Published in final edited form as:

J Mol Biol. 2018 April 13; 430(8): 1081–1083. doi:10.1016/j.jmb.2018.02.021.

Cys Links Heme: Stereo-orientation of Heme Transfer in Cytochrome *c* Biogenesis

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Heme is an essential prosthetic group and an important source of nutritional iron for microbes and man. Several prokaryotic heme uptake and transport systems have been identified and characterized [1], while only a handful have been identified in eukaryotes [2–4]. The weak and transient interactions between heme and their cognate transporters make it challenging to validate heme as the substrate, or identify binding sites within putative transporters. Covalent trapping of heme within transporters is an attractive strategy, as site-directed disulfide cross-linking approach has been used to probe conformational changes of transporters and protein–protein interactions for over 30 years [5]. More recently, cysteine-based covalent binding of modified nucleic acids to proteins was developed as a method for investigating protein/DNA interactions [6]. Heme can attach to proteins through both covalent bonds and non-covalent interactions. The best known examples are the *c*-type cytochromes, which form thioether bonds with heme via their conserved CXXCH motif [7]. Interestingly, previous studies have shown that introducing cysteines near heme-binding sites in soluble cytochrome *b* resulted in spontaneously covalent bond formation between heme and cytochrome *b*, converting it to a *bona fide* cytochrome *c* [8].

One of the most complex and best-studied heme transport systems is the cytochrome *c* biogenesis pathway, including systems I, II, and III [9,10]. System I, also called the Ccm system, is composed of eight integral membrane proteins, CcmABCDEFGH. Cytosolic heme is delivered to the periplasmic heme chaperone CcmE so that heme can be attached to apocytochrome *c*. First, CcmC interacts with heme to allow simultaneous binding of heme to both CcmC and CcmE. HoloCcmE is released from the complex upon the function of CcmABCD, an ABC transporter. Next, CcmE ferries the heme to CcmF/H for covalent attachment of heme to cytochrome *c* with thioreductants provided by CcmG. During the first process, a conserved histidine (H130) residue in CcmE forms an unusual covalent bond to the 2-vinyl group of heme [11,12]. Currently, only the structure of a truncated apoCcmE has been resolved [13]. A clear understanding of how the Ccm system transfers heme across the membrane for cytochrome *c* maturation is lacking. For example, what are the heme-binding sites in CcmC and CcmE during heme delivery? Does the conserved periplasmic WWD domain [14,15] identified in both Systems I and II directly bind heme?

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In this issue, Sutherland *et al.* solved these questions surrounding CcmE through adaption of a “cysteine trap” approach for understanding membrane transport. Based on the observations that cysteine substitution induces spontaneous conversion of cytochrome *b* to cytochrome *c*, the authors proposed a similar strategy to map heme-binding sites in membrane proteins: the transient interactions between heme and transporters/chaperones might be locked by crosslinking heme to specific cysteines near the endogenous heme-binding sites. This method was first applied to understand surface orientation of CcmE during heme transfer. Although H130 has been identified to bind heme, it is inadequate to determine the stereo-orientation of heme binding in CcmE. Thus, the authors substituted 16 residues adjacent to the two putative heme-binding pockets with cysteines in an H130A mutant background as H130 covalent links to the 2-vinyl group of heme. Each of these variants was purified in a CcmCDE complex by expressing in the *ccm*-deficient *Escherichia coli* mutant strain RK103, and subjected to UV-vis spectra, SDS-PAGE/Coomassie gels, heme stains, and CcmE immuno-blotting analysis. SDS-PAGE/heme stain results show that only CcmE (H130A/E132C) forms a covalent bond with heme. The single covalent linkage between E132C to a heme vinyl group was further confirmed by reduced pyridine hemochrome spectra. The authors also tested these cysteine substitutions in wild-type CcmE background. The fact that CcmE (E132C) retained 60% function implied that both H130 and E132C crosslinked to the 2-vinyl of heme, favoring an orientation that possesses a “4-vinyl pocket,” which is 10 Å from the 2-vinyl pocket.

CcmC directly interacts with heme and is required for loading heme to CcmE. It is not clear whether CcmC also transfers heme across the membrane, since CcmC does not have any conserved histidines in the transmembrane domain [16]. However, CcmC has two conserved periplasmic histidine residues and a conserved periplasmic WWD domain, which belongs to the heme-handling protein family (HHP) [17]. The authors first investigated whether the WWD domain directly interacted with heme by introducing cysteine substitutions in this region. A collection of 17 CcmCDE (H130A) complexes carrying engineered CcmC were analyzed for covalent heme-binding. While six variants exhibited various levels of potential crosslinking with heme, only the three stable variants CcmC (W114C) DE(H130A), CcmC (D126C)DE(H130A), and CcmC (R128C)DE(H130A) were further characterized. A loss of >75% activity in each of the three CcmCDE variants in the wild-type CcmE background confirmed the role of the WWD domain in delivering heme to CcmE. Pyridine hemochrome spectra revealed a single-covalent bond formation between heme and CcmC (W114C), whereas CcmC(D126C) and CcmC (R128C) only partially crosslinked with heme in the CcmE(H130A) background, indicating that these cysteine substitutions might only form covalent bond with a specific vinyl group of heme. The three stable variants were then examined in the wild-type CcmE background. Strikingly, the CcmC(W114C)DE(WT) variant displayed a unique migration and heme-staining pattern on SDS-PAGE, revealing the formation of a double crosslinked CcmC/heme/E(WT) co-complex. On the other hand, CcmC(D126C)DE(WT) and CcmC(R128C)DE(WT) variants showed similar heme-staining pattern to that observed in CcmCDE (WT), indicating that these two cysteine substitutions do not crosslink to the same heme vinyl groups as CcmC(W114C). Knowing that CcmE(H130) covalently bound to heme 2-vinyl, these results demonstrate that CcmC(W114C) must crosslink to the 4-vinyl group of heme.

The work by Sutherland *et al.* mapped heme-binding sites in CcmE and CcmC, providing the first direct evidence that heme directly interacts with the conserved WWD domain, and most importantly, proved that heme is stereochemically positioned and attached to CcmE during delivery from CcmC. Because the WWD domain is also present in other members of the cytochrome *c* biogenesis pathway, including CcmF (System I) and CcsBA (System II), heme might be orientated and transferred to apocytochrome *c* via the WWD domain in a similar manner. In fact, preliminary studies by the authors show that cysteine substitutions of the CcsBA WWD domain also result in specific covalent bonds with heme. While the crosslinking studies greatly facilitate our understanding of the structural basis of heme trafficking in cytochrome *c* biogenesis, many questions remain to be addressed. The CcmE proteins found in archaea and some sulfate-reducing bacteria (the *Desulfovibrio* species) have a cysteine instead of a histidine at H130 [18], and thus should form a thioether bond with heme, which is more stable than the unusual covalent bond in *E. coli* CcmE. Surprisingly, swapping the heme-binding motifs between *E. coli* (¹³⁰HDENY) and *Desulfovibrio* (¹²⁷CPSKY) resulted in a complete loss of function, although an *E. coli* CcmE (H130C) variant has been shown to covalently bind heme with lower yield [19]. The authors' experiments in this study also showed that cysteine substitutions in CcmC severely reduce Ccm complex function in the wild-type CcmE background, indicating the difficulty in breaking thioether bonds between cysteine and heme. It will be interesting to apply the cysteine-trapping strategy established in this work to investigate the heme transfer process in alternative cytochrome *c* biogenesis systems.

It should be realized that autonomous disulfide bond formation typically happens in an oxidative environment, that is, the periplasm of bacteria or the ER lumen of eukaryotic cells. To facilitate covalent bond formation in other locations, it is often necessary to add oxidizing agents or crosslinkers. Similarly, covalent bond formation between certain cysteine and heme occurs with limitations; that is, the CcmE (H130C) variant only crosslinks with heme under reducing conditions *in vitro* [19]. Therefore, the autonomy, specificity, and biocompatibility of this cysteine-trapping approach require further validation in different organisms beyond *E. coli*. In summary, the cysteine/heme crosslinking approach adds to the biochemical toolbox and will help reveal the molecular mechanisms of heme uptake, trafficking, and utilization in prokaryotes and eukaryotes. The double-crosslinking studies demonstrate the potential of using this method to explore interactions between hemoproteins and may aid in identifying unknown interacting partners by “trapping” them in ternary complexes.

Acknowledgments

Work in the Hamza lab is supported by funding from the National Institutes of Health DK85035 and DK074797.

References

1. Huang W, Wilks A. Extracellular heme uptake and the challenge of bacterial cell membranes. *Annu Rev Biochem.* 2017; 86:799–823. [PubMed: 28426241]
2. Quigley JG, Yang Z, Worthington MT, Phillips JD, Sabo KM, Sabath DE, et al. Identification of a human heme exporter that is essential for erythropoiesis. *Cell.* 2004; 118:757–766. [PubMed: 15369674]

3. Rajagopal A, Rao AU, Amigo J, Tian M, Upadhyay SK, Hall C, et al. Haem homeostasis is regulated by the conserved and concerted functions of HRG-1 proteins. *Nature*. 2008; 453:1127. [PubMed: 18418376]
4. Korolnek T, Zhang J, Beardsley S, Scheffer GL, Hamza I. Control of metazoan heme homeostasis by a conserved multidrug resistance protein. *Cell Metab*. 2014; 19:1008–1019. [PubMed: 24836561]
5. Falke JJ, Koshland DE. Global flexibility in a sensory receptor: a site-directed cross-linking approach. *Science*. 1987; 237:1596–1600. [PubMed: 2820061]
6. He C, Verdine GL. Trapping distinct structural states of a protein/DNA interaction through disulfide crosslinking. *Chem Biol*. 2002; 9:1297–1303. [PubMed: 12498882]
7. Barker PD, Ferguson SJ. Still a puzzle: why is haem covalently attached in c-type cytochromes? *Structure*. 1999; 7:R281–R290. [PubMed: 10647174]
8. Barker PD, Nerou EP, Freund SM, Fearnley IM. Conversion of cytochrome b562 to c-type cytochromes. *Biochemistry*. 1995; 34:15191–15203. [PubMed: 7578134]
9. Kranz R, Lill R, Goldman B, Bonnard G, Merchant S. Molecular mechanisms of cytochrome c biogenesis: three distinct systems. *Mol Microbiol*. 1998; 29:383–396. [PubMed: 9720859]
10. Kranz RG, Richard-Fogal C, Taylor JS, Frawley ER. Cytochrome c biogenesis: mechanisms for covalent modifications and trafficking of heme and for heme-iron redox control. *Microbiol Mol Biol Rev*. 2009; 73:510–528. [PubMed: 19721088]
11. Daltrop O, Stevens JM, Higham CW, Ferguson SJ. The CcmE protein of the c-type cytochrome biogenesis system: unusual in vitro heme incorporation into apo-CcmE and transfer from holo-CcmE to apocytochrome. *Proc Natl Acad Sci*. 2002; 99:9703–9708. [PubMed: 12119398]
12. Lee D, Pervushin K, Bischof D, Braun M, Thöny-Meyer L. Unusual heme–histidine bond in the active site of a chaperone. *J Am Chem Soc*. 2005; 127:3716–3717. [PubMed: 15771504]
13. Enggist E, Thöny-Meyer L, Güntert P, Pervushin K. NMR structure of the heme chaperone CcmE reveals a novel functional motif. *Structure*. 2002; 10:1551–1557. [PubMed: 12429096]
14. Beckman DL, Trawick DR, Kranz RG. Bacterial cytochromes c biogenesis. *Genes Dev*. 1992; 6:268–283. [PubMed: 1310666]
15. Hamel PP, Dreyfuss BW, Xie Z, Gabilly ST, Merchant S. Essential histidine and tryptophan residues in Ccs A, a system II polytopic cytochrome c biogenesis protein. *J Biol Chem*. 2003; 278:2593–2603. [PubMed: 12427766]
16. Richard-Fogal C, Kranz RG. The CcmC:heme:CcmE complex in heme trafficking and cytochrome c biosynthesis. *J Mol Biol*. 2010; 401:350–362. [PubMed: 20599545]
17. Lee JH, Harvat EM, Stevens JM, Ferguson SJ, Saier MH Jr. Evolutionary origins of members of a superfamily of integral membrane cytochrome c biogenesis proteins. *Biochim Biophys Acta Biomembr*. 2007; 1768:2164–2181.
18. Goddard AD, Stevens JM, Rao F, Mavridou DA, Chan W, Richardson DJ, et al. c-Type cytochrome biogenesis can occur via a natural Ccm system lacking CcmH, CcmG, and the heme-binding histidine of CcmE. *J Biol Chem*. 2010; 285:22882–22889. [PubMed: 20466730]
19. Stevens JM, Daltrop O, Higham CW, Ferguson SJ. Interaction of heme with variants of the heme chaperone CcmE carrying active site mutations and a cleavable N-terminal His tag. *J Biol Chem*. 2003; 278:20500–20506. [PubMed: 12657624]