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Formation of Cys-heme cross-link in K42C myoglobin under reductive conditions with molecular oxygen

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ABSTRACT

The structure and function of heme proteins are regulated by diverse post-translational modifications including heme-protein cross-links, with the underlying mechanisms not well understood. In this study, we introduced a Cys (K42C) close to the heme 4-vinyl group in sperm whale myoglobin (Mb) and solved its X-ray crystal structure. Interestingly, we found that K42C Mb can partially form a Cys-heme cross-link (termed K42C Mb-X) under dithiothreitol-induced reductive conditions in presence of O_2, as suggested by guanidine hydrochloride-induced unfolding and heme extraction studies. Mass spectrometry (MS) studies, together with trypsin digestion studies, further indicated that a thioether bond is formed between Cys42 and the heme 4-vinyl group with an additional mass of 16 Da, likely due to hydroxylation of the α-carbon. We then proposed a plausible mechanism for the formation of the novel Cys-heme cross-link based on MS, kinetic UV–vis and electron paramagnetic resonance (EPR) studies. Moreover, the Cys-heme cross-link was shown to fine-tune the protein reactivity toward activation of H_2O_2. This study provides valuable insights into the post-translational modification of heme proteins, and also suggests that the Cys-heme cross-link can be induced to form *in vitro*, making it useful for design of new heme proteins with a non-dissociable heme and improved functions.

1. Introduction

Using the same heme cofactor, Fe-protoporphyrin IX (Fig. 1A), heme proteins perform a large array of biological functions, including O_2 storage and delivery, electron transfer and catalysis [1–12]. The heme group is usually associated with the protein matrix non-covalently (*b*-type) such as in myoglobin (Mb) (Fig. 1B) with coordination, hydrogen(H)-bond and hydrophobic interactions, or covalently such as in cytochrome *c* (Cyt *c*) with one or two thioether bonds (*c*-type) [13]. Post-translational modifications (PTMs) of heme proteins by heme-protein cross-links have received much attention in the last decades, due to their crucial roles in regulating the structure and function of heme proteins [14–22]. A broad diversity of heme-protein cross-links have been discovered, such as Cys/SeCys-heme, Met-heme, His-heme, Trp/Tyr-heme, Glu/Asp-heme and Lys-heme cross-links, with bond types ranging from C—S, C—Se, C—N, C—C to C—O [14–16,18–22]. Meanwhile, the conditions required for the formation of these cross-links are not fully investigated, and the underlying mechanisms are not well understood.

In previous studies, Barker et al. [24] and Lin et al. [25] showed that by introduction of one Cys residue (N57C) or two Cys residues (N57C and S71C) close to the heme vinyl group(s) in Cyt b_5, one or two Cys-heme cross-links can be formed when the protein was expressed in *E. Coli*, with either a thioether bond as in Cyt *c* or an unusual [heme-CO-CH_2-S-CH_2-C_α] linkage, depending on the spatial position of each Cys. Barker et al. also showed that a *c*-type Cyt b_{562} can be engineered by introduction of two Cys residues (R98C/Y101C) in Cyt b_{562} to construct a CXXCH heme-binding motif [26]. Raven and co-workers showed that introduction of a Cys (S160C) close to the heme 2-vinyl group in ascorbate peroxidase (APX) led to the formation of a thioether bond in reconstitution of the apo-protein with heme *in vitro* under reductive conditions [27]. On the other hand, when a Met was introduced at the same position, S160M APX was able to form a sulfonium ion bond, with addition of a hydroxyl group at the C_α of heme vinyl group, by reconstitution of the apo-protein with heme and exposure to H_2O_2 [28]. In a recent study, we found that with a Tyr introduced to the heme

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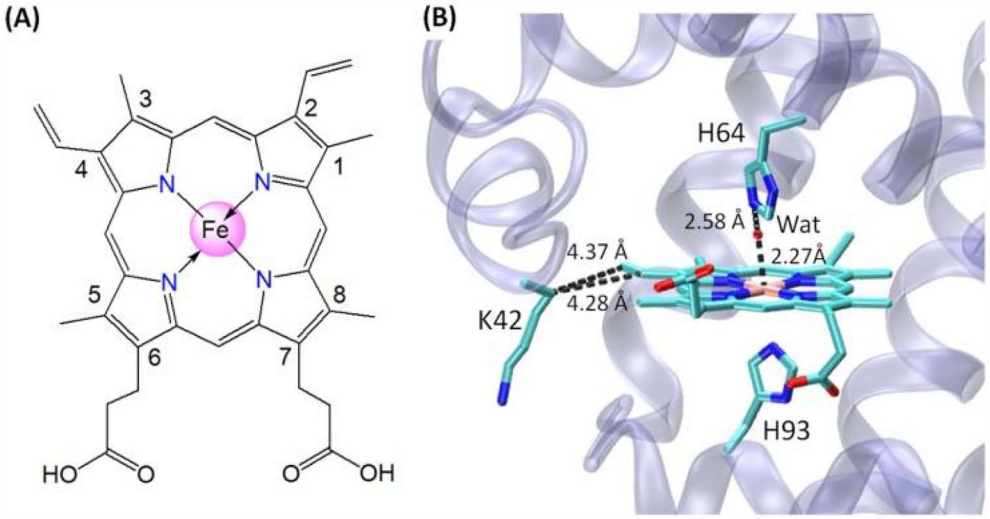

Fig. 1. (A) Chemical structure of heme, showing the substitutions. (B) X-ray crystal structure of the sperm whale met Mb (PDB entry code 1JP6 [23]), showing the distance between the C_B atom of Lys42 and the α-carbon (4.28 Å) and β-carbon (4.37 Å) of the heme 4-vinyl group, respectively.

distal pocket of sperm whale Mb, the F43Y Mb mutant formed a novel C—O bond between the Tyr hydroxyl group and heme 4-vinyl group *in vivo* [29]. We further showed that a double mutant L29H/F43Y Mb formed the novel Tyr-heme cross-link *in vitro* under dithiothreitol (DTT)-induced reductive conditions with molecular oxygen [30].

Owing to the reactivity of the thiol group, Cys residue is commonly utilized to covalently anchor metal complex in protein scaffolds for creating artificial metalloenzymes [1–13,31–34]. For example, Lu and co-workers attached a Mn-salen complex into apo-Mb *via* single or dual covalent linkages with Cys residues at different positions (T39C, Y103C, L72C or S108C), which confers peroxygenase activity with tunable reactivity and selectivity of sulfoxidation [35,36]. By inspection of the X-ray structure of sperm whale Mb, we noticed that Lys42 is close to the heme 4-vinyl group, with a short distance of ~4.3 Å between its C_B atom and the α- and β-carbons (Fig. 1B). We thus envisaged that if a Cys is introduced at this position, it might be able to form a Cys-heme cross-link *in vivo* or *in vitro* with the heme 4-vinyl group. As shown in this study, we found that although sperm whale K42C Mb was expressed as a protein without a Cys-heme cross-link, it can partially form the cross-link under DTT-induced reductive conditions in presence of O_2, which is distinct from the typical thioether bond in Cyt *c*. We provided evidences for the formation of the unusual Cys-heme cross-link and proposed a plausible mechanism.

2. Materials and methods

2.1. Protein preparation

Wild-type (WT) sperm whale Mb was expressed using the Mb gene of pMbt7–7 and purified using the procedure described previously [37]. The K42C Mb gene was constructed using the QuikChange Site Directed Mutagenesis Kit (Stratagene) with two primers, *i.e.*, forward, 5′-GAAA CTCTGGAATGTTTTGATCGTTTC-3′, and reverse, 5′-GAAACGATCAAA ACATTCCAGAGTTTC-3′. The mutation was confirmed by DNA sequencing assay. K42C Mb mutant was expressed in BL21(DE3) and purified using a similar procedure for WT Mb [37], without addition of dithiothreitol (DTT) in buffer solution during purification. After purification, the protein was concentrated to ~20 μM in air-saturated 100 mM potassium phosphate buffer (pH 7.0), and added DTT to a final concentration of 1 mM. The mixture was stirred overnight at 4 °C and then purified by using a PD-10 column. In preparation of control samples for MS experiments (Section 2.5), the buffer solution was degassed by purge of N_2 or by addition of imidazole to a final concentration of 0.2 M, with other procedure kept the same.

2.2. X-ray crystallography

Sperm whale K42C Mb with a high purity (A_{406nm}/A_{280nm} >4.0), were exchanged into 20 mM potassium phosphate (pH 7.0) and concentrated to ~2.0 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 [29]. 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 [38]. The structure was solved by the molecular replacement method and the 1.5 Å structure of L29H Mb (PDB entry 4IT8 [39]) was used as the starting model. Manual adjustment of the model was carried out using the program COOT [40] and the models were refined by PHENIX [41] and Refmac5 [42]. Stereochemical quality of the structures was checked by using PROCHECK [43]. All of residues locate in the favored and allowed region and none in the disallowed region.

2.3. UV–vis studies

UV–vis spectrum of ferric K42C Mb was recorded in 100 mM potassium phosphate buffer (pH 7.0) on a Agilent 8453 diode array spectrometer. The pyridine hemochrome spectrum was obtained by using 10 μM proteins in 19% (vol/vol) pyridine and 0.15 M NaOH, and the protein was reduced by a small amount of sodium dithionite. Protein concentration of K42C Mb was determined with an extinction coefficient of $\varepsilon_{408} = 160 \pm 5\ mM^{-1}{\cdot}cm^{-1}$, as calculated using the standard hemochromagen method [44]. Guanidine hydrochloride (Gdn·HCl)-induced unfolding of K42C Mb and K42C Mb-X were performed by titration of 10 μL protein solution to 2 mL Gdn·HCl stock solutions (0.5–5.0 M, pH 7.0) with a final protein concentration of ~10 μM. The samples were incubated 25 °C for 1 h before collecting the UV–vis spectra. The denaturation midpoints (C_m values) were 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.

2.4. Heme extraction studies

The heme group in K42C Mb and K42C Mb-X that was not