, by varying the guaiacol concentration using a fixed concentration of H₂O₂. The results are shown in Fig. 8B, and the parameters obtained from data fitting are summarized in Table 1. Compared to WT Mb, F46S Mb showed ~16.5-fold k_{cat} value and ~0.9-fold K_{m} value, resulting in ~18-fold catalytic efficiency ($k_{\text{cat}}/K_{\text{m}}$). For F46C/M55C Mb, it showed ~9.3-fold k_{cat} value of WT Mb. Although this value is lower than that for F46S Mb, F46C/M55C Mb exhibited much lower (~0.135-fold) K_{m} value, resulting in much higher catalytic efficiency, i.e., ~70-fold that of WT Mb. This activity is similar to other artificial peroxidases designed in Mb, such as F43H/H64L Mb (~40-fold)[47] and H64D Mb (~100-fold)[52]. These observations suggest that the substitution of Phe46 with serine facilitates H₂O₂ activation, and the formation of an intramolecular disulfide bond involving Cys46 further regulates the heme site to be more favorable for substrate binding due to rearrangement of both distal His64 and Arg45 (Fig. 2D).

In addition, the distal His-Arg pair is likely fine-tuned by the disulfide bond to be cooperative in peroxidase activity, as observed in native peroxidases[49].

(Table 1 should be here)

4. Conclusion

In summary, inspired by the presence of an intramolecular disulfide bond in one member of the globin family, Ngb, we introduced a pair of cysteines at the corresponding positions (46 and 55), instead of the entire C-D region of Ngb, into the scaffold of Mb. As predicted by molecular modeling, Cys46 and Cys55 formed an intramolecular disulfide bond, which was further confirmed experimentally by ESI-MS analysis and DTNB reaction, as well as CD spectrum. Spectroscopic studies showed that the designed F46C/M55C Mb adopts a major high-spin heme coordination state with a minor low-spin species, suggesting a controlling role of the disulfide bond in heme coordination state. Moreover, kinetic studies showed that substitution of bulky Phe46 with Ser46 facilitates H₂O₂ activation for the single mutant F46S Mb, whereas destabilizes the protein. Remarkably, the formation of an intramolecular disulfide bond involving Cys46 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 is presumably attributed to the formation and the fine-tuning effect of the Cys46-Cys55 disulfide bond, albeit remote from the heme active site. In addition to providing the structure-function information regulated by an intramolecular disulfide bond, this study suggests that construction of such a covalent bond can be applied to artificial heme protein design by fine-tuning both the structure and function.

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References:

- [1] B. Kuhlman, G. Dantas, G.C. Ireton, G. Varani, B.L. Stoddard, D. Baker, *Science* 302 (2003) 1364-1368.
- [2] Y. Lu, N. Yeung, N. Sieracki, N.M. Marshall, *Nature* 460 (2009) 855-862.
- [3] Z.-X. Huang, *FEBS J.* 277 (2010) 2911-2911.
- [4] W.J. Song, F.A. Tezcan, *Science* 346 (2014) 1525-1528.
- [5] A.G. Tebo, V.L. Pecoraro, *Curr. Opin. Chem. Biol.* 25 (2015) 65-70.
- [6] M. Dürrenberger, T.R. Ward, *Curr. Opin. Chem. Biol.* 19 (2014) 99-106.
- [7] Y.-W. Lin, E.B. Sawyer, J. Wang, *Chem. Asian. J.* 8 (2013) 2534-2544.
- [8] Y. Lin, J. Wang, Y. Lu, *Sci. China Chem.* 57 (2014) 346-355.
- [9] C. Hu, S.I. Chan, E.B. Sawyer, Y. Yu, J. Wang, *Chem. Soc. Rev.* 43 (2014) 6498-6510.
- [10] K. Oohora, T. Hayashi, *Curr. Opin. Chem. Biol.* 19 (2014) 154-161.
- [11] O. Shoji, Y. Watanabe, *J. Biol. Inorg. Chem.* 19 (2014) 529-539.
- [12] M. Bordeaux, V. Tyagi, R. Fasan, *Angew. Chem. Int. Ed. Engl.* 54 (2015) 1744-1748.
- [13] J. Du, M. Sono, J.H. Dawson, *Coord Chem Rev* 255 (2011) 700-716.
- [14] T. Matsuo, S. Hirota, *Bioorg. Med. Chem.* 22 (2014) 5638-5656.
- [15] V. Nanda, R.L. Koder, *Nat. Chem.* 2 (2010) 15-24.
- [16] Y.-W. Lin, *Proteins* 79 (2011) 679-684.

- [17] Y.-W. Lin, X.-G. Shu, K.-J. Du, C.-M. Nie, G.-B. Wen, *Comput. Biol. Chem.* 52 (2014) 60-65.
- [18] D.M. Moreno, M.A. Martí, P.M. De Biase, D.A. Estrin, V. Demicheli, R. Radi, L. Boechi, *Arch. Biochem. Biophys.* 507 (2011) 304-309.
- [19] N. Yeung, Y.W. Lin, Y.G. Gao, X. Zhao, B.S. Russell, L. Lei, K.D. Miner, H. Robinson, Y. Lu, *Nature* 462 (2009) 1079-1082.
- [20] Y. Hagihara, D. Saerens, *Biochim. Biophys. Acta* 1844 (2014) 2016-2023.
- [21] N.S. Reading, S.D. Aust, *Biotechnol. Prog.* 16 (2000) 326-333.
- [22] N.S. Reading, S.D. Aust, *Biochemistry* 40 (2001) 8161-8168.
- [23] M. Tiso, J. Tejero, S. Basu, I. Azarov, X. Wang, V. Simplaceanu, S. Frizzell, T. Jayaraman, L. Geary, C. Shapiro, C. Ho, S. Shiva, D.B. Kim-Shapiro, M.T. Gladwin, *J. Biol. Chem.* 286 (2011) 18277-18289.
- [24] B.G. Guimaraes, D. Hamdane, C. Lechauve, M.C. Marden, B. Golinelli-Pimpaneau, *Acta Crystallogr. D Biol. Crystallogr.* 70 (2014) 1005-1014.
- [25] H. Sugimoto, M. Makino, H. Sawai, N. Kawada, K. Yoshizato, Y. Shiro, *J. Mol. Biol.* 339 (2004) 873-885.
- [26] L. Astudillo, S. Bernad, V. Derrien, P. Sebban, J. Miksovská, J. Inorg. Biochem. 129 (2013) 23-29.
- [27] P. Beckerson, B.J. Reeder, M.T. Wilson, *FEBS Lett.* 589 (2015) 507-512.
- [28] L. Capece, M.A. Marti, A. Bidon-Chanal, A. Nadra, F.J. Luque, D.A. Estrin, *Proteins* 75 (2009) 885-894.
- [29] I. Boron, L. Capece, F. Pennacchietti, D.E. Wetzler, S. Bruno, S. Abbruzzetti, L. Chisari, F.J. Luque, C. Viappiani, M.A. Marti, D.A. Estrin, A.D. Nadra, *Biochim. Biophys. Acta* 1850 (2015) 169-177.
- [30] J.A. Sigman, B.C. Kwok, Y. Lu, *J. Am. Chem. Soc.* 122 (2000) 8192-8196.
- [31] Y.-W. Lin, N. Yeung, Y.-G. Gao, K.D. Miner, L. Lei, H. Robinson, Y. Lu, *J. Am. Chem. Soc.* 132 (2010) 9970-9972.
- [32] P. Urayama, G.N. Phillips Jr, S.M. Gruner, *Structure* 10 (2002) 51-60.
- [33] W. Humphrey, A. Dalke, K. Schulten, *J. Mol. Graph.* 14 (1996) 33-38, 27-38.
- [34] L. Kalé, R. Skeel, M. Bhandarkar, R. Brunner, A. Gursoy, N. Krawetz, J. Phillips, A. Shinozaki, K. Varadarajan, K. Schulten, *Journal of Computational Physics* 151 (1999) 283-312.
- [35] M. Morrison, S. Horie, *Anal. Biochem.* 12 (1965) 77-82.
- [36] P.W. Riddles, R.L. Blakeley, B. Zerner, *Methods Enzymol.*, Academic Press,

- 1983, pp. 49-60.
- [37] D.-J. Yan, W. Li, Y. Xiang, G.-B. Wen, Y.-W. Lin, X. Tan, *ChemBioChem* 16 (2015) 47-50.
 - [38] D.R. Doerge, R.L. Divi, M.I. Churchwell, *Anal. Biochem.* 250 (1997) 10-17.
 - [39] D.A. Baldwin, H.M. Marques, J.M. Pratt, *J. Inorg. Biochem.* 30 (1987) 203-217.
 - [40] H.H. Lai, T. Li, D.S. Lyons, G.N. Phillips, J.S. Olson, Q.H. Gibson, *Proteins: Structure, Function, and Bioinformatics* 22 (1995) 322-339.
 - [41] L. Yang, D. Yang, C. de Graaf, A. Moeller, G.M. West, V. Dharmarajan, C. Wang, F.Y. Siu, G. Song, S. Reedtz-Runge, B.D. Pascal, B. Wu, C.S. Potter, H. Zhou, P.R. Griffin, B. Carragher, H. Yang, M.W. Wang, R.C. Stevens, H. Jiang, *Nature communications* 6 (2015) 7859.
 - [42] S.M. Kelly, T.J. Jess, N.C. Price, *Biochim. Biophys. Acta* 1751 (2005) 119-139.
 - [43] S.M. Kelly, N.C. Price, *Curr. Protein Peptide Sci.* 1 (2000) 349-394.
 - [44] T. Burmester, B. Weich, S. Reinhardt, T. Hankeln, *Nature* 407 (2000) 520-523.
 - [45] J. Zeng, Y. Zhao, W. Li, X. Tan, G.-B. Wen, Y.-W. Lin, *J. Mol. Catal. B: Enzym.* 111 (2015) 9-15.
 - [46] J.-F. Du, W. Li, L. Li, G.-B. Wen, Y.-W. Lin, X. Tan, *ChemistryOpen* 4 (2015) 97-101.
 - [47] T. Matsui, S. Ozaki, E. Liong, G.N. Phillips, Jr., Y. Watanabe, *J. Biol. Chem.* 274 (1999) 2838-2844.
 - [48] L. Wan, M.B. Twitchett, L.D. Eltis, A.G. Mauk, M. Smith, *Proc. Natl. Acad. Sci. U. S. A.* 95 (1998) 12825-12831.
 - [49] T.L. Poulos, *Chem. Rev.* 114 (2014) 3919-3962.
 - [50] G.I. Berglund, G.H. Carlsson, A.T. Smith, H. Szoke, A. Henriksen, J. Hajdu, *Nature* 417 (2002) 463-468.
 - [51] B.C. Finzel, T.L. Poulos, J. Kraut, *J. Biol. Chem.* 259 (1984) 13027-13036.
 - [52] H. Sato, T. Hayashi, T. Ando, Y. Hisaeda, T. Ueno, Y. Watanabe, *J. Am. Chem. Soc.* 126 (2004) 436-437.

Legends to Figures:

Fig. 1. (A) Overall structure of WT Mb showing the heme site and the two residues (F46 and M55) to introduce a pair of disulfide bond; (B) Sequence alignment of the C-D region of Mb and Ngb. The conserved residues are highlighted in gray shadow and the double cysteines are highlighted in pink shadow, respectively.

Fig. 2. (A) Overlay of the modeling structure of F46C/M55C Mb (orange) with that of F46S Mb (pink) and WT Mb (gray), with helices A-H shown for comparison; (B) Detailed view of the heme site and the C-D region in simulated F46C/M55C Mb (orange) and overlaid with WT Mb (gray), showing the disulfide bond of C46-C55 and the double hydrogen bonds between R45, D60 and heme propionate groups (6-P or 7P), (C) Overlay of F46S Mb (orange) and WT Mb (gray), and (D) Overlay of F46C/M55C Mb (orange) and F46S Mb (orange).

Fig. 3. ESI-MS analysis of F46C/M55C Mb as purified (A) and that treated with reducing agent TCEP (B).

Fig. 4. (A) UV-Vis spectra of denatured F46C/M55C Mb, F46S/M55C Mb and WT Mb reacted with 1 eq. DTNB (10 μ M protein in 6 M Gdn-HCl, 100 mM KH_2PO_4 , pH 7.5, 25 $^\circ\text{C}$). The reaction of Cys and DTNB was shown as a control. The difference spectrum between F46C/M55C Mb and F46S/M55C Mb was also shown for clarification; (B) CD spectra of F46C/M55C Mb, F46S Mb and WT Mb (16 μ M protein in 100 mM KH_2PO_4 , pH 7.0, 25 $^\circ\text{C}$). The difference spectrum between F46C/M55C Mb and F46S Mb was also shown for clarification.

Fig. 5. (A) UV-Vis spectra of F46C/M55C Mb in ferric (black line) and ferrous (red line) states in 100 mM KH_2PO_4 , pH 7.0; (B) EPR spectrum of ferric F46C/M55C Mb collected at 10 K, 2 mW and 9.43 GHz. The spectra of F46S Mb and WT Mb were shown for comparison.

Fig. 6. (A) UV-visible spectra of Gdn-HCl-induced unfolding of F46C/M55C Mb, and (B) the normalized absorbance of Soret band versus Gdn-HCl concentrations. The results of F46S Mb and WT Mb were shown for comparison.

Fig. 7. (A) Stopped-flow spectra upon mixing 10 μM F46C/M55C Mb and 0.5 mM H_2O_2 in 100 mM KH_2PO_4 , pH 7.0, 25 $^\circ\text{C}$, for 10 sec. Inset, the single-exponential fit of the decay of Soret band at 411 nm; (B) Plots of observed rate constants versus H_2O_2 concentrations. The linear fit yields the apparent rate constant k_1 as the slope of the line. Control experiments of F46S Mb and WT Mb were shown for comparison.

Fig. 8. (A) Spectral changes at 470 nm for guaiacol (1 mM) oxidation by F46C/M55C Mb, F46S Mb and WT Mb (1 μM) with H_2O_2 (100 mM) (pH 7.0, 25 $^\circ\text{C}$); (B) Concentration-dependent initial rates for guaiacol (0.05-3 mM) oxidation at fixed H_2O_2 (100 mM). The data were fitted to the Michaelis-Menten equation.

Table 1: Kinetic parameters for H_2O_2 -dependent oxidation of guaiacol catalyzed by WT Mb and F46C/M55C Mb.

Figure1

Figure2

Figure3

Figure 4

Figure 5

Figure6

Figure7

Figure8

Table 1: Kinetic parameters for H₂O₂-dependent oxidation of guaiacol catalyzed by WT Mb, F46S Mb and F46C/M55C Mb.

Proteins	k_{cat} (s ⁻¹)	K_{m} (mM)	$k_{\text{cat}}/K_{\text{m}}$ (M ⁻¹ s ⁻¹)
WT Mb	0.4 ± 0.1	3.53 ± 0.05	110
F46S Mb	6.6 ± 0.1	3.14 ± 0.05	2100
F46C/M55C Mb	3.7 ± 0.1	0.48 ± 0.02	7700

Disulfide bond Design