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Review

The broad diversity of heme-protein cross-links: An overview

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ABSTRACT

Heme proteins perform a large array of biological functions using the same heme cofactor. A primary method of regulating these diverse functions is the heme-protein cross-link, an important post-translational modification. This review presents an overview of the broad diversity of heme-protein cross-links, including Cys/SeCys -heme, Met-heme, His-heme, Trp/Tyr-heme, Glu/Asp-heme and Lys-heme cross-links, which have been discovered in the last three decades, with bond type ranging from C-S, C-Se, C-N, C-C to C-O. Many advances have been made in revealing the mechanisms of heme-protein cross-link formation, as well as the structural and functional roles. Moreover, most of these cross-links have been successfully recreated in natural or *de novo* proteins. These tremendous progresses have not only enhanced our knowledge of how cross-links fine-tune the structure and function of natural heme proteins, but also provided us powerful strategies for design of artificial heme proteins with functionalities beyond those of natural heme proteins.

1. Introduction

Heme proteins are crucial for all biological systems as they perform a large array of functions, ranging from oxygen binding and delivery (e.g. myoglobin, Mb and hemoglobin, Hb), electron transfer (e.g. cytochrome *c*, cyt *c* and cytochrome b_5, cyt b_5), catalysis (e.g. peroxidase and cytochrome P450, CYP450) to signaling (e.g. CO censor CooA) [1–10]. It is remarkable that heme proteins use the same heme cofactor, Fe-protoporphyrin IX (Fig. 1), to confer such diverse functions. This is attributed to the variety of interactions between the heme cofactor and the protein polypeptide chain, such as the coordination of heme iron by various axial ligands (His, Met, Cys, water, *etc*) and the hydrogen-bonding interactions in the heme distal pocket [1–11]. In addition to these interactions, post-translational modifications (PTMs) also play key roles in tuning the structure and function of heme proteins. Examples include nitration of Tyr/Trp [12–14], glycosylation of Asn [15], cross-links between amino acids such as Tyr-His cross-link in heme-copper oxidase (HCO) [16,17], cross-links between the heme cofactor and the protein matrix [18–20], and even degradation of the heme cofactor yielding biliverdin and bilirubin [18].

Among these PTMs, the covalent cross-link between heme and protein matrix has attracted extensive attention during the last three decades, and it still continues to be a research hotspot in revealing the structure-property-reactivity-function (SPRF) relationship of heme proteins. With several substitutions on the heme porphyrin ring (e.g. 1-, 3-, 5- and 8-methyl groups, 2- and 4-vinyl groups and 6- and 7-propionate groups, Fig. 1), a broad diversity of heme-protein cross-links have been discovered between various amino acids in the protein matrix and the heme side chain as well as the heme porphyrin ring. This review summarizes the progresses from 1974 to 2015, and focuses on the diversity of heme-protein cross-links, the mechanisms of formation, the structural and functional roles and the potential applications. These progresses have not only enhanced our knowledge of how PTMs fine-tune the SPRF relationship of heme proteins, but also provided us new approaches for design of artificial heme proteins with advanced functions.

2. Cross-linking with heme vinyl group(s)

2.1. C–S bond

2.1.1. Thioether bond

One distinct characteristic of *c*-type cytochromes is that the heme cofactor is covalently linked to the protein matrix, commonly via two thioether bonds between the heme vinyl groups and the cysteine sulfurs of a classic CXXCH heme-binding motif, as exemplified in the well-known X-ray structure of horse heart cyt *c* (Fig. 2A, PDB ID: 1HRC [21]). The structure of the heme attached to an (A/F) XXCH motif through only one thioether bond has also been discovered for *C. fasciculata* mitochondrial cyt *c* (Fig. 2B, PDB entry 2W9K [22]). Moreover, an atypical heme in the cyt *b* subunit of cyt b_6f complex is covalently linked via one thioether bond, without an axial amino acid ligand (Fig. 2C PDB ID: 1Q90 [23]).

The thioether bond that distinguishes *b*-type and *c*-type cytochromes has attracted much attention over the last few decades. To gain insights into the mechanism of cyt *c* maturation, Barker *et al.* [24,25] showed

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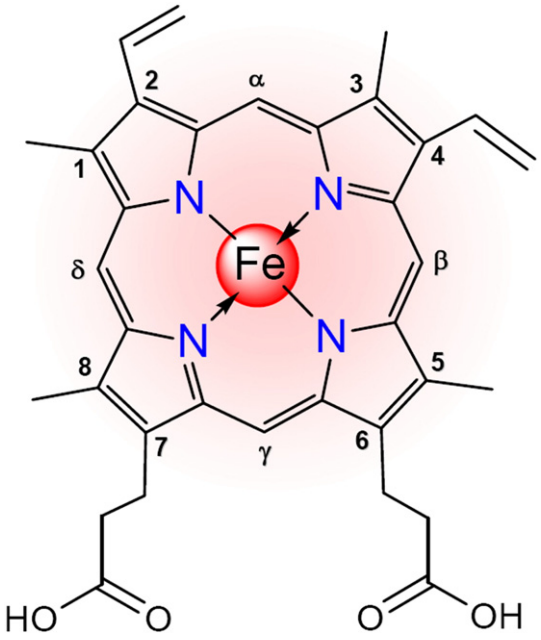

Fig. 1. Chemical structure of *b*-type heme, Fe-protoporphyrin IX.

that *b*-type cytochromes such as cyt b_5 and cyt b_{562} can be converted into cyt *c*-like proteins by introducing a Cys close to the heme 4-vinyl group in cyt b_5 (N57C mutation) [24], and by creating an artificial CXXCH heme-binding motif in cyt b_{562} (Arg98 and Tyr101 to Cys mutation) [25], respectively. Molecular modeling showed that a single thioether bond in N57C cyt b_5 does not alter the overall protein structure (Fig. 2D, [26]). The double thioether bonds in R98C/Y101C cyt b_{562}, termed cyt cb_{562}, were confirmed by an X-ray crystal structure (Fig. 2E, PDB ID: 2BC5 [27]). Due to the well-folded structure of four-helix bundle and enhanced stability with respect to cyt b_{562}, cyt cb_{562} was extensively used by Tezcan and co-workers [28–30] to create novel biomaterials, such as one-dimensional nanotubes and two- or three-dimensional crystalline arrays, by rational design of metal-binding sites on protein surface to introduce protein self-assembly. Moreover, when a heme-binding motif of CX_3CH or CX_4CH was constructed in cyt b_{562}, cyt cb_{562} could also be maturated with heme attached correctly through two thioether bonds, indicating these motifs are the substrate of cyt *c* maturation (Ccm) apparatus [31]. Meanwhile, with an unnatural heme-binding motif of CX_nCH (n = 1, 5, or 6), an extra sulfur was incorporated into one thioether bond of the maturated cyt cb_{562}, resulting in formation of an aberrant persulfide linkage [32].

Huang and co-workers [33], including the author, further showed that a cyt *c*-like cyt b_5 can be obtained by introducing two cysteine residues (N57C and S71C mutations) close to the two heme vinyl groups without constructing the typical CXXCH motif of cyt *c*. Remarkably, in the double mutant of N57C/S71C cyt b_5, Cys57 forms the typical thioether linkage with the heme 4-vinyl group and Cys71 forms an unusual [heme-CO-CH_2-S-CH_2-C_α] linkage with the heme 2-vinyl group, with the formation depending on the spatial position of each Cys (Fig. 3). The observation is further supported by the crystal structure of cyt rc_{552} (PDB ID: 1QYZ, Fig. 2F), where Cys11 forms the unusual Cys-heme linkage with heme 2-vinyl group, likely by a nucleophilic or a free radical mechanism involving O_2 (Scheme 1) [34]. Similar to that for cyt rc_{552}, N57C/S71C cyt b_5 was overexpressed in *E. coli* where the cross-links formed spontaneously without the involvement of Ccm apparatus. The protein has a reduced hemochrome spectrum in pyridine with an α-band identical to that of cyt *c* (550 nm), which is blue-shifted from that of WT Mb (556 nm) (Fig. 4A). With heme covalently attached, cyt *c*-like N57C/S71C cyt b_5 exhibits an enhanced (~350-fold) peroxidase activity in its partially unfolded form induced by guanidine hydrochloride (Gdn · HCl), compared to that of wild-type (WT) cyt b_5, where heme releases upon unfolding (Fig. 4B). These studies have enhanced our understanding of the mechanism of Cys-heme cross-link formation *in vivo*, and also laid down the groundwork for functional protein design based on the scaffold of cyt *c*-like cyt b_5.

It should be noted that although cyt *c* maturation in cells is a protein-catalyzed process in different biogenesis systems [35–40], Daltrop *et al.* [41] demonstrated that a *c*-type cytochrome can form spontaneously *in vitro* from heme and apo-protein via a *b*-type intermediate under reducing conditions. Moreover, the thioether bond can form with heme derivatives containing other metals such as

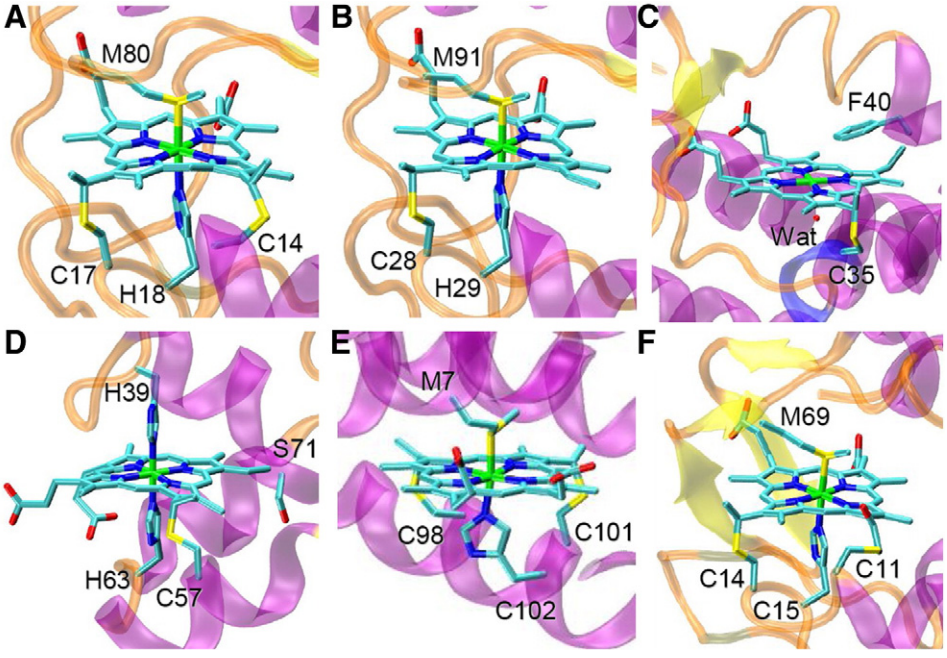

Fig. 2. Covalently linked heme by thioether bond(s) in horse heart cyt *c* (A), *C. fasciculata* mitochondrial cyt *c* (B), cyt *b* subunit of cyt b_6f complex (C), N57C cyt b_5 mutant (D), R98C/Y101C cyt b_{562} mutant (E) and cyt rc_{552} (F).