Molecular Basis Behind Inability of Mitochondrial Holocytochrome c Synthase to Mature Bacterial Cytochromes

DEFINING A CRITICAL ROLE FOR CYTOCHROME c α HELIX-1*

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Mitochondrial holocytochrome c synthase (HCCS) is required for cytochrome c (cyt c) maturation and therefore respiration. HCCS efficiently attaches heme via two thiethioether to CXXCH of mitochondrial but not bacterial cyt c even though they are functionally conserved. This inability is due to residues in the bacterial cyt c N terminus, but the molecular basis is unknown. Human cys c with deletions of single residues in α helix-1, which mimic bacterial cyt c, are poorly matured by human HCCS. Focusing on ΔM13 cyt c, we co-purified this variant with HCCS, demonstrating that HCCS recognizes the bacterial-like cytochrome. Although an HCCS-WT cyt c complex contains two covalent links, HCCS-ΔM13 cyt c contains only one thiether attachment. Using multiple approaches, we show that the single attachment is to the second thiole of C15SQC18H, indicating that α helix-1 is required for positioning the first cysteine for covalent attachment, whereas the histidine of CXXCH positions the second cysteine. Modeling of the N-terminal structure suggested that the serine residue (of CSQCH) would be anchored where the first cysteine should be in the CXXCH motif, indicating that the second cysteine is positioned by the conserved C15SQC18H heme attachment motif (Fig. 2, blue asterisks), a residue at the sequence position equivalent to Lys8 and Ile10 found in human cyt c (see Fig. 2A, blue asterisks). Furthermore, following an important Phe residue found in human (Phe31) and some bacterial cytochromes (21, 27), a residue at the sequence position corresponding to Met13 in human cyt c (see Fig. 2A) is missing in certain bacterial cytochromes. Substitution of the glutamate residues and insertion of an alanine residue at the corresponding positions in Rhodobacter capsulatus cyt c2 resulted in its maturation by HCCS (27, 28), thereby suggesting a critical role of the α helix-1 adjacent to the CXXCH motif in mediating interactions between the substrate and enzyme.

Cytochrome c (cyt c)3 biogenesis involves the covalent attachment of a heme cofactor to two cysteine residues found in the conserved CXXCH motif where its His residue acts as an axial ligand to the heme iron (1–3). Although the mitochondrial-type monoheme cyt c is structurally and functionally conserved in prokaryotes and eukaryotes (1, 3, 4), the three systems responsible for its biogenesis are distinct (5). For most eukaryotes, cyt c maturation (i.e. heme attachment) occurs in the mitochondrial intermembrane space, catalyzed by a single enzyme known as holocytochrome c synthase (HCCS) or System III (6–8). Fig. 1 summarizes a four-step model for biogenesis by HCCS (blue) including regions in cyt c (green) important for maturation. In bacteria and archaea, dedicated multicomponent pathways known as Systems I (CCM) and/or II (CcsBA) traffic and attach the heme (9–16). In prokaryotes, many c-type cytochromes exist in addition to monoheme proteins (17–20). These are all matured by Systems I and II, consistent with the proposal that only the CXXCH motif is recognized (21–24).

Previous genetic studies have shown that bacterial cytochromes are incompatible substrates for HCCS-mediated maturation (25, 27, 28, 30). However, it has also been shown by two independent groups that making three mutations in the N-terminal α helix-1 region of bacterial cytochromes can convert them into a substrate that is recognized and matured by HCCS (27, 28), c-type cytochromes from the alphaproteobacteria Rhodobacter and Paracoccus have glutamate residues at the sequence positions equivalent to Lys8 and Ile10 found in human cyt c (see Fig. 2A, blue asterisks). Furthermore, following an important Phe residue found in human (Phe31) and some bacterial cytochromes (21, 27), a residue at the sequence position corresponding to Met13 in human cyt c (see Fig. 2A) is missing in certain bacterial cytochromes. Substitution of the glutamate residues and insertion of an alanine residue at the corresponding positions in Rhodobacter capsulatus cyt c2 resulted in its maturation by HCCS (27), thereby suggesting a critical role of the α helix-1 adjacent to the CXXCH motif in mediating interactions between the substrate and enzyme.

The N-terminal α helix-1 of human cyt c consists of an 11-residue sequence (Val4–Lys14) immediately followed by the conserved C15XXC18H19 heme attachment motif (Fig. 2, A and B). α helix-1 includes the Lys8, Ile10, and Phe11 residues that appear to be important for HCCS-mediated maturation due to their putative roles in substrate recognition by HCCS (21, 25, 27, 28). Cys15 and Cys18 supply the reactive thiole side chains that, respectively, form thioether attachments to the 2- and 4-vinyl groups of heme (2, 3). Recently, it was shown that His19 is recognized as one of two axial heme ligands in the HCCS-cyt c complex (31); the other is His144 of HCCS (27). Furthermore, cyt c His19 plays crucial roles in facilitating thioether formation at the active site of HCCS (31).

Previous results have led to the proposal of a four-step model (see Fig. 1) for HCCS-mediated cyt c maturation (27, 31–33).

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First, HCCS non-covalently binds to reduced heme with His\textsuperscript{154} serving as an axial ligand. Second, heme-bound HCCS recognizes, binds to, and positions apocytochrome adjacent to the heme with His\textsuperscript{19} from cyt c acting as the second axial ligand. Third, heme spontaneously forms two thioether bonds to the apocytochrome cysteines at the CXXCH motif. Fourth, the heme-attached cyt c is released from HCCS.

The exact roles of the N-terminal helix-1 residues (e.g. Phe\textsuperscript{11}) in recognition (step 2) and/or thioether attachment (step 3) are unknown. In this study, we engineered human cyt c variants (e.g. deletion of the Met\textsuperscript{13} residue (ΔM13)) to reflect the sequence properties of bacterial cyt c with resultant poor maturation by HCCS. We examined the biochemical basis behind the inability of HCCS to mature the ΔM13 cyt c variant. We discovered that substrate recognition by HCCS is not defective with the ΔM13 cyt c variant. Rather, the N-terminal α helix-1 of cyt c is responsible for aligning the Cys\textsuperscript{15} thiol for covalent attachment to the heme 2-vinyl. The ΔM13 cyt c variant does not possess a thioether at the Cys\textsuperscript{15} position when bound at the HCCS active site. However, in the ΔM13 cyt c variant, His\textsuperscript{19} forms the axial heme ligand and positions the Cys\textsuperscript{18} thiol for heme 4-vinyl attachment. These results provide mechanistic insight into step 2 (and consequently step 3) of HCCS-mediated cytochrome c maturation and are depicted in an updated model in Fig. 1. We confirmed these conclusions by engineering a cyt c substrate with a repositioned “Cys\textsuperscript{15}” thiol in

**FIGURE 1. Current model of HCCS-mediated cytochrome c maturation.** The enlarged illustration shown in the center of the model depicts events that take place in step 2, which are studied here and described in the text.
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Results

Residue Spacing in α Helix-1 of Cyt c Is Important for HCCS-mediated Maturation—To determine how sequence variations in the N-terminal region of bacterial c-type cytochromes affect the HCCS-mediated biogenesis of eukaryotic cyt c, we co-expressed human HCCS with human cyt c variants that are analogous to bacterial cytochromes (Fig. 2, A–C). Ser16 and Gln17 were substituted with alanine residues (S16A/Q17A) because the equivalent positions in bacterial cytochromes are variable. These substitutions resulted in near-wild-type (WT) levels of cyt c (Fig. 2C) and thus do not appear to be involved in maturation by HCCS. Changing Lys8 and Ile10 in human cyt c to glutamate (K8E/I10E) resulted in a marked increase in cyt c yield (Fig. 2C). We have not investigated this effect further because ΔM13, which mimics the shifted residue spacing found in bacterial cytochromes (Fig. 2A), resulted in at least a 10-fold decrease in maturation by HCCS (Fig. 2C). The decrease in cyt c was confirmed by UV-visible spectra of purified soluble ΔM13 cyt c when compared with WT cyt c from 1-liter cultures (Fig. 2D). This purified cyt c is termed “released cyt c” because it is released from membrane-bound HCCS into the soluble fraction as described previously (27). Levels of released cyt c for the S16A/Q17A variant were the same as WT, whereas the levels of the K8E/I10E and K8E/I10E/ΔM13 variants were 1.8-fold more and 10-fold less than WT, respectively. Other N-terminal α helix-1 deletion variants of cyt c (i.e. ΔI12, ΔK14, ΔM13/ΔS16, and ΔM13/CSAQG) also were poorly matured as determined by UV-visible spectroscopy (Fig. 2, A and E). Combining the Met13 deletion with the Lys8/Ile10 substitutions (K8E/I10E/ΔM13) resulted in a dramatic reduction of holocytochrome c product (Fig. 2C), effectively negating the increase in cyt c yield gained by the K8E/I10E double mutation. These data indicate that the spatial organization of residues in the N-terminal α helix-1 is of major importance for maturation by HCCS and the major basis behind the inability of HCCS to mature bacterial c-type cytochromes.

Cyt c Variants Containing N-terminal Residue Deletions Form Single Thioether Complexes with HCCS—Following co-expression, a fraction of cyt c can be co-purified in complex with membrane-bound HCCS (27, 31, 32), effectively trapping a transitional intermediate during the maturation process. For example, single cysteine or histidine (from the CXXCH motif) the ΔM13 background, creating a unique CXCH motif, which now was matured by HCCS.

FIGURE 2. Spatial context of cyt c sequence required for efficient HCCS-mediated maturation. A, ClustalW-generated amino acid sequence alignment of N-terminal region from bacterial (Rhodobacter capsulatus (UniProt ID P00096) and Paracoccus denitrificans (UniProt ID P00094)) and eukaryotic (Homo sapiens (UniProt ID P99999)) cyts c along with the cyt c variants constructed for this study. Identical residues are shaded in light blue. Blue asterisks (*) denote residue positions in human cyt c that were substituted with glutamate. Red asterisks (**) denote residue positions in human cyt c that were deleted or substituted for this study. Residue numbering represents that of full-length human cyt c sequence with the initiating methionine as position 1. B, PyMOL-generated structure of human cyt c N terminus (modified from Protein Data Bank code 3ZCF chain D) with residues from Gly1 to His19 shown. Residues in α helix-1 and the heme attachment site are color-coded as follows: Phe1, pink; Ile12, gray; Met13, magenta; Lys14, cyan; Cys15 and Cys18, yellow; His19, blue. Heme is depicted in red. C, heme stain of released cyt c purified from 1-liter cultures. Spectra are offset for clarity. Abs, absorbance.
substitutions are trapped in complex with HCCS, whereas the triple mutant (in which both cysteines and the histidine residue are changed) is not (31). We used this co-purification approach to determine whether ΔM13 cyt c was defective in recognition by HCCS (step 2) or perhaps another step in maturation. HCCS complexes containing cyt c exhibit spectral characteristics that provide insight into the heme environment within the complex (31). To determine whether cyt c α helix-1 deletion variants interact with HCCS, we purified recombinantly expressed HCCS from Escherichia coli membranes and probed for co-purified cyt c (Fig. 3, A–C). Similar to WT cyt c (Fig. 3A, lane 1), each deletion variant co-purified with HCCS in a heme-attached state (Fig. 3A). The heme stain shows significant holocytochrome c in all cases co-purifying with HCCS. This was confirmed by total protein stain (Fig. 3B) and by immunoblotting with antibodies that specifically recognize cyt c (Fig. 3C). However, when we evaluated the UV-visible spectra of the WT, ΔM13, and ΔK14 complexes (Fig. 3D, panels i–iii), complexes with deletion variants exhibited spectral features similar to a C15S cyt c complex (Refs. 27 and 31 and Fig. 3D, panel iv). When co-purified with HCCS, C15S cyt c is only able to form a single covalent attachment (at Cys15) with heme, yielding a split α absorption peak at wavelength 555–560 nm upon chemical reduction (Refs. 27 and 31 and Fig. 3D, panel iv, red line). Complexes with C18A cyt c form a single thioether with heme at Cys18 and yield an α peak at ~560 nm (27, 31). The above results suggest that ΔM13 cyt c may only have Cys18 covalently attached when in complex with HCCS. These results were further supported by dithionite-reduced pyridine hemochrome spectra for each of the complexes, which showed α peak maxima at 552.8, 552.4, and 552.5 nm for ΔM13, ΔK14, and the C15S cyt c complexes, respectively (Fig. 3E), values indicative of a single thioether (31, 34, 35). Likewise, other deletion variants co-purified with HCCS (Fig. 3, A–C) and showed similar α peak maxima values in the reduced pyridine (Fig. 3F), all diagnostic for the presence of a single covalent attachment (31, 34, 35). As expected, the complex with WT cyt c exhibited an α peak maximum at 550.8 nm in the pyridine hemochrome spectrum (Fig. 3F), a value that is consistent with heme containing two covalent attachments (31, 32, 36). Collectively, these data suggest that, once bound to HCCS, cyt c variants containing deletions in α helix-1 readily form only a single thioether with heme, although both reactive cysteines in the C15°XXC18°H19° heme attachment motif are intact. Furthermore, based on the spectral similarities of these variants with the C15S cyt c mutant complex, it is possible that formation of the single thioether takes place at the Cys18 posi-
tion. All complexes exhibit spectra demonstrating that the His^{19} axial ligand is formed (with the HCCS His^{154} as the other axial ligand) (data not shown). This includes the shift in the Soret peak (i.e. of reduced versus oxidized) and increased absorption of the reduced peaks as discussed previously (31).

**Deletion of Cyt c Met^{13} Abrogates Formation of Cys^{15} Thioether Linkage in the HCCS Complex**—We focused on Met^{13} cyt c to test the hypothesis that HCCS complexes with cyt c deletion variants in α helix-1 form only a single covalent attachment to heme at Cys^{18}. We generated single cysteine substitutions (of CXXCH) in the M13 cyt c background (Fig. 2A). Substituted residues in this background are denoted with wild-type numbering of the corresponding residue to maintain clarity for the residue order, and tick marks (•) are used to imply altered spacing from wild type. When we co-expressed HCCS with either ΔM13/C15′A or ΔM13/C18′A cyt c variants, purified complexes were obtained (Fig. 4, A–C), indicating that a single cysteine substitution combined with the deletion of Met^{13} did not abolish recognition/interaction of the cytochrome with HCCS. However, the ΔM13/C18′A cyt c variant no longer possessed covalently attached heme (Fig. 4B, last lane). These data were further validated by UV-visible and reduced pyridine hemochrome spectra of the purified complexes (Fig. 4, D and E). Spectral properties of ΔM13/C15′A cyt c were nearly identical to those of the HCCS complex with ΔM13 cyt c (compare Fig. 4D, panel i, with Fig. 3D, panel ii), whereas the ΔM13/C18′A complex appeared similar to recombinant HCCS alone (Fig. 4D, panel ii, and Refs. 27, 31, and 32). Recombinant HCCS is purified with endogenous heme that is non-covalently bound (27, 31, 32), and the absence of any covalent linkages to the heme present in the ΔM13/C18′A complex was confirmed by the reduced pyridine hemochrome absorption maximum value of 555.5 nm (Fig. 4E, red line). The presence of a single thioether in the ΔM13/C15′A complex was confirmed by its pyridine hemochrome spectrum (Fig. 4E, black line), which again mimicked the complex with ΔM13 (Fig. 3E, red line), both exhibiting α absorption maxima around 552 nm. These data
demonstrate that the ΔM13 cyt c variant forms a single thioether with heme at the Cys18 position in the C15XXC18H19 heme attachment site of cyt c; thus, the Cys15 side chain is free in the complex with HCCS and not covalently attached.

Deletion of Met13 Places Cys15 Out of Position Relative to Phe11 in N-terminal Helix-1—Based on our data, we hypothesized that the Cys15 residue in ΔM13 cyt c was spatially repositioned from the heme 2-vinyl when bound at the active site of HCCS, thereby preventing its covalent attachment. To gain more insight into the potential structural abnormalities elicited by this deletion, we used PEP-FOLD (37, 38) to predict the N-terminal structure of human cyt c (from Asp3 to His19), including helix-1 and the heme attachment motif (Fig. 5). The helical character in the structural prediction of the WT cyt c N-terminal region appeared similar to that found in the cyt c solved structure (compare Fig. 5A with Fig. 2B). PEP-FOLD structures displayed in Fig. 5 anchor Phe11 in the same position; thus, the remaining residues are displayed relative to anchored Phe11. In the predicted structure for the N-terminal region of ΔM13 cyt c (Fig. 5B), it is clear that Cys15 is displaced one residue away from its WT location relative to Phe11. Rather than a backbone hydrogen bond between Phe11 and Cys15 (a bonding pattern that occurs in α helices (39)), in the ΔM13 cyt c α helix-1, the backbone hydrogen bond would link Phe11 to Ser16. A PEP-FOLD model in which Ser16 is substituted with cysteine (in a ΔM13/C15’A background) (Fig. 5C) shows that the S16C residue is now positioned where a covalent attachment might occur more frequently, that is if the α helix-1 (including Phe11) anchors the first cysteine residue next to 2-vinyl of heme at the HCCS active site.

Engineering an Unconventional CXCH Heme Attachment Site Partially Restores Thioether Formation at the Proximal Cysteine Residue in a ΔM13/C15’A Cyt c Variant—The theoretical PEP-FOLD models and crystal structure of human cyt c both indicate some flexibility of the loop between the two cysteines (Figs. 2B and 5A–C). Thus, if cyt c α helix-1 is anchoring Cys15 and the His19 axial ligand positions the Cys18 thiol in the ΔM13 background, substituting Ser16 with cysteine may facilitate thioether attachment. To make sure the native Cys15 is unable to form a covalent bond, we used the ΔM13/C15’A variant as a starting point. We engineered a S16C substitution in the ΔM13/C15’A cyt c variant background (Fig. 2A) (thus creating a CXCH motif) to determine whether the predicted PEP-FOLD model (Fig. 5C) and the binding data (Figs. 3 and 4) described above would again “anchor” the new cysteine residue. To compare an alternate residue spacing arrangement, we also engineered a cysteine substitution at Lys14 in the ΔM13/C15’A cyt c variant background (Fig. 2A), thus creating a unique CXXXCH heme attachment motif. To determine whether these variants could stably interact with HCCS, we purified complexes and evaluated whether cyt c co-purified (Fig. 6, A–C). The ΔM13/C15’A/K14’C variant was undetectable in the com-

FIGURE 5. Three-dimensional models depicting the positioning of cyt c Cys15 for thioether formation (within the active site of HCCS). PEP-FOLD-predicted models of the N-terminal region (Asp3–His19) from human-derived WT (A), ΔM13 (B), and ΔM13/C15’A/S16’C (C) cys c are depicted. PyMOL was used to display each predicted model. Select residues are shown for each structure, including the cysteine residues in yellow and His19 in blue. Phe11 is used to anchor each structure.
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FIGURE 6. Creation of a non-canonical CXCH heme attachment site partially restores formation of the first thioether in ΔM13/C15’A cyt c variant. Purified HCCS complexes were separated by SDS-PAGE, and heme stain (A), SYPRO Ruby blot stain (B), and immunoblot with anti-cyt c antibodies (C) of HCCS co-purified with the indicated cyt c variants are shown. Blue asterisks (*) refer to a contaminating band present in the co-purified HCCS complex with ΔM13/C15’A/K14’C, which is not recognized by cyt c antisera. Red asterisks (†) refer to signal from loading dye front. D, UV-visible absorption spectra of HCCS co-purified with ΔM13/C15’A/K14’C (panel i) or ΔM13/C15’A/S16’C (panel ii) cyt c. Spectra of purified proteins are shown in black, and spectra of purified proteins reduced with sodium dithionite are shown in red. The α/β peaks in the reduced spectra are enlarged 3-fold for clarity. Absorption maxima values are indicated. E, UV-visible absorption spectrum of HCCS-matured ΔM13/C15’A (black), ΔM13/C15’A/S16’C (red), and ΔM13/C15’A/K14’C (blue) cyt c purified from 1-liter cultures. Absorption maxima values are indicated. F, sodium dithionite-reduced pyridine hemochrome spectrum of released ΔM13/C15’A/S16’C cyt c purified from 1-liter culture. The absorption maximum of α peak is indicated. Abs, absorbance; WB, Western blotting.

plex (Fig. 6, A–C), suggesting that the CXXXCH cyt c substrate variant is not recognized by HCCS. However, ΔM13/C15’A/S16’C cyt c could be detected in complex with HCCS with heme attached (Fig. 6, A–C). The UV-visible absorption spectrum of the HCCS complex with ΔM13/C15’A/K14’C cyt c appeared similar to that expected for HCCS alone (Fig. 6D, panel i, and Refs. 27, 31, and 32), and the reduced pyridine spectrum of HCCS co-expressed with this variant confirmed that only b-type heme was present (data not shown), thus confirming that this variant does not interact with HCCS (Fig. 6, A–C). Absorption spectra for the HCCS complex with ΔM13/C15’A/S16’C cyt c exhibited broad α/β peaks upon reduction (Fig. 4D, panel ii) with α absorption maxima values similar to those of the complex with ΔM13/C15’A cyt c (Fig. 4D, panel i) (see “Discussion”). The UV-visible spectrum of released S16’C variant appeared strikingly similar to that of WT cyt c, exhibiting the c-type cytochrome signature α absorption peak at 549 nm, whereas the low levels of released ΔM13/C15’A showed a 552-nm maximum (Fig. 6E). Moreover, the S16’C cyt c (CXCH variant) was released at a level 2.5-fold higher than the ΔM13/C15’A parent (Fig. 6E), suggesting that two thioethers are formed, thus facilitating its release (i.e. step 4 of cyt c maturation). Accordingly, the reduced pyridine hemochrome of the released ΔM13/C15’A/S16’C cyt c gave an α peak maximum at 550 nm, indicating that both thioethers formed at the CXCH site (Fig. 6F).

N-terminal Region of Cyt c Protected from Protease Cleavage When in Complex with HCCS—Results presented above indicate that the cyt c α helix-1 and CXCH motif are directly bound to the HCCS active site. As a complementary approach, we initiated a biochemical analysis of the HCCS-WT cyt c complex to establish which regions of cyt c are bound. Upon resolv- ing HCCS-WT cyt c complexes by SDS-PAGE, we observed heme-stainable polypeptides migrating below full-length cyt c (Fig. 7A, arrows). These polypeptides could be detected by protein stain when increased amounts of co-complex were loaded (Fig. 7B, arrow). We hypothesized that these small peptides are degradation products of complexed cyt c with natural protection from proteolysis. Notably, matured cyt c (released from HCCS) that is purified from the soluble fraction does not contain this truncated fragment when resolved by SDS-PAGE (data not shown). To determine what region of cyt c was “protected” while in complex with HCCS, we isolated the polypeptides (Fig. 7B, arrow) for mass spectrometry analysis. Mass spectrometry of tryptic digests identified the N-terminal portion (Ile10–Lys45) of cyt c containing the heme attachment site (Fig. 7C, tryptic peptides detected in red) (Note that peptides containing heme (i.e. as expected with the peptide containing the CSQCH
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A

\[ \text{HCSS:WT cyt c co-complex} \]

\[ \begin{align*}
\text{protein} & : 5 & 15 & 30 \text{ ug} \\
\text{Holo HCSS} & & & \\
\text{Heme stain} & & & \\
\text{Holo cyt c} & & & \\
\text{c} & & & \\n\end{align*} \]

B

\[ \text{Sypro blot stain} \]

\[ \text{HCSS} \]

\[ \text{c} \]

\[ \text{N-terminal region protected from protease degradation when bound to HCSS.} \]

C

\[ \text{Human cytochrome c} \]

\[ \begin{align*}
1 & \text{MGDVEKGKIKI FIMKCSQCHT VEGKGGHKGTG 30} \\
31 & \text{PNLHGLGFRK TQAPQGSYT AANKNKIIW 60} \\
61 & \text{GEDTLMEYLE NPKKYIPGTTK MIFVG1KKKE 90} \\
91 & \text{ERADLIAFLK KATNE 105} \\
\end{align*} \]

FIGURE 7. Cyt c N-terminal region protected from protease degradation when bound to HCSS. The indicated amounts of purified HCSS complexes were separated by SDS-PAGE, and heme stain (A) and SYPRO Ruby blot stain (B) of HCSS co-purified with WT cyt c are shown. Arrows with asterisks (*) represent cleaved cyt c products. The cleaved cyt c polypeptide was excised from the polyacrylamide gel, treated with trypsin, and processed for mass spectrometry analysis. C, cyt c sequence in red represents tryptic peptides identified by mass spectrometry from the excised band. Sequence included in N-terminal \(\alpha\) helix-1 is underlined. The heme attachment site (CSQSH) is shown in bold.

motif) are typically poorly detected by mass spectrometry (40, 41); however, the presence of heme within the truncated fragment was confirmed by heme stain (Fig. 7A, top arrow). Importantly, the \(\alpha\) helix-1 tryptic peptide was detected in the protected fragment (Fig. 7C, underlined sequence). We conclude that the cyt c N-terminal \(\alpha\) helix-1 is likely bound at the active site of HCSS, consistent with the \(\Delta M13\) cyt c results.

Discussion

We established that the N-terminal \(\alpha\) helix-1 of cyt c plays a major role in steps 2 and 3 (Fig. 1) of HCSS-mediated maturation by positioning Cys15 of the C\(^{15}\)XXC\(^{18}\)H\(^{19}\) motif for heme attachment. Specifically, the spatial context of the cyt c N-terminal \(\alpha\) helix-1 sequence relative to the CXXCH motif is crucial for optimal heme attachment to the polypeptide, which, in large part, explains why bacterial cytochromes are not matured by HCSS. Although the bacterial CCM system (i.e. System I) can successfully mature a wide range of mono- and multiheme cytochromes, containing simply a CXXCH motif (where \(X_n = 1-4\) intervening residues) (21, 24, 42, 43), additional structural features of eukaryotic cytochromes contribute to the strict substrate specificity of HCSS. For example, previous genetic studies have suggested that the conserved phenyalanine residue in cyt c \(\alpha\) helix-1 is critical for HCSS maturation by serving as a determinant for substrate recognition (step 2) because substitution of this residue can result in poor cyt c yields (21, 25) (Phe\(^{11}\) in human cyt c the equivalent of this position in bacterial cytochromes is not fully conserved (21, 44)). Here we show deficiencies in cyt c production with the human \(\Delta M13\) cyt c variant containing residue spacing similar to some bacterial cytochromes. However, our data indicate that poor substrate recognition of this variant is not the basis for defective maturation because complex formation with HCSS is unaffected (Fig. 3). Conclusive evidence was obtained that thioether formation (step 3) at the Cys\(^{15}\) position is specifically impacted by the altered residue spacing caused by the deletion of Met\(^{13}\) (and other \(\alpha\) helix-1 residue deletions) (Fig. 4). Accordingly, we discovered that changing the position of the proximal cysteine in the context of the Met\(^{13}\) deletion (i.e. \(\Delta M13/C15' A/S16' C\) partially restored thioether formation (Fig. 6) by correcting the spatial orientation of the cysteine with the residues in the N-terminal \(\alpha\) helix (Fig. 5).

The human HCSS recognizes both cyt c and cyt c\(_1\) of the membrane cytochrome \(b_c\) complex III (6, 45). Some lower eukaryotes such as Saccharomyces cerevisiae have two related synthases: HCSS that recognizes cyt c and HCC,\(S\) that recognizes cyt c\(_1\) (8, 46). Hamel and co-workers (45) have shown in S. cerevisiae that human HCSS (termed CCHL for cytochrome c heme lyase) matures yeast cyt c and cyt c\(_1\), so it has broader specificity. They also showed that, when overexpressed, the yeast HCSS matures cyt c\(_1\) and that suppressor substitutions of the cyt c\(_1\) heme attachment motif, CAACH (second alanine), allow recognition by yeast HCSS (45, 47). Our study shows there is little impact on substrate recognition by human HCSS with serine or glutamine substitutions in the human cyt c CSQCH motif (Fig. 2) in keeping with broader substrate specificity for the human HCSS. Our results are consistent with the genetic analyses in yeast by Hamel and co-workers (45) that suggest that the CXXCH motif, as well as the region N-terminal to this site, are important determinants for HCSS maturation of cyt c. Here we define that the cyt c \(\alpha\) helix-1 is a component of the recognition elements but is specifically needed for positioning the first cysteine residue at the HCSS active site. A PEPFOLD analysis of the human cyt c\(_1\) region (IRRGFQVKQVCASCH) where Tyr replaces the Phe\(^{11}\) of cyt c yields a similar \(\alpha\) helix region before the CXXCH motif (data not shown). We have previously shown that a Tyr can replace the cyt c\(_1\) Phe\(^{11}\) with normal HCSS-mediated maturation unlike an alanine substitution (27). It is likely that the mechanisms for heme attachment by HCSS described here for cyt c are applicable to cyt c\(_1\).

\(\Delta M13\) cyt c, as well as all other \(\alpha\) helix-1 deletion variants reported here, are poorly released from HCSS with yields between 5 and 10% of WT cytochrome c (Fig. 2, C and D), reminiscent of other HCSS-matured, single thioether cyt c variants (e.g. C15S and C18A (27, 48)). Accordingly, poorly released cyt c variants are co-purified in complex with HCSS at
higher levels than WT cyt c (e.g. Fig. 4, A–C). These data further support the contention that formation of both thioureas is required for efficient release (i.e. step 4) of the cytochrome product from the HCCS active site (27). Because the HCCS complex with the engineered CXCH heme attachment site (i.e. ΔM13/C15’A/S16’C cyt c) exhibited some spectral characteristics similar to the complex containing the single thiourea M13/C15’A cyt c parent, we cannot conclude whether both cysteines are thiourea-attached. However, we also do not know what spectral features to expect for a HCCS complex with a CXCH cyt c covalent link. We base our conclusion of a repositioned S16’C on the fact that HCCS releases 2.5–3-fold more ΔM13/C15’A/S16’C cyt c relative to ΔM13/C15’A (Fig. 6E) and other deletion variants. We propose that the positioning of the cysteine substitution at Ser16’T renders the variant more competent to form both thioureas at the HCCS active site, causing further distortion of the heme (31), which facilitates increased release of the CXCH cyt c. We note that the low levels of released and purified cyt c from each deletion variant exhibited a peak absorption maxima at 550 nm (Fig. 2, D and E), similar to that expected for a cytochrome with two thioureas like WT (27, 31, 49). Although ΔM13 cyt c, for example, contains an intact heme attachment site (CSQCH), only a single thiourea (at Cys18’) is present in complex with HCCS (Figs. 3 and 4). It is possible that only rarely doubly attached ΔM13 cyt c variants are ultimately released from HCCS. Alternatively, it is also possible that the low level of ΔM13 cyt c that is released forms the second thiourea upon folding and/or while free in the cell. In either scenario, the conclusions here remain the same.

It is noteworthy that the ΔM13/C15’T/A/S16’C cyt c variant containing the unconventional CXCH heme attachment site was biosynthesized by HCCS at significant levels (~22% of WT levels). A previous study using recombinant yeast HCCS (termed CCHL) attempted to mature an engineered cyt c variant from horse heart containing a CXCH heme attachment site but could not detect product (21). Our results are consistent with that study because efficient maturation and release of the CXCH variant required “repositioning” of the first cysteine and shortening of α helix-1 mediated by the deletion of Met13. Thus, two regions in mitochondrial cyt c form the basis for recognition and thiourea attachment by HCCS: the α helix-1 and the CXCH motif where the histidine plays multiple critical roles as described previously (31). We demonstrate in this work that both of these substrate maturation determinants can be modulated and potentially exploited to produce other unconventional cyt-c-type cytochromes.

### Experimental Procedures

#### Construction of Strains and Plasmids

Plasmids used in this study, pRGK403 (pGEX-HCCS: N-terminal GST-tagged human WT HCCS), pRGK405 (pBAD CYCS: WT human cytochrome c (CYCS)), and pRGK417 (pBAD CYCS (C15S cytochrome c)) have been described previously (27). Oligonucleotide primer sequences and plasmids are reported in Table 1. Nucleotide substitutions were engineered using the QuickChange II site-directed mutagenesis kit (Agilent Technologies) according to the manufacturer’s specifications. All cloning steps were confirmed by sequencing. Verified clones were transformed into the E. coli Δcm strain RK103 (50) or C43 (51).

#### Bacterial Protein Extraction Reagent Functional Assay

E. coli strains were grown overnight and used to inoculate 5 ml of LB supplemented with appropriate antibiotics. These cultures were grown at 37 °C with shaking at 200 rpm for 3 h followed by induction with 0.1 mm isopropyl-1-thio-β-d-galactopyranoside and 0.8% arabinose (w/v) for an additional 3 h. Cells were harvested by centrifugation at 4,500 × g, and the cell pellet was lysed in 200 μl of Bacterial Protein Extraction Reagent (Thermo Scientific). 100 μg of extracted protein was resolved by SDS-PAGE, transferred to nitrocellulose, and analyzed by heme stain. Heme stained cytochrome c signals were quantified using Image Studio Lite Version 5.2 software (LI-COR Biosciences).

### Table 1

| Primer pair | Oligo ID | Sequence (5’–3’) | Constructed plasmid |
|-------------|---------|------------------|---------------------|
| 1          | CYCS_ΔM13 primer1 | TTTTCAACGGTGTGGCACTGAGCGGAACACTTAATAAAAATCTTCTTGCCTTTCTCAACATCA | pBAD CYCS (ΔM13) |
| 2          | CYCS_ΔM13 primer2 | TTTTCAACGGTGTGGCACTGAGCGGAACACTTAATAAAAATCTTCTTGCCTTTCTCAACATCA | pBAD CYCS (ΔM13) |
| 3          | CYCS_K8E/I10E primer1 | TTTTCAACGGTGTGGCACTGAGCGGAACACTTAATAAAAATCTTCTTGCCTTTCTCAACATCA | pBAD CYCS (ΔM13) |
| 4          | CYCS_K8E/I10E primer2 | TTTTCAACGGTGTGGCACTGAGCGGAACACTTAATAAAAATCTTCTTGCCTTTCTCAACATCA | pBAD CYCS (ΔM13) |
| 5          | CYCS_ΔM13 primer1 | TTTTCAACGGTGTGGCACTGAGCGGAACACTTAATAAAAATCTTCTTGCCTTTCTCAACATCA | pBAD CYCS (ΔM13) |
| 6          | CYCS_ΔM13 primer2 | TTTTCAACGGTGTGGCACTGAGCGGAACACTTAATAAAAATCTTCTTGCCTTTCTCAACATCA | pBAD CYCS (ΔM13) |
| 7          | CYCS_ΔM13 primer1 | TTTTCAACGGTGTGGCACTGAGCGGAACACTTAATAAAAATCTTCTTGCCTTTCTCAACATCA | pBAD CYCS (ΔM13) |
| 8          | CYCS_ΔM13 primer2 | TTTTCAACGGTGTGGCACTGAGCGGAACACTTAATAAAAATCTTCTTGCCTTTCTCAACATCA | pBAD CYCS (ΔM13) |
| 9          | CYCS_ΔM13 primer1 | TTTTCAACGGTGTGGCACTGAGCGGAACACTTAATAAAAATCTTCTTGCCTTTCTCAACATCA | pBAD CYCS (ΔM13) |
| 10         | CYCS_ΔM13 primer2 | TTTTCAACGGTGTGGCACTGAGCGGAACACTTAATAAAAATCTTCTTGCCTTTCTCAACATCA | pBAD CYCS (ΔM13) |
| 11         | CYCS_ΔM13 primer1 | TTTTCAACGGTGTGGCACTGAGCGGAACACTTAATAAAAATCTTCTTGCCTTTCTCAACATCA | pBAD CYCS (ΔM13) |
| 12         | CYCS_ΔM13 primer2 | TTTTCAACGGTGTGGCACTGAGCGGAACACTTAATAAAAATCTTCTTGCCTTTCTCAACATCA | pBAD CYCS (ΔM13) |
Mechanistic Role of α Helix-1 in Cytochrome c Maturation

HCCS Protein Expression and Purification—GST-HCCS proteins were co-expressed with cytochrome c (WT or variant) and purified from E. coli Δccm (RK103 or C43) as described previously (27). Briefly, 100 ml of starter cultures were grown overnight at 37 °C with shaking and used to inoculate 1 liter of LB supplemented with the appropriate antibiotics. Following 1 h of growth of the 1-liter cultures at 37 °C with shaking at 120 rpm, the cultures were induced with 0.1 mM isopropyl-1-thio-β-d-galactopyranoside for expression of pGEX-HCCS. For co-expression of the pBAD CYCS (WT or variants), the cultures were induced with 0.2% arabinose (w/v) 2 h after the induction of HCCS expression. Cells were harvested by centrifugation at 4,500 × g, resuspended in PBS with 1 mM PMSF, and sonicated. The crude sonicate was cleared by centrifugation at 24,000 × g for 20 min, and the membrane fraction was isolated by ultracentrifugation at 100,000 × g for 45 min. Membrane pellets were solubilized in 50 mM Tris (pH 8), 150 mM NaCl, 1% Triton X-100 on ice for 1 h. Solubilized membranes were loaded onto glutathione-agarose (Pierce) for an overnight batch pulldown of GST-HCCS protein (with any co-purified cytochrome c). Bound GST-HCCS complexes were eluted with 20 mM reduced glutathione in 50 mM Tris (pH 8), 150 mM NaCl, 0.02% Triton X-100 and concentrated in an Amicon Ultra Centrifugal Filter (Millipore) with a 100,000 molecular weight cutoff, and the total protein concentration was determined using the Bradford reagent (Sigma).

Cytochrome c Purification—The purification of HCCS-released cytochrome c was performed as described previously (31). Briefly, Δccm E. coli carrying plasmids for GST-HCCS and cytochrome c were inoculated into 100 ml of LB supplemented with the appropriate antibiotics, grown overnight at 37 °C with shaking, and used to inoculate 1 liter of LB. Following 1 h of growth of the 1-liter cultures at 37 °C with shaking at 120 rpm, the cultures were induced with 0.1 mM isopropyl 1-thio-β-d-galactopyranoside for expression of pGEX-HCCS. 2 h after the induction of HCCS expression, arabinose was added to 0.2% (w/v) to induce the expression of pBAD CYCS overnight. Cells were harvested by centrifugation at 4,500 × g, resuspended in 50 mM Tris (pH 8), 150 mM NaCl, with 1 mM PMSF, and sonicated. The crude sonicate was cleared by centrifugation at 24,000 × g for 20 min, and the soluble fraction was isolated by ultracentrifugation at 100,000 × g for 45 min. The supernatant was loaded onto CM Sepharose Fast Flow resin (GE Healthcare) for an overnight batch pulldown of positively charged proteins (including cytochrome c). Bound proteins were eluted with 20 mM reduced glutathione in 50 mM Tris (pH 8), 500 mM NaCl and concentrated in an Amicon Ultra Centrifugal Filter with a 3,000 molecular weight cutoff, and total protein concentration was determined using the Bradford reagent.

Heme Stains, SYPRO Ruby and Coomassie Protein Staining, and Immunoblotting—Heme stains and SYPRO Ruby blot staining were performed as described previously (31, 52). Briefly, to preserve the heme signal, protein samples were prepared for SDS-PAGE with loading dye at 1:1 (v/v) that did not contain reducing agents, and the samples were left unboiled. Following electrophoresis, proteins were transferred to nitrocellulose membranes, and the chemiluminescence signal for the heme stain was developed using the SuperSignal Femto kit (Thermo Scientific) and detected with either the ImageQuant LAS4000 Mini detection system (Fujifilm-GE Healthcare) or the Odyssey Fc Imaging System (LI-COR Biosciences). Following heme staining, membranes were washed in PBS and treated with fixing solution (7% acetic acid, 10% methanol (v/v)) for 15 min. The membranes were washed in deionized water, stained with SYPRO Ruby protein blot reagent (Molecular Probes) for 15 min, and washed again in deionized water. SYPRO-stained proteins were visualized with the ImageQuant LAS4000 Mini detection system using the Y515-Di filter or with the Odyssey Fc Imaging System. The membranes were then blocked in PBS containing 5% milk protein (w/v) for 1 h. Blots were probed for 1 h with a 1:2,000 dilution of antiserum against cytochrome and then washed with PBS. Cyt c antiserum was raised (Cocalico Biologials) against horse heart apocyt c. This cyt c antiserum reacts well with human cyt c. Protein A-peroxidase (Sigma) was used as a secondary label for detection. Following several washes with PBS, the chemiluminescence signal for anti-cytochrome c was developed using the Immobilon Western kit (Millipore) and detected by either the ImageQuant LAS4000 Mini detection system or the Odyssey Fc Imaging System. For Coomassie stains, proteins resolved by SDS-PAGE were stained in Coomassie solution (50% methanol (v/v), 10% acetic acid (v/v), 0.25% Coomassie Brilliant Blue (Sigma) (w/v)) for 1 h. Background staining was removed with several applications of a destaining solution (50% methanol (v/v), 10% acetic acid (v/v)). Stained proteins were imaged with the LAS-1000 Plus detection system (Fujifilm-GE Healthcare).

UV-Visible Absorption Spectroscopy—UV-visible absorption spectra were recorded with a Shimadzu UV-1800 spectrophotometer at room temperature. All spectra were obtained in the same buffer in which the proteins were purified (for membrane protein complexes, 50 mM Tris (pH 8), 150 mM NaCl, 0.02% Triton X-100; for cytochrome c, 50 mM Tris (pH 8), 500 mM NaCl). Spectra of chemically reduced proteins were generated by the addition of solid sodium dithionite (sodium hydrosulfite) to the purified sample. Pyridine hemochrome extraction of purified proteins was performed as described previously (36). Briefly, 0.5 M NaOH and pyridine were added to 100 μg of purified protein to yield final concentrations of 100 mM NaOH and 20% pyridine (v/v). Samples were chemically reduced with the addition of solid sodium dithionite (sodium hydrosulfite), and UV-visible spectra were recorded from 500 to 600 nm.

Three-dimensional Models—Three-dimensional models of the human cytochrome c N terminus (WT and variants) were generated by PEP-FOLD (37, 38) in Protein Data Bank format and subsequently illustrated in PyMOL (PyMOL Molecular Graphics System, Version 1.8, Schrödinger, LLC). Of the five PEP-FOLD-generated peptide predictions for each cytochrome variant, the selected peptide models represent the lowest energy conformation and thus the most probable structural arrangement. The x-ray crystal structure for recombinant human cytochrome c (Protein Data Bank code 3ZCF) (53) was obtained from the Research Collaboratory for Structural Bioinformatics Protein Data Bank and modified in PyMOL.

In-gel Trypsin Digestion and Peptide Extraction for Mass Spectrometry—100 μg of purified GST-HCCS-WT cyt c complexes was resolved by SDS-PAGE on a 15% polyacrylamide gel. Resolved proteins were stained with Coomassie solution for 1 h
and destained in destaining solution overnight. The Coomassie-stained band containing the protein of interest was excised from the gel and cut into 1-mm² pieces. The gel pieces were washed with vigorous agitation at room temperature with 200 μl of Milli-Q (EMD Millipore) water for 5 min, 200 μl of acetonitrile for 10 min, and 200 μl of 50 mM NH₄HCO₃, 50% acetonitrile for 15 min. Washes with the NH₄HCO₃, 50% acetonitrile solution were repeated until the Coomassie stain was completely removed. The gel pieces were washed once more with 200 μl of acetonitrile for 5 min with vigorous agitation at room temperature, then incubated in 30 μl of trypsin digestion buffer (6% 100 ng/μl trypsin, 47% Milli-Q water, 47% 100 mM NH₄HCO₃) for 10 min, and covered with no more than 20 μl of 50 mM NH₄HCO₃. The sample was incubated at 37 °C overnight after which the sample was incubated with 25 μl of 1% formic acid, 2% acetonitrile at room temperature with vigorous agitation for 20 min. The supernatant containing the extracted peptides was transferred to a clean tube and dried down in a refrigerated SpeedVac centrifuge for 30 min. The dried peptide sample was resuspended in 10 μl of 0.1% formic acid, vortexed, cleaned with micro-C₁₈ reversed phase ZipTips (EMD Millipore), and eluted in 12 μl of 70% acetonitrile, 0.1% formic acid solution, which was loaded into a 96-well plate reader in an LTQ-Orbitrap Velos Pro (Thermo) mass spectrometer. The collected data were analyzed using Mascot software (Matrix Science) for protein identification and Scaffold (Proteome Software) to validate identified proteins.

Author Contributions—S. E. B. designed and performed experiments, analyzed data, and wrote the manuscript. J. H. performed experiments. R. G. K. designed experiments, analyzed data, and co-wrote the manuscript with S. E. B.

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