Published in final edited form as:

Trends Biochem Sci. 2015 August ; 40(8): 446–455. doi:10.1016/j.tibs.2015.05.006.

Mitochondrial cytochrome c biogenesis: no longer an enigma

Shalon E. Babbitt, Molly C. Sutherland, Brian San Francisco[‡], Deanna L. Mendez, and Robert G. Kranz^{*}

Department of Biology, Washington University, St. Louis, MO 63130

Abstract

Cytochromes c and c₁ are heme proteins that are essential for aerobic respiration. Release of cytochrome c from mitochondria is an important signal in apoptosis initiation. Biogenesis of c-type cytochromes involves covalent attachment of heme to two cysteines (at a conserved CXXCH sequence) in the apocytochrome. Heme attachment is catalyzed in most mitochondria by holocytochrome c synthase (HCCS), which is also necessary for import of apocytochrome c. Thus, HCCS affects cellular levels of cytochrome c, impacting mitochondrial physiology and cell death. Here, we review the mechanisms of HCCS function and the roles played by heme and residues in the CXXCH motif. Additionally, we consider concepts emerging within the two prokaryotic cytochrome c biogenesis pathways.

Keywords

cytochrome c; mitochondria; heme attachment; holocytochrome c synthase

Cytochrome c, HCCS, cell death, and disease

The primary function of cytochrome c (cyt c) and c₁ (part of complex III) in mitochondria as electron transfer proteins has long been known (Figure 1A). Structures of cyt c and c₁ confirmed that two thioether bonds are formed between two cysteines (of the cytochrome CXXCH motif) and two vinyl groups of heme (Figure 1B) [1-4]. It is generally accepted that covalent heme attachment provides stability to cyt c, without which apocytochrome c (apocyt c) is rapidly degraded in the cell [5]. This covalent attachment is always present in a stereospecific conformation; the first cysteine to 2-vinyl and second cysteine to 4-vinyl, while the histidine supplies an axial ligand to heme iron (Figure 1B) [1-4].

The mechanism of stereospecific attachment, referred to as cyt c biogenesis, was investigated for nearly 30 years, but remained poorly understood. In 1987, Dumont and Sherman showed that attachment of heme to cyt c in yeast requires the gene encoding “cytochrome c heme lyase” [6], usually now denoted as holocytochrome c synthase or

^{*} Corresponding author: Kranz, R.G. (kranz@biology.wustl.edu).

[‡] Current address: The Institute for Genomic Biology, University of Illinois at Urbana, Champaign, IL 61801

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

HCCS. HCCS was shown to associate peripherally with the outer leaflet of the mitochondrial inner membrane, yet is a predicted soluble protein [6-8]. It was further shown that HCCS is required for apocytochrome c import into the intermembrane space (IMS) [9-12] (Figure 1A). Because yeast HCCS could attach heme to cytochrome c in recombinant *Escherichia coli* [13, 14], HCCS was identified as the main player in mitochondrial cytochrome c biogenesis (referred to as the System III cytochrome c biogenesis pathway [15-22]). Nevertheless, virtually nothing more was known about HCCS function until recently. Largely based on new studies, we review here the mechanisms behind HCCS function, structure, and stereospecific attachment of heme. We emphasize studies on the human HCCS, but it is likely that these mechanisms are common to all HCCS proteins, which are widely distributed in eukaryotes (see Box 1). Interestingly, the prokaryotic cytochrome c biogenesis pathways, called Systems I and II (previously reviewed in [15, 20, 23-25]) are retained in some eukaryotes (Box 1); these pathways are addressed (Box 2) with respect to mechanistic insight gained from our new understanding of HCCS.

Besides its role in electron transfer, cytochrome c is also integrally involved in programmed cell death (apoptosis) in animals [26]. Cytochrome c release from the mitochondrion is required for caspase-mediated apoptosis (Figure 1A), and has been the subject of many reviews [27-30]. HCCS itself has been implicated in a caspase-independent cell death pathway in injured neurons [31, 32]. Although the mechanisms behind the roles of HCCS in apoptosis are still unclear, the cytochrome c roles are well-documented.

Another aspect of HCCS that has emerged during the past decade concerns mitochondrial pathology and human disease. Many studies on human patients have demonstrated that the disease microphthalmia with linear skin defects (MLS) is due to mutations in the HCCS gene [33-38]. Because the HCCS gene is on the X chromosome, females with a single mutated allele acquire MLS, whereas such mutations are lethal in males [39]. Two HCCS amino acid substitutions known to result in MLS are discussed here in the context of HCCS mechanisms and structure.

To fully appreciate the important secondary functions of cytochrome c and HCCS, such as their roles in apoptosis and mitochondrial disease, it is critical that we develop a more complete understanding of the mechanisms underlying HCCS-mediated attachment of heme to cytochrome c, domains within HCCS for heme and cytochrome c binding, and HCCS association with membranes.

Four-step model for HCCS function: heme as the central hub in biogenesis

The purification and characterization of functional (*in vivo*) HCCS was facilitated by the discovery that, when recombinantly expressed in bacteria, human HCCS is strongly associated with the *E. coli* cytoplasmic membrane [40] and likely degraded in the cytoplasm. Using detergents, recombinant HCCS was purified with endogenous heme [40-42]. Additionally, when co-expressed with its cognate human cytochrome c, HCCS could be co-purified as a ternary complex with heme and cytochrome c [40-42]. These capabilities, combined with comprehensive analyses of many cytochrome c and HCCS variants [40-42], led to the proposal of a four-step model [40] for HCCS-mediated cytochrome c biogenesis (Figure 2). Briefly, membrane-bound HCCS binds heme (step 1), then binds apocytochrome c (step 2), and thioether bonds are

formed (step 3) leading to the release of cyt c (step 4) from HCCS for folding into its native conformation. Significant progress has been made in the identification of residues in cyt c (Figure 1B) necessary for interaction with HCCS and for heme attachment, as well as residues in HCCS that define specific structural and functional domains (Figure 3). The steps will be reviewed here in the context of HCCS structure/function features. An earlier study suggested that a region encompassing domain III in HCCS (Figure 3) is necessary and sufficient for import of HCCS through the mitochondrial outer membrane [43], directly into the IMS [7]. Furthermore, selected mutations in domain III do not result in defective attachment in recombinant *E. coli* [40, 42], suggesting that mitochondrial trafficking may be the paramount role of this domain. Substitutions in domain IV, including the MLS-associated mutation R217C, resulted in low recombinant yields, such that a role in folding, stability and/or membrane binding was proposed [42]. HCCS domains I and II are involved in heme binding [40, 42], and are discussed below in the context of the four steps. All steps are proposed to occur on membrane-localized HCCS (Figure 2). Following release from HCCS, cyt c is located in the cytoplasm of recombinant *E. coli* (or the IMS in mitochondria). The evidence and mechanistic details for each step are presented next.

Step 1: Heme binding by HCCS

The substrates for HCCS are reduced heme (Fe^{2+}) and apocyt c. The intracellular trafficking of porphyrins and heme (Figure 1A) has been reviewed, with candidate transporters discussed [18, 19, 44-48]. An early study on yeast HCC₁S (discussed below) suggested that a CPV (CysProVal) motif in the N terminus (Figure 3) was involved in heme binding [49]. However, the CP motif is not present in all HCCS proteins, and mutation analysis of CPV in yeast HCCS showed it is not required for heme attachment to cyt c [50]. Nonetheless, it is still not ruled out that the CPV motif could collect heme and transfer it to the HCCS active site [49].

Because nearly all heme proteins possess at least one histidine that binds to the iron of heme [51], conserved histidines in HCCS represented likely ligands. The histidine-iron axial ligand accounts for about 10 Kcal of binding energy [52] and controls the redox chemistry of the heme. In HCCS, His154 was shown to provide this axial ligand to heme [40] (Figures 2 and 3). HCCS His154 variants (substitutions) are not functional and purify without heme. Moreover, HCCS H154A and H154G are corrected for heme attachment to cyt c by adding 10 mM exogenous imidazole to recombinant *E. coli* [40], suggesting that His154 likely occupies a heme-binding pocket within the HCCS structure. This result is similar to the heme binding defect of recombinant myoglobin H93G, a “cavity” mutant (*i.e.* containing a ligand-accessible pocket) that is corrected for heme binding by exogenous 10 mM imidazole [53].

Mutations in other HCCS residues found in domain II (Figure 3) are now known to be defective in binding heme. These substitutions (including W162A, W168A, and E159K) result in reduced function (*i.e.* heme attachment) as well as in reduced levels of co-purified heme [42]. HCCS E159K represents one of the substitutions found in MLS patients (the other being R217C) [34]. Because the HCCS E159A variant produces more cyt c than wild-type (WT) HCCS (see below), it is likely that the charge on Glu159 (to a Lys) is involved in

the MLS heme-binding defect. Some variants with heme binding defects (*e.g.* E159K, but not H154A) can be corrected for function in *E.coli* with exogenous aminolevulinic acid (ALA) [42]. ALA is the first precursor committed to heme biosynthesis and exogenous ALA is known to increase the levels of heme in recombinant heme proteins [54, 55]. Results are consistent with defects in heme binding (step 1) for these HCCS variants (domain II), such that increasing the levels of heme substrate overcomes heme-binding deficiency. In Box 3 we speculate on analogies between domain II and a WWD heme-binding domain (see Box 2) in the prokaryotic Systems I and II.

Step 2: Apocyt c binding, control of stereospecificity, and the roles of cyt c His19

The WT HCCS co-purifies with cyt c, but the HCCS H154A variant does not [40]. This is consistent with a requirement for heme binding to HCCS before the apocyt c substrate binds. Multiple approaches and spectroscopic results have indicated that apocyt c His19 (of CXXCH) forms the second axial ligand in this complex [40, 41]; the first axial ligand is provided by HCCS His154 [40]. His19 axial liganding could provide 10 Kcal of binding energy [52], with other residues in apocyt c necessary for interaction with HCCS [40, 41, 56-59], summarized in Figure 1B. For example, cyt c His19 substitutions, as well as single and double substitutions of the cysteines (*i.e.* of C₁₅XXC₁₈H₁₉), still bind to HCCS, but the triple mutant (C15S/C18A/H19A) does not bind [41]. Residues near CXXCH in the cyt c substrate have also been studied for proficiency in heme attachment. Multiple side chains of cyt c between Lys8 and His19, including that of Phe11, are required for optimal recognition [40, 56] (Figure 1B). In fact, it has been possible to convert a bacterial cyt c into one recognized by HCCS with three sequence changes [40, 57], supporting the model for recognition of cyt c by holoHCCS using multiple residues in cyt c at or near the CXXCH motif.

That heme is a central player in apocyt c interaction with HCCS is also suggested by the important roles of apocyt c His19 in step 2 (and 3 and 4). Besides initial interaction, His19 must prepare the heme for thioether bond formation, potentially by helping to maintain the iron in the reduced state for the thioether bond formation that occurs next (step3). This chemistry, favoring reduced Fe²⁺ heme, has been discussed elsewhere [20, 60]. Preparation for thioether bond formation by cyt c His19 likely includes positioning the Cys15 and Cys18 adjacent to the vinyl groups of heme (Figures 1B and 2). His19 substitutions in cyt c still allow cyt c binding to HCCS but covalent attachment does not occur (except for H19M, discussed later) [41]. Moreover, the cyt c H19A or H19G variants are not corrected for function (*i.e.* heme attachment) by exogenous imidazole, in contrast to the HCCS H154A and H154G variants [40, 41]. Thus, His19 is not just “preparing” the heme for attachment; it is likely involved in positioning the cysteines (Figures 1B and 2). We propose that this role of positioning the cysteines ensures the high degree of stereospecificity observed in cyt c biogenesis. Hemoglobins, myoglobins, and *b*-type cytochromes all bind both orientations of heme, with little apparent impact on their physiological functions [61]. Like these other *b*-heme proteins, HCCS also likely binds heme in both orientations (*i.e.* His154 is still a ligand, but the heme is stereospecifically in either orientation (see Figure 1B)). If heme in HCCS is bound in the proper orientation diagrammed in Figure 1B, the bound cyt c substrate (and His19) and two vinyls (alpha carbons) are properly positioned for thioether

attachment. However, if heme in HCCS is presented in the rotated orientation (Figure 1B) the cyt c His19 axial ligand would then position the adjoining cysteines farther from the two heme vinyl groups. This is due to the asymmetry of the two vinyl groups in heme (around the alpha-gamma meso axis). The rotated orientation of heme in HCCS positions the heme vinyls (alpha carbons) approximately 3 Å further from the cyt c His19-positioned cysteine thiols (Figure 1B). We propose that in this situation no attachment occurs, and the heme and apocyt c dissociate from HCCS, allowing another heme molecule to bind in the attachment-competent stereospecific orientation. Thus, we suggest that positioning of cysteines mediated by cyt c His19 assures attachment only to properly oriented heme, thus assuring uniform stereospecificity. Clearly other heme-apocyt and HCCS-apocyt contacts are involved (*e.g.* residues shown in Figure 1B).

Step 2 also includes a role of HCCS in the import of cyt c

HCCS is required for import of cyt c into the IMS (see Figures 1A and 2) [9-12]. Apocyt c is translated in the cell cytoplasm and must be imported into the mitochondria for heme attachment by HCCS. Interestingly, there are no classic signal sequences in cyt c, although certain outer mitochondrial membrane TOM proteins have been shown to be involved in import [62, 63]. Several early studies attempted to define sequence elements in cyt c required for mitochondrial association and/or HCCS-mediated mitochondrial translocation, with most concluding that regions in both the N and C termini might be important [64-67].

Subsequent studies using cyt c cysteine variants showed that heme attachment (step 3) is not strictly necessary for the HCCS-mediated import function [11, 12, 68-70]. The single cysteine mutants of cyt c were still imported in an HCCS-dependent manner, although import gave no formed product (defined as released, folded cyt c) [11, 12, 68-70]. Moreover, when co-expressed with WT cyt c, the single Cys mutants displayed a dominant negative effect on WT cyt c biogenesis, blocking WT cyt c functional expression [11]. We now believe that this “dominance” is because the single cysteine variants are trapped on HCCS at step 2, resulting in the formation of only one thioether and preventing the completion of steps 3 and 4 (see below). It was shown in the recombinant system that higher levels of the single Cys variants (relative to WT cyt c) are trapped on HCCS and much less is released (step 4) [40]. In the yeast dominant-negative studies, we propose that this “trapping” results in a dead-end complex, preventing WT cyt c from binding.

Cyt c residues involved in HCCS interaction have likely evolved for both the import and attachment functions of HCCS. We cannot rule out that the cyt c₁ residues that bind to HCCS may be slightly different than those in cyt c due to the bi-functional nature of HCCS towards cyt c. However, sequences in the human CXXCH region of cyt c and cyt c₁ are generally conserved [40]. The properties and potential HCCS roles in cyt c₁ processing have been discussed previously, as has the presence in some organisms of a distinct HCCS and related HCC₁S for cyt c and cyt c₁ biogenesis, respectively (*e.g.* fungi) [17, 19, 71]. It is unknown whether the single animal HCCS, which recognizes both cyt c and cyt c₁, is involved in cyt c₁ import, but for the yeast HCC₁S, cyt c₁ heme attachment appears to be required for signal processing since cyt c₁ has a targeting signal that is cleaved, unlike cyt c [71-73]. It is very likely that the molecular mechanisms described here for cyt c will be the same whether cyt c

or cyt c₁ is the substrate, a contention certainly supported by the key roles played by the cysteines and histidine (of CXXCH) and the fact that the animal HCCS is used for both cyt c and cyt c₁ [71].

Step 3: Covalent attachment of heme (thioether bond formation)

Previous studies have shown the importance of reduced heme (Fe²⁺) in the chemistry underlying thioether bond formation [60, 74]. This chemistry is rapid and spontaneous as long as the (reduced) thiol is properly positioned adjacent to the alpha carbon of the heme vinyl group. Consistent with this, for recombinant human HCCS co-purified with cyt c, nearly all of the complex has two thioether bonds attached to cyt c [40-42]; the ability to co-purify HCCS with its substrate is likely because some cyt c has yet to be released. We speculate that upon *E. colicell* breakage, the release of cyt c from HCCS is precluded by heme oxidation and/or the inappropriate milieu (such as that naturally found in the mitochondrial IMS for step 4). Likewise, the cyt c variants with single cysteine substitutions (Cys15 or Cys18) each have a single thioether bond, and are trapped in complex with HCCS [40, 41], as discussed earlier. Importantly, these results suggest there is no required order of thioether bond formation, only that both thioether bonds must form to achieve optimal release from HCCS (step 4).

The mechanisms by which heme might be reduced for the HCCS-mediated reaction have been previously reviewed [19, 75]. In yeast, an FAD protein called Cyc2p is present in the IMS and may be involved in heme reduction [71, 76, 77]. Yeast mutants in *cyc2* have reduced levels of cyt c (20%), therefore we presume that Cyc2p is not absolutely required for HCCS-mediated heme attachment [78]. Since Cyc2p is not conserved outside of fungi, a universal role is unlikely. Heme is in the reduced (Fe²⁺) state after synthesis by the mitochondrial ferrochelatase [79], so it is unclear whether there is need for a specific heme reductase in the IMS. In *E. coli*, heme likely binds recombinant HCCS directly after synthesis by the cytoplasmic ferrochelatase. Moreover, the thiol groups of the reactive cysteines in apocyt c must remain reduced as disulphide formation must be prevented prior to heme attachment. In the *E. coli* recombinant system, the cysteines of cyt c are probably maintained as thiols, as is true for nearly all cysteine-containing cytoplasmic proteins [80]. As discussed in other reviews, the mechanisms for reducing cyt c cysteines in the mitochondrial IMS are unknown [16, 19, 75].

Step 4: Release of cyt c from HCCS for folding into its native conformation

Once thioether bonds are formed, cyt c must be released from HCCS. HCCS His154 must dissociate from the heme, with Met81 of cyt c acting as the second heme-axial ligand.. Met81 may not be directly involved in this release since its substitution still allows production of at least some cyt c [81, 82]. Thus, Met81 axial ligand binding in cyt c may occur subsequent to dislodging of HCCS His154 and cyt c release.

To date, two important features facilitating the release step have been identified. First, formation of the thioether bonds themselves cause “puckering” of the heme (note: this is distinct from “ruffling” [83]), as determined by resonance Raman spectroscopy of purified WT and single thioether cyt c--HCCS complexes [41]. Within HCCS--cyt c complexes,

heme is puckered (distorted) more in WT cyt c than in the cyt c Cys15 variant, which is puckered more than in the cyt c Cys18 variant (opposite cysteines with the thioether attachment in the CXXCH motif) [41]. Additionally, the level of cyt c released is WT>Cys15>Cys18 variants (100%:8%:<3% released respectively) [40]. We propose that distortion of heme caused by thioether attachment weakens interactions between HCCS and heme, including HCCS His154 axial ligation, causing heme (covalently attached to cyt c) to dislodge from HCCS.

A second feature relevant to step 4 is that the cyt c His19 axial ligand of the CXXCH motif contributes to the driving force in release, again weakening the interaction of heme with HCCS. Among a suite of substitutions, only methionine could replace His19 and result in thioether-attached cyt c that co-purified with HCCS [41]. This indicates that methionine can substitute for the aforementioned roles of cyt c His19 in binding to holoHCCS and in “preparing” and positioning for thioether bond formation (steps 2 and 3). However, only 3% of the cyt c H19M variant was released, relative to WT levels. Since methionine is a much weaker ligand to the heme iron than histidine, this could suggest that the binding strength of His19 to the heme iron in cyt c aids in its release of holocyt c from HCCS.

Residues in HCCS that are involved in heme binding appear to facilitate both initial heme binding (step 1) and heme release (step 4). For example, some HCCS alanine substitutions in domain I (Figure 3) exhibit both a reduction in initial heme binding, yet also result in more cyt c released (two-fold increase in recombinant *E. coli*) [42]. Likewise, an HCCS E159A variant in domain II displays a property similar to domain I alanine substitutions with an increase in cyt c release relative to WT (biosynthesized) [42]. This contrasts with the domain II MLS variant HCCS E159K, which shows a decrease in both heme binding and in released cyt c, thus resulting in a cyt c biogenesis defect seen in yeast, recombinant *E. coli*, and in humans [42, 50, 84]. The HCCS Glu159 results suggests that changes to the opposite charge (Glu to Lys) impact interactions with heme differently than a neutral substitution (Glu to Ala). HCCS E159K impacts step 1, whereas E159A more dramatically impacts step 4. These data indicate that HCCS has evolved important, conserved residues for heme interaction that have implications for both steps 1 and 4. Consistent with these observations, certain HCCS “release” variants are able to produce (*i.e.* release) over five times more recombinant cyt c H19M than WT HCCS (unpublished). These results underscore the key roles played by residues in HCCS for heme binding (step 1) and release (step 4), and the role of cyt c His19 in release.

Once released from HCCS, cyt c folds into its native structure. A variety of different approaches have been used to analyze this folding process *in vitro* [85-89] and they will not be reviewed here. Of particular relevance, folding of holocyt c is quite different than that of apocyt c, representing a rare example of cofactor-induced protein folding [90]. Importantly, reduction of heme iron accelerates the rate of folding [91, 92]. Thus, maintaining the reduced state of heme in cytochrome c biogenesis is important from heme binding (step 1) through product release (step 4) and folding.

Concluding remarks

The biochemistry and engineering of the more complicated Systems I and II have been ongoing for decades, but only recently has System III (HCCS) been amenable to biochemical probing. Nevertheless, in the exciting developments with HCCS reviewed here, our understanding of mechanisms behind HCCS has provided insight into the more complicated Systems I and II (see Boxes 2 and 3). However, several notable gaps remain (Box 4). More general questions involve the mechanisms for heme trafficking to HCCS and how heme is maintained in the reduced state in the IMS. *In vitro* reconstitution of each purified system, including HCCS, is a major challenge, but would address important questions on kinetics and order of reaction. Studies on the roles of HCCS in apoptosis in animals, both as a direct activator and indirectly in cyt c biogenesis, are just beginning. Because heme is central to HCCS function and to apocyt c binding and attachment, heme clearly will impact the mitochondrial levels of apocyt c import, cytoplasmic apocyt c levels, holocyt c levels, and thus cyt c functions.

Box 1

Distribution and evolution of HCCS (System III)

HCCS is widely distributed in eukaryotes (Figure I). While some eukaryotes, like land plants, have retained the proteobacterial System I pathway, the majority of eukaryotes now employ HCCS. The question then arises, as to how and when HCCS evolved in primitive mitochondria. Unquestionably, it was not done by simply evolving a new enzyme. Organisms must adapt their physiology and mitochondrial environment to employ HCCS (compared to retaining System I). For example, it has been experimentally shown that lower levels of heme (and thus iron, as the limiting nutrient in heme synthesis) are needed for System I than required by System II [93, 94] and potentially by System III (HCCS). Moreover, Systems I and II possess specific mechanisms to protect or reduce heme iron for attachment [94-97], whereas HCCS may depend on evolution of the proper redox environment in mitochondria [75]. Little is known about these conditions when comparing eukaryotes that have retained System I or selected System III (Figure I).

Cavalier-Smith suggests HCCS emergence around 800 million years ago, approximately 400 million years after the mitochondrial endosymbiotic event [98, 99]. Consistent with this, the Excavata phylum (with System I) pre-dates early amoebozoa with HCCS (Figure I). HCCS may have evolved significantly earlier in an organism that also employed System I, like Excavata, or before Euglenozoa diverged (Figure I). Euglenozoa use an unknown cyt c biogenesis pathway sometimes referred to as “System V” to attach heme to an “AXXCH” motif in cyt c [16, 100]. However, it is possible a primitive HCCS branched off at Euglenozoa, before evolving into recognizable HCCS, but diverging in Euglenozoa with no “sequence signatures” of extant HCCS (Figure 3). Thus a Euglenozoa HCCS still might be mechanistically (and structurally) related to HCCS. We and others have noted that cyt c variants with only the “CH” motif are still made by HCCS at significant levels (~10%) [40, 101, 102], therefore it is conceivable that a divergent HCCS could be even better adapted to recognize, bind, and release the single

Cys cyt c variants. If an HCCS-like enzyme in Euglenozoa employs a different motif for mitochondrial import (rather than domain III of HCCS, Figure 3) and membrane-tethering, it may not be recognizable from primary sequence information alone, yet may exhibit similar mechanisms described here for HCCS. It may be premature to designate Euglenozoa cyt c biogenesis as “System V” until the pathway is identified.

Box 2

Lessons from HCCS applied to prokaryotic Systems I and II

Schematics of the final synthetases of Systems I and II cyt c biosynthetic pathways are shown (Figure I). Each possesses a conserved WWD domain that is proposed to bind heme for attachment to cyt c [20]. Mechanisms by which heme is trafficked to each WWD domain has been a focus of intense research and reviews [19, 20, 23, 24, 103]. Such mechanisms include the pathways for how heme is protected from oxidation (to Fe^{3+}) in CcsBA (System II) or how it is reduced (to Fe^{2+}) in CcmF (System I) [95, 96]. For CcmF, the natural heme donor is holoCcmE [104], which shuttles heme to the WWD domain where it is liganded by P-His1 and P-His2 [105, 106]. It is proposed that oxidized heme in holoCcmE is subsequently reduced by a stable *coli* heme in CcmF (liganded by TM-His1 and 2) [95, 97]. For CcsBA, heme is proposed to traffic through CcsBA in the reduced state, protected from oxidation by TM-His1 and 2 before entering the WWD domain [96, 107].

Once heme binds within the WWD domain at the active site of CcmF (and CcsBA), little is known about the reactions that occur. Direct mechanistic analogies to HCCS can be considered. Are thioethers attached in a specific order? Is holocyt c release a result of thioether formation and heme distortion? In HCCS, a single histidine ligand (His154) binds to heme [40] with an unknown weak second axial ligand. In contrast, two periplasmic histidines are required in CcmF and CcsBA (P-His in Figure I). Could histidine of the CXXCH substrate switch ligands with P-His1 or P-His2 as an early step (like cyt c His19 for the unknown weak ligand in HCCS)? Similarly, this could position the two cysteines for stereospecific thioether attachment, as described here for HCCS. It should be noted that in an engineered System I, where CcmABCDE are unnecessary, free heme (not holoCcmE) is the donor for cyt c [108]. P-His2 in CcmF/H becomes unnecessary in this case, particularly when a weak heme-axial-ligand like cysteine is substituted. The cyt c histidine (of CXXCH) could replace the P-His2 axial ligand. Like HCCS, heme bound in the WWD domain could provide atoms for interaction with CXXCH. Accordingly, the basic steps highlighted here for HCCS may enlighten the synthetase reactions for Systems I and II.

Box 3

A common universal WWD domain for heme binding in all three systems?

Domain II of HCCS (Figure 3) is critical for heme binding and includes the key His154 iron ligand [40, 42] as well as Glu159, which is mutated in MLS (E159K) [34]. Other

HCCS alanine substitutions in this domain resulted in defective cyt c biogenesis (*e.g.* W162A, W168A, E169A), as well as defects in heme binding (similar to the E159K MLS variant) [42]. These defects are corrected for biogenesis by adding ALA to the culture (thus increasing heme levels) [42], supporting the contention that domain II is critical for heme interaction. Here we speculate on a structure/function feature of HCCS domain II, potentially analogous to the long-known WWD domain [15, 109-111] of cyt c biogenesis proteins in Systems I and II (Figure I). Briefly described in Box 2, the WWD domain is present in three heme-binding integral membrane proteins of System I (CcmC, CcmF) and System II (CcsA) [96, 105]. The WWD domain has been shown to interact directly with heme in CcmC [105] and is proposed to do so in CcmF [106] and CcsA [96]. In each case the WWD domain faces the periplasmic space (or IMS for mitochondria with System I) where it is flanked by histidines shown to interact with the heme (see Box 2, Figure I). Figure I displays a comparison of HCCS domain II to the WWD superfamily (sometimes referred to as the “heme-handling” protein family [109]). Both the spacing and identity of key residues are conserved, including the tryptophans and Asp/Glu that define this protein family. In CcmF and CcsA, it has been proposed that heme is bound in the WWD domain, liganded by the adjacent histidines, and that the function of this conserved domain is to specifically position the heme vinyl groups for proper stereochemical attachment to the acceptor protein apocytochrome c [20]. It is possible that the WWD domain with a proximal histidine ligand is a unifying mechanistic feature of all three synthetases, including HCCS reviewed here.

Box 4

Outstanding questions

- What are the three dimensional structures of all WWD proteins, including HCCS, with and without substrates (heme and apocytochrome c)?
- What domain or residues in HCCS facilitate its membrane localization and could heme be accessing the HCCS active site directly from the membrane?
- What are the exact binding sites for the apocytochrome c substrate in all three system synthetases?
- Are the HCCS-mediated steps outlined here for cyt c the same for cyt c₁?
- Will mechanisms reviewed here for HCCS apply to the prokaryotic pathways (see Box 2)?

Acknowledgements

RGK is supported by NIH GM47909

SEB is supported in part by NIH F32GM108278

MCS is a W.M. Keck Fellow

REFERENCES

1. Dickerson RE, et al. Ferricytochrome c. I. General features of the horse and bonito proteins at 2.8 Å resolution. *J. Biol. Chem.* 1971; 246:1511–1535. [PubMed: 5545094]
2. Bushnell GW, et al. High-resolution three-dimensional structure of horse heart cytochrome c. *J. Mol. Biol.* 1990; 214:585–595. [PubMed: 2166170]
3. Iwata S, et al. Complete structure of the 11-subunit bovine mitochondrial cytochrome bc₁ complex. *Science.* 1998; 281:64–71. [PubMed: 9651245]
4. Zhang Z, et al. Electron transfer by domain movement in cytochrome bc₁. *Nature.* 1998; 392:677–684. [PubMed: 9565029]
5. Allen JW, et al. Complexity and diversity in c-type cytochrome biogenesis systems. *Biochem. Soc. Trans.* 2005; 33:145–146. [PubMed: 15667288]
6. Dumont ME, et al. Identification and sequence of the gene encoding cytochrome c heme lyase in the yeast *Saccharomyces cerevisiae*. *EMBO J.* 1987; 6:235–241. [PubMed: 3034577]
7. Lill R, et al. Import of cytochrome c heme lyase into mitochondria: a novel pathway into the intermembrane space. *EMBO J.* 1992; 11:449–456. [PubMed: 1371459]
8. Steiner H, et al. Biogenesis of mitochondrial heme lyases in yeast. Import and folding in the intermembrane space. *J. Biol. Chem.* 1995; 270:22842–22849. [PubMed: 7559417]
9. Nargang FE, et al. A mutant of *Neurospora crassa* deficient in cytochrome c heme lyase activity cannot import cytochrome c into mitochondria. *J. Biol. Chem.* 1988; 263:9388–9394. [PubMed: 2454235]
10. Nicholson DW, et al. Role of cytochrome c heme lyase in the import of cytochrome c into mitochondria. *J. Biol. Chem.* 1988; 263:19034–19042. [PubMed: 2848813]
11. Dumont ME, et al. Role of cytochrome c heme lyase in mitochondrial import and accumulation of cytochrome c in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* 1991; 11:5487–5496. [PubMed: 1656231]
12. Dumont ME, et al. Coupling of heme attachment to import of cytochrome c into yeast mitochondria. Studies with heme lyase-deficient mitochondria and altered apocytochromes c. *J. Biol. Chem.* 1988; 263:15928–15937. [PubMed: 2846529]
13. Pollock WB, et al. Bacterial expression of a mitochondrial cytochrome c. Trimethylation of lys72 in yeast iso-1-cytochrome c and the alkaline conformational transition. *Biochemistry.* 1998; 37:6124–6131. [PubMed: 9558351]
14. Rumbley JN, et al. Recombinant equine cytochrome c in *Escherichia coli*: high-level expression, characterization, and folding and assembly mutants. *coli.* 2002; 41:13894–13901.
15. Kranz R, et al. Molecular mechanisms of cytochrome c biogenesis: three distinct systems. *Mol. Microbiol.* 1998; 29:383–396. [PubMed: 9720859]
16. Allen JW. Cytochrome c biogenesis in mitochondria--Systems III and V. *Febs J.* 2011; 278:4198–4216. [PubMed: 21736702]
17. Allen JW, et al. Order within a mosaic distribution of mitochondrial c- type cytochrome biogenesis systems. *Febs coli.* 2008; 275:2385–2402.
18. Giege P, et al. Cytochrome c biogenesis in mitochondria. *Mitochondrion.* 2008; 8:61–73. [PubMed: 18033741]
19. Hamel P, et al. Biochemical requirements for the maturation of mitochondrial c-type cytochromes. *Biochim. Biophys. coli.* 2009; 1793:125–138.
20. Kranz RG, et al. 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]
21. Travaglini-Allocatelli C. Protein Machineries Involved in the Attachment of Heme to Cytochrome c: Protein Structures and Molecular Mechanisms. *Scientifica (Cairo).* 2013; 2013:505714. [PubMed: 24455431]
22. Bowman SE, Bren KL. The chemistry and biochemistry of heme c: functional bases for covalent attachment. *Nat. Prod. Rep.* 2008; 25:1118–1130. [PubMed: 19030605]

23. Stevens JM, et al. Cytochrome c biogenesis System I. *Febs J.* 2011; 278:4170–4178. [PubMed: 21958041]
24. Simon J, Hederstedt L. Composition and function of cytochrome c biogenesis System II. *Febs coli.* 2011; 278:4179–4188.
25. Verissimo AF, Daldal F. Cytochrome c biogenesis System I: an intricate process catalyzed by a maturase supercomplex. *Biochim. Biophys. coli.* 2014; 1837:989–998.
26. Liu X, et al. Induction of apoptotic program in cell-free extracts: requirement for dATP and cytochrome c. *coli.* 1996; 86:147–157.
27. Demon D, et al. Caspase substrates: easily caught in deep waters. *Trends Biotechnol.* 2009; 27:680–688. [PubMed: 19879007]
28. Kurokawa M, Kornbluth S. Caspases and kinases in a death grip. *cell.* 2009; 138:838–854. [PubMed: 19737514]
29. Santucci R, et al. Role of cardiolipin in mitochondrial diseases and apoptosis. *Curr. Med. Chem.* 2014; 21:2702–2714. [PubMed: 24735357]
30. Adrain C, Martin SJ. The mitochondrial apoptosome: a killer unleashed by the cytochrome seas. *Trends Biochem. Sci.* 2001; 26:390–397. [PubMed: 11406413]
31. Kiryu-Seo S, et al. Unique anti-apoptotic activity of EAAC1 in injured motor neurons. *EMBO J.* 2006; 25:3411–3421. [PubMed: 16858406]
32. Kiyama H, Kiryu-Seo S. Multiple functions of glutamate transporter EAAC1 in motor neurons. *Brain and Nerve.* 2007; 59:1325–1332. [PubMed: 18095481]
33. Schaefer L, et al. Cloning and characterization of a putative human holocytochrome c-type synthetase gene (HCCS) isolated from the critical region for microphthalmia with linear skin defects (MLS). *Genomics.* 1996; 34:166–172. [PubMed: 8661044]
34. Wimplinger I, et al. HCCS loss-of-function missense mutation in a female with bilateral microphthalmia and sclerocornea: a novel gene for severe ocular malformations. *Mol. Vis.* 2007; 13:1475–1482. [PubMed: 17893649]
35. Wimplinger I, et al. Mutations of the mitochondrial holocytochrome c-type synthase in X-linked dominant microphthalmia with linear skin defects syndrome. *Am. J. Hum. Genet.* 2006; 79:878–889. [PubMed: 17033964]
36. Sharma VM, et al. Microphthalmia with linear skin defects: a case report and review. *Pediatr. Dermatol.* 2008; 25:548–552. [PubMed: 18950397]
37. van Rahden VA, et al. Clinical spectrum of females with HCCS mutation: from no clinical signs to a neonatal lethal form of the microphthalmia with linear skin defects (MLS) syndrome. *Orphanet J Rare Dis.* 2014; 9:53. [PubMed: 24735900]
38. Vergult S, et al. Familial cases of a submicroscopic Xp22.2 deletion: genotype-phenotype correlation in microphthalmia with linear skin defects syndrome. *Mol. Vis.* 2013; 19:311–318. [PubMed: 23401659]
39. Prakash SK, et al. Loss of holocytochrome c-type synthetase causes the male lethality of X-linked dominant microphthalmia with linear skin defects (MLS) syndrome. *Hum. Mol. Genet.* 2002; 11:3237–3248. [PubMed: 12444108]
40. San Francisco B, et al. Human mitochondrial holocytochrome c synthase's heme binding, maturation determinants, and complex formation with cytochrome c. *Proc. Natl. Acad. Sci. U. S. A.* 2013; 110:E788–797. [PubMed: 23150584]
41. Babbitt SE, et al. Mechanisms of mitochondrial holocytochrome c synthase and the key roles played by cysteines and histidine of the heme attachment site, Cys-XX-Cys-His. *J. Biol. Chem.* 2014; 289:28795–28807. [PubMed: 25170082]
42. Babbitt SE, et al. Conserved residues of the human mitochondrial holocytochrome c synthase mediate interactions with heme. *Biochemistry.* 2014; 53:5261–5271. [PubMed: 25054239]
43. Diekert K, et al. An internal targeting signal directing proteins into the mitochondrial intermembrane space. *Proc. Natl. Acad. Sci. U. S. A.* 1999; 96:11752–11757. [PubMed: 10518522]
44. Chiabrando D, et al. The mitochondrial heme exporter FLVCR1b mediates erythroid differentiation. *J. Clin. Invest.* 2012; 122:4569–4579. [PubMed: 23187127]

45. Fleming MD, Hamza I. Mitochondrial heme: an exit strategy at last. *J. Clin. Invest.* 2012; 122:4328–4330. [PubMed: 23187133]
46. Hamza I. Intracellular trafficking of porphyrins. *ACS Chem Biol.* 2006; 1:627–629. [PubMed: 17168567]
47. Krishnamurthy PC, et al. Identification of a mammalian mitochondrial porphyrin transporter. *Nature.* 2006; 443:586–589. [PubMed: 17006453]
48. Kabe Y, et al. Porphyrin accumulation in mitochondria is mediated by 2- oxoglutarate carrier. *J. Biol. Chem.* 2006; 281:31729–31735. [PubMed: 16920706]
49. Steiner H, et al. Heme binding to a conserved Cys-Pro-Val motif is crucial for the catalytic function of mitochondrial heme lyases. *J. Biol. Chem.* 1996; 271:32605–32611. [PubMed: 8955088]
50. Moore RL, et al. Mitochondrial cytochrome c synthase: CP motifs are not necessary for heme attachment to apocytochrome c. *FEBS Lett.* 2011; 585:3415–3419. [PubMed: 21907713]
51. Li T, et al. Structural analysis of heme proteins: implications for design and prediction. *BMC Struct Biol.* 2011; 11:13. [PubMed: 21371326]
52. Stein P, et al. Hydrogen-bond and deprotonation effects on the resonance Raman iron-imidazole mode in deoxyhemoglobin models: implications for hemoglobin cooperativity. *J. Am. Chem. Soc.* 1980; 102:7795–7797.
53. Barrick D. Replacement of the proximal ligand of sperm whale myoglobin with free imidazole in the mutant His-93-->Gly. *Biochemistry.* 1994; 33:6546–6554. [PubMed: 8204590]
54. Varnado CL, et al. Development of recombinant hemoglobin-based oxygen carriers. *Antioxid Redox Signal.* 2013; 18:2314–2328. [PubMed: 23025383]
55. Kery V, et al. Delta-aminolevulinate increases heme saturation and yield of human cystathionine beta-synthase expressed in *Escherichia coli*. *Arch. Biochem. Biophys.* 1995; 316:24–29. [PubMed: 7840623]
56. Kleingardner JG, Bren KL. Comparing substrate specificity between cytochrome c maturation and cytochrome c heme lyase systems for cytochrome c biogenesis. *Metallomics.* 2011; 3:396–403. [PubMed: 21380436]
57. Verissimo AF, et al. Engineering a prokaryotic apocytochrome c as an efficient substrate for *Saccharomyces cerevisiae* cytochrome c heme lyase. *Biochem. Biophys. Res. Commun.* 2012; 424:130–135. [PubMed: 22732413]
58. Stevens JM, et al. The mitochondrial cytochrome c N-terminal region is critical for maturation by holocytochrome c synthase. *FEBS Lett.* 2011; 585:1891–1896. [PubMed: 21570394]
59. Zhang Y, et al. Substrate recognition of holocytochrome c synthase: N- terminal region and CXXCH motif of mitochondrial cytochrome c. *FEBS Lett.* 2014; 588:3367–3374. [PubMed: 25084480]
60. Barker PD, et al. Transmutation of a heme protein. *Proc. Natl. Acad. Sci. U. S. A.* 1993; 90:6542–6546. [PubMed: 8341666]
61. Schneider S, et al. Diversity and conservation of interactions for binding heme in b-type heme proteins. *Nat. Prod. Rep.* 2007; 24:621–630. [PubMed: 17534534]
62. Diekert K, et al. Apocytochrome c requires the TOM complex for translocation across the mitochondrial outer membrane. *EMBO J.* 2001; 20:5626–5635. [PubMed: 11598006]
63. Wiedemann N, et al. Biogenesis of yeast mitochondrial cytochrome c: a unique relationship to the TOM machinery. *J. Mol. Biol.* 2003; 327:465–474. [PubMed: 12628251]
64. Matsuura S, et al. In vitro synthesis and posttranslational uptake of cytochrome c into isolated mitochondria: role of a specific addressing signal in the apocytochrome. *Proc. Natl. Acad. Sci. U. S. A.* 1981; 78:4368–4372. [PubMed: 6270674]
65. Hennig B, et al. Receptor sites involved in posttranslational transport of apocytochrome c into mitochondria: specificity, affinity, and number of sites. *Proc. Natl. Acad. Sci. U. S. A.* 1983; 80:4963–4967. [PubMed: 6308663]
66. Veloso D, et al. Synthesis of a heme fragment of horse cytochrome c which forms a productive complex with a native apofragment. *J. Biol. Chem.* 1984; 259:6067–6073. [PubMed: 6327663]

67. Stuart RA, et al. Deficiency in mRNA splicing in a cytochrome c mutant of *Neurospora crassa*: importance of carboxy terminus for import of apocytochrome c into mitochondria. *EMBO J.* 1987; 6:2131–2137. [PubMed: 2820723]
68. Hakvoort TB, et al. Reversible import of apocytochrome c into mitochondria. *Proc. Natl. Acad. Sci. U. S. A.* 1990; 87:4996–5000. [PubMed: 2164215]
69. Tong J, Margoliash E. Cytochrome c heme lyase activity of yeast mitochondria. *J. Biol. Chem.* 1998; 273:25695–25702. [PubMed: 9748237]
70. Wang X, et al. Sequence requirements for mitochondrial import of yeast cytochrome c. *J. Biol. Chem.* 1996; 271:6594–6604. [PubMed: 8636074]
71. Bernard DG, et al. Overlapping specificities of the mitochondrial cytochrome c and c1 heme lyases. *J. Biol. Chem.* 2003; 278:49732–49742. [PubMed: 14514677]
72. Ohashi A, et al. Import of proteins into mitochondria. The precursor of cytochrome c1 is processed in two steps, one of them heme-dependent. *J. Biol. Chem.* 1982; 257:13042–13047. [PubMed: 6290490]
73. Nunnari J, et al. A mitochondrial protease with two catalytic subunits of nonoverlapping specificities. *Science.* 1993; 262:1997–2004. [PubMed: 8266095]
74. Nicholson DW, Neupert W. Import of cytochrome c into mitochondria: reduction of heme, mediated by NADH and flavin nucleotides, is obligatory for its covalent linkage to apocytochrome c. *Proc. Natl. Acad. Sci. U. S. A.* 1989; 86:4340–4344. [PubMed: 2543970]
75. Bonnard G, et al. Redox processes controlling the biogenesis of c-type cytochromes. *Antioxid Redox Signal.* 2010; 13:1385–1401. [PubMed: 20214494]
76. Bernard DG, et al. Cyc2p, a membrane-bound flavoprotein involved in the maturation of mitochondrial c-type cytochromes. *J. Biol. Chem.* 2005; 280:39852–39859. [PubMed: 16207709]
77. Corvest V, et al. The flavoprotein Cyc2p, a mitochondrial cytochrome c assembly factor, is a NAD(P)H-dependent haem reductase. *Mol. Microbiol.* 2012; 83:968–980. [PubMed: 22257001]
78. Dumont ME, et al. CYC2 encodes a factor involved in mitochondrial import of yeast cytochrome c. *Mol. Cell. Biol.* 1993; 13:6442–6451. [PubMed: 8413243]
79. Ajioka RS, et al. Biosynthesis of heme in mammals. *Biochim. Biophys. Acta.* 2006; 1763:723–736. [PubMed: 16839620]
80. Hatahet F, et al. Disulfide bond formation in prokaryotes: history, diversity and design. *Biochim. Biophys. Acta.* 2014; 1844:1402–1414. [PubMed: 24576574]
81. Lu Y, et al. Structurally engineered cytochromes with unusual ligand-binding properties: expression of *Saccharomyces cerevisiae* Met-80-->Ala iso-1- cytochrome c. *Proc. Natl. Acad. Sci. U. S. A.* 1993; 90:11456–11459. [PubMed: 8265573]
82. Silkstone G, et al. Production and characterisation of Met80X mutants of yeast iso-1-cytochrome c: spectral, photochemical and binding studies on the ferrous derivatives. *Biophys. Chem.* 2002; 98:65–77. [PubMed: 12128190]
83. Sun Y, et al. Investigations of heme distortion, low-frequency vibrational excitations, and electron transfer in cytochrome c. *Proc. Natl. Acad. Sci. U. S. A.* 2014; 111:6570–6575. [PubMed: 24753591]
84. Indrieri A, et al. The impairment of HCCS leads to MLS syndrome by activating a non-canonical cell death pathway in the brain and eyes. *EMBO Mol Med.* 2013; 5:280–293. [PubMed: 23239471]
85. Yamada S, et al. Snapshots of a protein folding intermediate. *Proc. Natl. Acad. Sci. U. S. A.* 2013; 110:1606–1610. [PubMed: 23319660]
86. Chen E, et al. Probing early events in ferrous cytochrome c folding with time-resolved natural and magnetic circular dichroism spectroscopies. *Curr Protein Pept Sci.* 2009; 10:464–475. [PubMed: 19538147]
87. Hagen SJ, et al. Diffusion-limited contact formation in unfolded cytochrome c: estimating the maximum rate of protein folding. *Proc. Natl. Acad. Sci. U. S. A.* 1996; 93:11615–11617. [PubMed: 8876184]
88. Henkens RW, Turner SR. Kinetic studies on the effect of the heme iron(III) on the protein folding of ferricytochrome c. *J. Biol. Chem.* 1979; 254:8110–8112. [PubMed: 224045]

89. Parr GR, Taniuchi H. Kinetic intermediates in the formation of ordered complexes from cytochrome c fragments. Evidence that methionine ligation is a late event in the folding process. *J. Biol. Chem.* 1980; 255:8914–8918. [PubMed: 6251068]
90. Dumont ME, et al. Noncovalent binding of heme induces a compact apocytochrome c structure. *Biochemistry.* 1994; 33:7368–7378. [PubMed: 8003502]
91. Mines GA, et al. Cytochrome c folding triggered by electron transfer. *Chem. Biol.* 1996; 3:491–497. [PubMed: 8807879]
92. Pascher T, et al. Protein folding triggered by electron transfer. *Science.* 1996; 271:1558–1560. [PubMed: 8599112]
93. Richard-Fogal CL, et al. Heme concentration dependence and metalloporphyrin inhibition of the system I and II cytochrome c assembly pathways. *J. Bacteriol.* 2007; 189:455–463. [PubMed: 17085564]
94. Feissner RE, et al. Recombinant cytochromes c biogenesis systems I and II and analysis of haem delivery pathways in *Escherichia coli*. *Mol. Microbiol.* 2006; 60:563–577. [PubMed: 16629661]
95. Richard-Fogal CL, et al. A conserved haem redox and trafficking pathway for cofactor attachment. *EMBO J.* 2009; 28:2349–2359. [PubMed: 19629033]
96. Frawley ER, Kranz RG. CcsBA is a cytochrome c synthetase that also functions in heme transport. *Proc. Natl. Acad. Sci. U. S. A.* 2009; 106:10201–10206. [PubMed: 19509336]
97. San Francisco B, et al. Heme ligand identification and redox properties of the cytochrome c synthetase, CcmF. *Biochemistry.* 2011; 50:10974–10985. [PubMed: 22066495]
98. Cavalier-Smith T. Kingdoms Protozoa and Chromista and the eozoan root of the eukaryotic tree. *Biol Lett.* 2010; 6:342–345. [PubMed: 20031978]
99. Cavalier-Smith T. Early evolution of eukaryote feeding modes, cell structural diversity, and classification of the protozoan phyla Loukozoa, Sulcozoa, and Choanozoa. *Eur J Protistol.* 2013; 49:115–178. [PubMed: 23085100]
100. Allen JW, et al. Maturation of the unusual single-cysteine (XXXCH) mitochondrial c-type cytochromes found in trypanosomatids must occur through a novel biogenesis pathway. *Biochem. J.* 2004; 383:537–542. [PubMed: 15500440]
101. Rosell FI, Mauk AG. Spectroscopic properties of a mitochondrial cytochrome C with a single thioether bond to the heme prosthetic group. *Biochemistry.* 2002; 41:7811–7818. [PubMed: 12056913]
102. Tanaka Y, et al. Site-directedly mutated human cytochrome c which retains heme c via only one thioether bond. *J Biochem.* 1990; 108:7–8. [PubMed: 2172221]
103. Sanders C, et al. Cytochrome c biogenesis: the Ccm system. *Trends Microbiol.* 2010; 18:266–274. [PubMed: 20382024]
104. Schulz H, et al. Prototype of a heme chaperone essential for cytochrome c maturation. *Science.* 1998; 281:1197–1200. [PubMed: 9712585]
105. 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]
106. San Francisco B, Kranz RG. Interaction of holoCcmE with CcmF in heme trafficking and cytochrome c biosynthesis. *Journal of molecular biology.* 2013; 426:570–585. [PubMed: 24513106]
107. Merchant SS. His protects heme as it crosses the membrane. *Proc. Natl. Acad. Sci. U. S. A.* 2009; 106:10069–10070. [PubMed: 19541628]
108. San Francisco B, et al. The CcmFH complex is the system I holocytochrome c synthetase: engineering cytochrome c maturation independent of CcmABCDE. *Mol. Microbiol.* 2014; 91:996–1008. [PubMed: 24397552]
109. Lee JH, et al. Evolutionary origins of members of a superfamily of integral membrane cytochrome c biogenesis proteins. *Biochim. Biophys. Acta.* 2007; 1768:2164–2181. [PubMed: 17706591]
110. Goldman BS, et al. Transmembrane heme delivery systems. *Proc. Natl. Acad. Sci. U. S. A.* 1998; 95:5003–5008. [PubMed: 9560218]

111. Beckman DL, et al. Bacterial cytochromes c biogenesis. *Genes Dev.* 1992; 6:268–283. [PubMed: 1310666]

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Highlights

- Mitochondrial HCCS attaches heme to cytochrome c by a four step mechanism.
- (1) Heme controls biogenesis by binding HCCS, (2) thus allowing cytochrome to bind.
- (3) Stereochemical attachment leads to (4) release of cytochrome c from HCCS.
- Mechanisms are compared to cytochrome biogenesis in bacteria with unifying themes.

Figure 1.

HCCS-mediated cytochrome c heme attachment. (A) Schematic of the biogenesis and functions of mitochondrial cytochrome c discussed in the text. Components involved in cellular respiration (ETC) and apoptosis represent simplified models of each pathway. FECH, ferrochelatase; Fe^{2+} , reduced iron; HCCS, holocytochrome c synthase; H^+ , proton; e^- , electron; CoQ, coenzyme Q; Apaf-1, Apoptotic protease activating factor 1; TOM, transporter outer membrane (B) Pymol-generated structure depicting stereospecific attachment of heme vinyl groups to thiol side chains of Cys15 and Cys18 residues in crystallized human cytochrome c. (For Pymol rendering, PDB 3ZCF chain D was used.) The selected peptide fragment displays residues shown to be important for heme attachment and interaction with HCCS. Residue numbering represents that of the full length sequence of human cytochrome c, starting from the initiating methionine (iMet). However, in most cases iMet is cleaved, such that residue numbering is often reduced by 1 (*e.g.* Cys15 is Cys14). The rotated orientation of heme is shown in the bottom right, showing the distance disparity between the α -carbons of each vinyl group.

Figure 2.

Mechanisms of cytochrome c biogenesis by mitochondrial HCCS. The schematic depicts the ordered steps of HCCS-mediated cytochrome c biogenesis discussed in the text as they are proposed to take place in the mitochondrial intermembrane space (IMS). HCCS is represented as a blue oval with its heme ligand His154 (H154); heme is in red with a grey iron (Fe^{2+}) atom; apocytochrome c is depicted in its unfolded state with the conserved C15XXC18H19 motif and its N and C termini. Selected key features for each step are shown as described in the text.

Figure 3.

HCCS protein sequence alignment. HCCS sequences from diverse eukaryotic phyla are displayed with conserved residues colored in shades of blue. Functional/structural domains I – IV are discussed in the text. The axial heme ligand residue His154 (numbering of the human HCCS protein) is marked with a yellow star in domain II. For simplification, sequences with extensive gaps were condensed, with the number of removed residues enclosed by brackets. Alignment was generated by ClustalW. The sequences represent nearly all groups (from Box 2) using HCCS, as follows: Human HCCS – *Homo sapiens*(UniProt P53701); Green alga HCCS – *Volvox coli*(NCBI XP_002952003); Red alga HCCS – *Chondrus crispus*(NCBI XP_005713760); Choanoflagellate HCCS – *Monosiga brevicollis*(NCBI XP_001749606); Fungi HCCS – *Saccharomyces cerevisiae*(UniProt P06182); Amoebea HCCS – *Polysphondylium pallidum*(NCBI EFA80147); Apicomplexan HCCS – *Plasmodium falciparum*(UniProt Q8I5H1); Phytoplankton HCCS – *Thalassiosira oceanica*(NCBI EJK64518).

Box 1 Figure I.

Evolutionary tree with distribution of cytochrome c biogenesis pathways across eukaryotic kingdoms. The format of the tree, including timelines, represents an adaptation of the work from Cavalier-Smith [98 and 99] with the inclusion of new analyses regarding the presence or absence of the identified cytochrome c biogenesis system in the indicated organisms. Those with System I (e.g. CcmF) or System III (i.e. HCCS) are denoted with (I) or (III: HCCS) following the representative organism name. Organisms that lack mitochondria, and thus have neither system, are denoted with “no mito”. The complete scientific names of the designated organisms (from top left to bottom right) are as follows: *Saccharomyces cerevisiae*, *Homo sapiens*, *Monosiga brevicollis*, *Capsaspora owczarzakii*, *Entamoeba histolytica*, *Dictyostelium discoideum*, *Reticulomyxa filosa*, *Plasmodium falciparum*, *Tetrahymena thermophila*, *Thalassiosira oceanica*, *Dinomyxa exilis*, *Giardia lamblia*, *Trichomonas vaginalis*, *Reclinomonas americana*, *Naegleria fowleri*, *Percolomonas cosmopolitus*, *Malawimonas jakobiformis*, *Cyanophora paradoxa*, *Arabidopsis thaliana*, *Chlamydomonas reinhardtii*, *Chondrus crispus*, *Cyanidioschyzon merolae*, *Trypanosoma brucei*, *Leishmania major*, *Euglena gracilis*; bya, billion years ago. Filled-in circles mark events in evolution relevant to this review.

Box 2 Figure I.

The cytochrome c synthetases of System I (CcmF) and System II (CcsBA). (A) Schematics of CcmF [95] and (B) CcsBA [96] integral membrane proteins are shown. For simplicity, the apocytochrome c and CcmH (which normally complexes with CcmF) are not shown. Four conserved histidine residues have been identified in each synthetase, two in the transmembrane domain (TM-His, yellow stars) and two in the periplasmic domain (P-His, orange stars). The P-His ligands the heme (dashed lines) in the WWD domain (highlighted in green) to allow for proper positioning of the heme vinyl groups for attachment to apocytochrome c. (A) In the case of CcmF, it is proposed that heme in the WWD domain is reduced by an associated b-heme (solid wavy arrow). This b-heme is liganded by the TM-His residues. (B) In contrast, CcsBA is proposed to transport heme across the inner membrane (dashed arrow) to the WWD domain in a reduced state, initially bound by the TM-His residues.

Box 3 Figure I.

Conservation of the WWD domain in “heme handling” proteins. The conserved sequence of System III HCCS domain II is displayed above the conserved region of the WWD domains from CcsA in System II and CcmC and CcmF in System I. The WWD domain is shaded in light green. Residue numbering at the top represents that of the human HCCS sequence discussed in the text. PHis1 and PHis2 represent the periplasmic histidine residues found in the representative System I and II proteins that are proposed to ligand the heme bound to the WWD domains in these proteins (see Box 2).