Mechanisms of Mitochondrial Holocytochrome c Synthase and the Key Roles Played by Cysteines and Histidine of the Heme Attachment Site, Cys-XX-Cys-His*

Shalon E. Babbitt, Brian San Francisco, Deanna L. Mendez, Gudrun S. Lukat-Rodgers, Kenton R. Rodgers, Eric C. Bretsnyder, and Robert G. Kranz

From the Department of Biology, Washington University, St. Louis, Missouri 63130 and the Department of Chemistry and Biochemistry, North Dakota State University, Fargo, North Dakota 58102

Background: Cytochrome c is covalently attached to heme at a conserved CXXCH motif by holocytochrome c synthase (HCCS). The residues in the heme attachment motif mechanistically contribute to the HCCS-mediated maturation of cytochrome c. Efficient heme attachment is coordinated by the conserved histidine residue in the motif.

Conclusion: Insights into the mechanism of cytochrome c biogenesis broadens our understanding of mitochondrial biology.

Mitochondrial cytochrome c assembly requires the covalent attachment of heme by thioether bonds between heme vinyl groups and a conserved CXXCH motif of cytochrome c/c1. The enzyme holocytochrome c synthase (HCCS) binds heme and apocytochrome c substrate to catalyze this attachment, subsequently releasing holocytochrome c for proper folding to its native structure. We address mechanisms of assembly using a functional Escherichia coli recombinant system expressing human HCCS. Human cytochrome c variants with individual cysteine, histidine, double cysteine, and triple cysteine/histidine substitutions (of CXXCH) were co-purified with HCCS. Single and double mutants form a complex with HCCS but not the triple mutant. Resonance Raman and UV-visible spectroscopy support the proposal that heme puckering induced by both thioether bonds facilitates release of holocytochrome c from the complex. His-19 (of CXXCH) supplies the second axial ligand to heme in the complex, the first axial ligand was previously shown to be from HCCS residue His-154. Substitutions of His-19 in cytochrome c to seven other residues (Gly, Ala, Met, Arg, Lys, Cys, and Tyr) were used with various approaches to establish other roles played by His-19. Three roles for His-19 in HCCS-mediated assembly are suggested: (i) to provide the second axial ligand to the heme iron in preparation for covalent attachment; (ii) to spatially position the two cysteinyl sulfurs adjacent to the two heme vinyl groups for thioether formation; and (iii) to aid in release of the holocytochrome c from the HCCS active site. Only H19M is able to carry out these three roles, albeit at lower efficiencies than the natural His-19.

Respiration in eukaryotes depends on the mitochondrial electron transport chain, composed of proteins and co-factors that convert electron flow (oxidation/reduction) into a proton gradient (1). This proton gradient is used to synthesize ATP, for transport, and for other processes essential to the cell (1). Two of the proteins in the chain are cytochrome c (cyt c) and cytochrome c1 (cyt c1) (of the bc1 complex, complex III). These cytochromes have heme covalently attached to the proteins at the heme binding site CXXCH, where cysteines form thioether bonds to the two heme vinyl groups (2). The histidine in the CXXCH motif acts as an axial ligand to the heme iron (with Met-81 in human cyt c supplying the second axial ligand) (2).

Over two decades ago, studies in yeast showed that the gene encoding holocytochrome c synthase (HCCS) is required for this covalent attachment in mitochondria (3). However, because the enzyme has been refractory to recombination over-expression and purification, little has emerged on the mechanisms underlying HCCS function. Although fungi have two related HCCS proteins, one for attaching heme to cyt c (3) and one for cyt c1 (4, 5), animals possess a single HCCS that recognizes both cyt c-type cytochromes (6, 7). In addition to respiration, cyt c and HCCS are critical to programmed cell death (i.e. apoptosis) in animals (8, 9). Recently, the human HCCS gene was shown to be mutated in the genetic disease microphthalmia with linear skin defects (10). Thus, it is important to understand the mechanisms of HCCS function and cyt c assembly in mitochondria (which is also referred to as the system III cyt c biogenesis pathway (11)).

Very recently, the system III pathway was reconstituted (12) in Escherichia coli Δccm, a strain lacking the endogenous system I genes (ccmA-H) (13). The human HCCS, engineered as an N-terminal GST fusion protein, was able to attach heme to its cognate human cyt c when both were recombiantly expressed in this background. It was demonstrated that human GST-HCCS is membrane localized (12), although no transmembrane helices are predicted in HCCS (6, 14). HCCS was purified with endogenous heme, and this heme required HCCS residue His-154 for binding. A model describing four steps in the HCCS pathway for cyt c assembly was proposed (Fig. 1A): step 1 is the

*This work was supported, in whole or in part, by National Institutes of Health Grants RO1 GM47909 (to R. G. K.) and RO1 AI072719 (to K. R. R.).
1Present address: The Institute for Genomic Biology, University of Illinois at Urbana-Champaign, Urbana-Champaign, IL 61801.
2To whom correspondence should be addressed: 1 Brookings Dr., Campus Box 1137, St. Louis, MO 63130. Tel.: 314-935-4278; E-mail: kranz@biology.wustl.edu.

3The abbreviations used are: HCCS, holocytochrome c synthase; cyt c, cytochrome c; ccm, cytochrome c maturation; RR, resonance Raman; LS, low spin; HS, high spin; Sc, 5-coordinate; 6c, 6-coordinate.
binding of heme with His-154 as an axial ligand; step 2 is the binding of apocytochrome c substrate; step 3 is formation of the two thioether bonds; step 4 is the release of holocytochrome c from the active site of HCCS. The model is based on specific observations in the recombinant system. For example, when expressed with its cognate cytochrome c, recombinant WT HCCS co-purified as a complex with its holocytochrome c, but the HCCS H154A mutant did not co-purify (step 2). When both thioether bonds are formed (step 3), the holocytochrome c is released to the cytoplasmic fraction (step 4). If only one thioether bond is formed (e.g. in single cysteine variants of the CXXCH), the cytochrome c is trapped as a complex and not released (Fig. 1A). A structural schematic indicating where residues such as Cys-15, Cys-18, and His-19 (of CXXCH) might be positioned in the HCCS active site in the complex is shown in Fig. 1B.

One of the proposals for step 2 is that His-19 of the apocytochrome c binds to the heme iron, replacing an unknown ligand from HCCS (Fig. 1, A and B). In the present study we have characterized the multiple roles of His-19 in our 4-step model. We have also further characterized the roles of the two cysteines, including a comprehensive analysis of HCCS complexes with cytochrome c substituted at Cys-15, Cys-18, His-19, and double and triple substitutions in the conserved heme attachment site. Resonance Raman (RR) spectroscopy was used to interrogate the heme environment in the HCCS complexes. The RR and UV-visible spectra are consistent with His-19 acting as the second axial ligand in the bis-His HCCScytochrome c complex (step 2). These data support the proposal that perturbations of heme (e.g. puckering) caused by the formation of both thioether bonds (step 3) lead to release of holocytochrome c from the HCCS active site (step 4).

**EXPERIMENTAL PROCEDURES**

**Construction of Strains and Plasmids**—Plasmids used in this study, pRGK403 (pGEX-HCCS: N-terminal GST-tagged WT human HCCS), pRGK405 (pBADcycs: WT human cytochrome c (CYCS)), pRGK417 (pBADcycs (C15S)), pRGK418 (pBADcycs (C18A)), pRGK419 (pBADcycs (H19A)), and pRGK420 (pGEX-HCCS (H154A)), have been described previously (12). Other oligonucleotide primer sequences and derived plasmids are reported in Table 1. Nucleotide substitutions were engineered using the QuikChange 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* Δ*ccm* strain RK103 (15).

**Imidazole Complementation and Bacterial Protein Extraction Reagent Functional Assay—*E. coli* strains were grown overnight and used to inoculate 5 ml of LB broth supplemented with appropriate antibiotics. These cultures were grown in the presence or absence of 10 mM imidazole (pH 7) at 37 °C with shaking at 200 rpm for 3 h, followed by induction with 0.1 mM D-thiogalactopyranoside 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 B-PER reagent (Thermo Scientific). Total protein was quantified by absorbance at 280 nm using a Nanodrop 1000 spectrophotometer (Thermo Scientific) and 100 μg of extracted protein was resolved by SDS-PAGE, transferred to nitrocellulose membranes, and analyzed by heme stain.

**Heme Stains and Sypro Ruby Protein Blot Staining**—Heme stains were performed as described previously (16). 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 chemiluminescent signal for the heme stain was developed using the SUPERSIGN Femto kit (Thermo Scientific) and detected with the ImageQuant LAS4000 Mini detection system (Fujifilm-GE Healthcare). 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.

**Protein Expression and Purification**—GST-HCCS proteins were expressed (with or without cytochrome c variants) and purified from the *E. coli* Δ*ccm* strain RK103 as described previously (12). Briefly, 100-ml starter cultures were grown overnight at 37 °C with shaking and used to inoculate 1 liter of LB broth supplemented with the appropriate antibiotics. Following 1 h growth of the 1-liter cultures at 37 °C with shaking at 120

---

**TABLE 1**

| Oligo ID | Sequence (5’-3’) | Constructed plasmid |
|---------|------------------|---------------------|
| 1 CYCS_C15S_C18A_Fwd | TTA TGA AGA GCT CCC AGA GCC ACA CCG TTG AAA AGA GAG CTC CCA GAG CGC CAC CGT TGA AAA GGG A | pBAD CYCS (C15S,C18A) |
| 2 CYCS_C15S_C18A_H19A_Fwd | GAA GAG CTC CCA CCG CAC CCC GCC ACC TGT GTA AAA AGA GAG | pBAD CYCS (C15S,C18A,H19A) |
| 3 CYCS_H19C_Fwd | AGT AAG GCC GCA CCG CCC CCC AGT GCC GCA CCG TGT AAA AGA GAG | pBAD CYCS (H19C) |
| 4 CYCS_H19G_Fwd | ATG AAG TTG GCC CAC TGG GAA CAC TGT GTA AAA AGA GAG | pBAD CYCS (H19G) |
| 5 CYCS_H19K_Fwd | ATG AAG TTG GCC CAC TGG GAA CAC TGT GTA AAA AGA GAG CTC CCA GAG CGC CAC CGT TGA AAA GGG A | pBAD CYCS (H19K) |
| 6 CYCS_H19M_Fwd | ATG AAG TTG GCC CAC TGG GAA CAC TGT GTA AAA AGA GAG | pBAD CYCS (H19M) |
| 7 CYCS_H19R_Fwd | CTG CTG TCT TCA GCT AGT GGT GTG CGA CAC TGG GAA CAC TGT GTA AAA AGA GAG CTC CCA GAG CGC CAC CGT TGA AAA GGG A | pBAD CYCS (H19R) |
| 8 CYCS_H19Y_Fwd | CTG CTG TCT TCA GCT AGT GGT GTG CGA CAC TGG GAA CAC TGT GTA AAA AGA GAG CTC CCA GAG CGC CAC CGT TGA AAA GGG A | pBAD CYCS (H19Y) |
---

[321x704]/H11032

**ASBMB**

**VOLUME 289 • NUMBER 42 • OCTOBER 17, 2014**
rpm, the cultures were induced with 0.1 mM isopropyl β-D-1-thiogalactopyranoside for expression of pGEX-HCCS for 5 h. For co-expression of the pBAD-cycS (cytochrome c 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 ultracentrifugation 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 pull-down of GST-HCCS protein (with or without the co-purified cytochrome c variants). Bound GST-HCCS protein or co-complexes were eluted with 20 mM reduced glutathione in 50 mM Tris (pH 8), 150 mM NaCl, 0.02% Triton X-100, concentrated in an Amicon Ultra Centrifugal Filter (Millipore) with either a 100,000 or 30,000 cutoff, and the total protein concentration was determined using the Bradford reagent (Sigma).

Cytochrome c Purification—Δc3cm 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 broth. Following 1 h growth of the 1-liter cultures at 37 °C with shaking, the cultures were induced with 0.1 mM isopropyl β-D-1-thiogalactopyranoside 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 (cytochrome c variants) overnight. 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 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 pull-down of positively charged proteins (including cytochrome c). Bound proteins were eluted with 50 mM Tris (pH 8), 150 mM NaCl, concentrated in an Amicon Ultra Centrifugal Filter (Millipore) with a 10,000 cutoff, and the total protein concentration was determined using a spectrophotometer at room temperature as described previously (17). 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, and 0.02% Triton X-100; for cytochrome c, 50 mM Tris (pH 8) and 500 mM NaCl). Chemically reduced spectra were generated upon the addition of solid sodium dithionite (sodium hydrosulfite) to the purified sample. Where specified, imidazole (1 mM, pH 7) was added to purified protein samples at 100 mM prior to the recording of spectra.

Reduced Pyridine Hemochrome—Pyridine extractions of purified proteins were performed as described previously (18). 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 dithionite (sodium hydrosulfite) and UV-visible spectra were recorded from 500 to 600 nm.

Resonance Raman (RR) Spectroscopy—All samples for RR were prepared in 50 mM Tris (pH 8), 150 mM NaCl, and 0.02% dodecyl maltoside. The extent of heme loading varied in the co-purified HCCS/cytochrome c complexes as follows: HCCS-WT cytochrome c, 11%; HCCS-C15S cytochrome c, 16%; HCCS-C18A cytochrome c, 22%; HCCS-H19M cytochrome c, 3%; and HCCS-H19A cytochrome c, 4%. The RR samples for ferric HCCS-WT cytochrome c, HCCS-C15S cytochrome c, and HCCS-C18A cytochrome c were 33 μM in heme; the HCCS-cytochrome c H19M and H19A mutants were 28 and 25 μM in heme, respectively. Ferrous HCCS/cytochrome c complexes were prepared under nitrogen from the ferric samples by addition of a 50-fold excess of sodium dithionite in a buffered solution.

Resonance Raman spectra were recorded with 413.1-nm excitation from a Kr+ laser or 514.5 nm emission from an Ar+ laser using the 135° backscattering geometry for collection of Raman-scattered light. The spectrometer was calibrated against Raman frequencies of toluene, dimethylformamide, acetone, and methylene bromide. Spectra were recorded at ambient temperature from the samples in spinning 5-mm NMR tubes. UV-visible absorbance spectra were recorded from RR samples before and after spectral acquisition to assess whether sample integrity had been compromised by exposure to the laser beam. Laser power at ferric and ferrous samples ranged from 6 to 9 milliwatt; no spectral artifacts due to photo-induced chemistry were observed with these irradiation powers.

RESULTS

HCCS Recognition of Cytochrome c Variants Possessing Single Cys, Double Cys, and Triple Cys/His Substitutions, and Analysis of Complexes Formed—Single Cys and His substitutions in the CXXCH site of cyt c still form complexes with the human HCCS. Using various approaches, we and others have suggested that Phe-11, Ile-10, and Lys-8 (Fig. 1B) need to be properly positioned within the predicted α-helix for HCCS-mediated synthesis of cyt c (12, 19, 20). We hypothesized that these residues along with the CXXCH motif comprise important contacts for apocytochrome c binding to HCCS and subsequent heme attachment. We wanted to further characterize the HCCS-cyt c complexes with single cysteine substitutions and examine the recognition and other properties of cyt c with multiple substitutions. We focus in this report on the cysteines and histidine.

Human HCCS was co-expressed and purified in the presence of its cognate cyt c variants (wild type (WT), single Cys, double Cys, and triple Cys/His). The WT cyt c, single Cys and double Cys variants all co-purify with HCCS, as shown by Sypro Ruby staining (Fig. 2A) and heme staining (Fig. 2B). As shown previously the single Cys substitutions have ~2-fold more cyt c trapped in the complex than WT cyt c (12). The double Cys mutant (which would not be detected by the heme stain because it cannot covalently link to the heme) exhibits the highest level of cyt c in the complex. We estimate this to be at least 4-fold higher than WT (see Fig. 2A, lane 4). This suggests that the double Cys mutant is fully trapped in complex with HCCS,
FIGURE 1. Model of HCCS-mediated cytochrome c assembly. Taken from San Francisco et al. (12). A, a four-step model for assembly, as discussed in the text. B, diagram of a PEP-FOLD generated structure for the first 20 residues of the human cytochrome c, with key residues described in the text, manually modeled with heme (in pink), and displayed using PyMol. The blue oval represents HCCS with the heme iron ligand His-154.

FIGURE 2. Characterization of purified complexes of HCCS with selected cytochrome c variants. A, Sypro Ruby-stained blot, and B, heme stain of WT HCCS co-purified with the indicated cytochrome c variants (lanes 1–5) or HCCS alone (lane 6). UV-visible absorption spectra of WT HCCS co-purified with C, WT cytochrome c; D, C15S cytochrome c; E, C18A cytochrome c; F, C15SC18A cytochrome c (double mutant); G, C15SC18AH19A cytochrome c (triple mutant); and H, HCCS alone (in the absence of cytochrome c, but with heme bound). Spectra of purified proteins are shown in black. Spectra of purified protein chemically reduced with sodium dithionite are shown in red. The α and β peaks in panel D were enlarged 3-fold for clarity. Arrows depict the wavelength (nm) of absorption (Abs) maxima.
more so than the single Cys mutants or WT. We previously showed that small amounts of Cys-15 (~8%) and Cys-18 (~3%) substituted cytochromes were released from the complex into the cytoplasmic fraction (12). It is possible that the double mutant is not released, facilitating the trapping of more complex. The triple mutant could not be co-purified (Fig. 2A, lane 5), showing no apocytc upon purification of HCCS, similar to HCCS expressed and purified in the absence of cyt c expression (lane 6). This suggests that multiple residues (e.g. Lys-8, Ile-10, Phe-11, Cys-15, Cys-18, and His-19 (12, 19, 20)) form contacts with HCCS and that in this case, it was necessary to remove three of the contacts to prevent stable binding to HCCS.

UV-visible absorption spectra of the complexes (Fig. 2, C–G) and HCCS alone (Fig. 2H) provided information on the state of heme at the active site of HCCS. Previously we have shown that HCCS alone shows a Soret maximum at ~423 nm (12), and that this spectrum does not change (i.e. red-shift toward a more reduced state) when the reductant sodium dithionite is added under ambient atmospheric conditions (Fig. 2H, red line). The HCCS complexes with cyt c WT, C15S, and C18A variants each are purified in the oxidized state with a blue-shifted Soret maximum (412–415 nm). Upon reduction, these show red-shifted Soret maxima (420–424 nm) and α maxima between 553 and 562 nm (Fig. 2, C–E), as shown previously (12). HCCS complexes with the double Cys variant were again purified in the oxidized state and when reduced, a red-shifted Soret (426 nm) and α maximum at 561 nm were observed (Fig. 2F). The changes observed upon chemical reduction in WT, Cys-15, Cys-18, and double Cys are consistent with the proposal that in each of these complexes His-19 provides the second axial ligand to the heme iron. HCCS complexes with the triple substitution, which does not appear to bind the apocytc substrate, shows spectra (Fig. 2G) nearly identical to HCCS only (Fig. 2H). This is consistent with a lack of apocytc substrate binding.

Heme stains of the single Cys complexes, as shown previously (12), exhibit evidence of covalent bonding to the heme within the complex (Fig. 2B), but spectra were unlike those expected with a single covalent linkage. C15S complexes showed split α maxima (555 and 560 nm) and C18A showed an α maximum at 562 nm (Fig. 2, D and E). Single Cys covalent linkages often, but not always, show α maxima around 555 nm in their reduced native state (21, 22). Pyridine hemochrome spectra (Fig. 3, A and B) of the complexes, reduced with dithionite, show that heme in the WT complex likely has two covalent linkages (549 nm), the single cysteines have one covalent linkage (553 nm), and that the double Cys, triple substitution, and HCCS only (no cyt c) show no covalent linkages and thus are b-type heme (556 nm). The unique spectra of the purified complexes are most likely a reflection of the unique heme environment at the active site in each HCCS complex.

FIGURE 3. Pyridine hemochrome spectra (A and B) and Soret region absorptions with and without imidazole (C–H) of HCCS/cyt c complexes (or HCCS alone). A, sodium dithionite-reduced pyridine hemochrome spectra of WT HCCS co-purified with WT cytochrome c (black), C15S cytochrome c (pink), or C18A cytochrome c (blue). B, sodium dithionite-reduced pyridine hemochrome spectra of WT HCCS co-purified with WT cytochrome c (black), C15S,C18A cytochrome c (green), C15S,C18A,H19A (orange), and alone (in the absence of cytochrome c) (red). UV-visible absorption spectra showing the Soret peak of WT HCCS co-purified with: C, WT cytochrome c; D, C15S cytochrome c; E, C18A cytochrome c; F, C15S,C18A cytochrome c; G, C15S,C18A,H19A cytochrome c; and H, alone (in the absence of cytochrome c). Spectra of purified protein are shown in black. Spectra of purified protein in the presence of 100 msi imidazole are shown in purple. Arrows depict the wavelength (nm) of absorption (Abs) maxima.
Mechanisms of Mitochondrial Cytochrome c Biogenesis

We have shown previously that HCCS has an axial ligand provided by residue His-154 and an unidentified second axial ligand (12). This second ligand can be replaced in vitro by imidazole, whereupon the HCCS is air oxidized, resulting in a blue-shifted Soret maximum, from 424 to 414 nm (Fig. 3H). To further examine whether the His-19 residue is replacing one of the axial ligands (from HCCS) in all complexes where His-19 is present, we added 100 mM imidazole to the complexes. Soret maxima in the HCCS complexes with WT, C15S, C18A, and double Cys mutants were not shifted upon imidazole addition (Fig. 3, C–F), consistent with the proposal that the His-19 imidazole side chain already occupies the second axial ligand position. Soret maxima of the triple Cys/His mutant complex (Fig. 3G) and HCCS alone (Fig. 3H) were both blue shifted 8–10 nm upon addition of imidazole. Thus the second axial ligand to heme in these two proteins can be provided by exogenous imidazole, thereby facilitating oxidation.

Mechanisms of Recognition, Attachment, and Release Orchestrated by the Cytochrome c His-19 Residue (of CXXCH)—In heme proteins like myoglobin, when the proximal histidine ligand (His-93) is replaced with a glycine, heme no longer binds to the recombinant H93G protein in vivo (23). However, adding 10 mM exogenous imidazole during growth allows the H93G myoglobin to again bind heme in vivo (23). Imidazole binds to the H93G “cavity” and facilitates the binding of heme. Our group has used this “imidazole correction” to analyze many of the heme-binding proteins (with histidines) in cyt c assembly systems I, II, and III (12, 17, 24). (System III is the HCCS, whereas systems I and II are in prokaryotes.) These proteins with conserved histidines, shown to bind heme, are no longer functional when substituted with alanine or glycine. Indeed, the HCCS H154A mutant was restored by exogenous imidazole for its cyt c assembly function in recombinant E. coli (12). Likewise, if the sole function of the cyt c His-19 is to bind heme iron in HCCS, then imidazole should correct the function of cyt c H19A or H19G variants. (That is, exogenous imidazole should bind to the heme and prepare it for thioether bond formation.) Neither the H19A nor H19G variant was corrected for heme attachment (cyt c assembly) by exogenous imidazole (Fig. 4). As shown previously (12), the HCCS H154A variant is corrected for function (Fig. 4). We propose that in addition to providing the ligand to heme in complex with HCCS, an additional role of His-19 is to spatially position the two cysteine thiol s adjacent to the heme vinyls for thioether formation. Free imidazole would serve the first role (as it does with HCCS H154A) but would be unable to position the adjacent CXXC motif because, not being bonded to the CXXC motif, free imidazole binding cannot constrain the CXXC conformation to template its regiospecific cross-linking reactions with the heme vinyl groups.

Whereas the apocytc c substrates are still recognized by HCCS when cysteines or histidine are substituted, the specific roles of His-19 clearly involve more than just recognition. That is, His-19 is required for (i) liganding heme iron (in complexes with HCCS) and (ii) positioning the CXXC for attachment (Fig. 1, steps 2 and 3). We wanted to determine whether other possible side chains that can coordinate to heme iron (e.g. Met, Tyr, and Cys) would substitute for these two roles of His-19 in the cyt c assembly. We examined HCCS complexes with cyt c containing H19G, Ala, Met, Cys, Tyr, Arg, and Lys substitutions. Each complex was purified from membrane fractions using Triton X-100 and glutathione columns. SDS-PAGE and StryoRuby staining for protein showed approximately equal levels of GST-HCCS and cytochrome c (Fig. 5A). The staining for heme in these complexes showed that for both WT (Fig. 5B, lane 1) and H19M cyt c (lane 6), significant levels of heme was covalently attached to the cyt c. For all other His-19 substitutions, the majority of heme remained with the HCCS. (Although SDS-PAGE typically dissociates heme that is non-covalently bound to a heme protein (e.g. with b-hemes), the SDS-PAGE conditions used here employ room temperature solubilization in SDS sample buffer, no DTT, and it has been shown previously that with these conditions some b-heme remains with the HCCS polypeptide (12).) UV-visible spectra of complexes were also recorded. Under ambient atmospheric conditions, it was possible to chemically reduce only the H19M complex to yield an a and b absorption (Fig. 5D) like the WT (Fig. 5C). However, the Soret regions of complexes with all substitutions looked similar, at ~424 nm, regardless of oxidation state. Because these Soret absorption results were similar to the HCCS only preparations, we hypothesized that possibly the H19M is a mixture of c-heme and b-heme, whereas the other substitutions are largely b-heme (i.e. non-covalently linked). In this case, the Soret of H19M reflects the b-heme species. We recorded pyridine hemeochrome spectra from heme extractions of the reduced complexes to identify the heme types. Although the WT complex yielded a hemochrome maximum at 551 nm (Fig. 5C, inset), indicative of mostly c-type, the H19M was 553 nm (Fig. 5D, inset), thus a mixture, and the other His-19 substitutions at 555 nm (Fig. 5, E–J), thus mostly b-hemes. Consistent with H19M complexes representing a mixture of b- and c-type hemes, the resonance Raman spectra also suggested it has similarities to H19A (b-heme) and WT (c-heme) (see below). We conclude that the methionine substitution may satisfy the two roles of His-19 described above, albeit with less efficiency.

![Figure 4: Cytochrome c His-19 is not just a heme iron ligand like the His-154 of HCCS: functional correction of HCCS H154A but not cytochrome c H19A or H19G by exogenously added imidazole.](image-url)
Thus, the H19M was able to at least partially accomplish steps 2 and 3 (Fig. 1A), therefore we determined whether it also could fulfill step 4, the “release” step, yielding a biosynthesized cyt c with axial bis-met ligation. Recombinant cultures, expressing the HCCS and cyt c genes were harvested and fractionated, and the soluble fractions (containing released cyt c) were studied further. Proteins were partially purified over cation exchange columns because the isoelectric point (pI) of the human cyt c is nearly at pH 10 (based on pI calculations of the full-length protein). Fractions eluted with high salt were analyzed by UV-visible spectra (Fig. 6A) and by SDS-PAGE and heme stains (Fig. 6B) to quantitate levels of released holocyt c. Only the H19M substitution yielded biosynthesized cyt c, with a spectrum distinct from the WT, including a split Soret band. Both the WT and H19M are purified in the reduced state, yielding nearly identical α (550 nm) and β (520 nm) absorption maxima (Fig. 6A). The amounts of H19M purified ranged from 1 to 5% of the WT holocyt c yield, whereas all other His-19 variants yielded less than 0.1%, the limit of detection in these experiments. A previous report has suggested that arginine can replace the His-19 axial ligand to some extent (25). We did not produce any H19R product in our study. We propose that the Met-19-iron interaction is less effective (than His-19-iron) at “pulling” the heme from the HCCS His-154 ligand (step 4).

**FIGURE 5.** Methionine substitution of cytochrome c His-19 is capable of facilitating the covalent attachment of heme to cytochrome c. A, Sypro Ruby-stained blot, and B, heme stain of WT HCCS co-purified with WT cytochrome c (lane 1) or the indicated cytochrome c His-19 variants (lanes 2–8). UV-visible absorption spectra of WT HCCS co-purified with: C, WT cytochrome c; D, H19M cytochrome c; E, H19C cytochrome c; F, H19K cytochrome c; G, H19A cytochrome c; H, H19G cytochrome c; I, H19R cytochrome c; and J, H19Y cytochrome c. Spectra of purified protein are shown in black. Spectra of purified protein chemically reduced with sodium dithionite are shown in red. The inset spectra in C–J represent sodium dithionite-reduced pyridine hemochrome spectra of each respective complex. Arrows depict the wavelength (nm) of absorption (Abs) maxima.
Mechanisms of Mitochondrial Cytochrome c Biogenesis

Therefore, these results suggest that His-19 also plays a key role in the release step 4 (Fig. 1A).

Resonance Raman Spectroscopy of the HCCS Complexes—To provide further insight into the heme environments in its purified ternary complexes with HCCS and each of the cyt c constructs, we examined the complexes by RR spectroscopy. The RR signature of heme is responsive to structural and conformational changes that it undergoes during biochemical transformations, such as those associated with the biosynthesis of holocytochrome c. Cross-linking of the heme 2- and 4-vinyl groups to the Cys residues of the CXXCH motif in cyt c lowers the heme symmetry by inducing equilibrium out-of-plane distortion of the heme and modifying the vinyl substituents (28) (Scheme 1).

We have recorded high and low frequency, Soret (or B)-excited RR spectra of the complexes in both their ferrous and ferric states. Spectra of the ferrous form of HCCS, which is thought to be the active form, are shown in Fig. 7, along with the Q-excited spectrum of the WT complex in the core size marker region. Interpretation of the spectra of the ferric complexes, shown in Fig. 8, is consistent with that of the ferrous complexes. To the best of our knowledge, the RR spectra reported here for C15S and C18A mutants provide the first RR spectroscopic signatures of hemes cross-linked to cyt c through a single thioether bridge.

In-plane Skeletal and Substituent Modes—High-frequency RR spectra of HCCS in complex with heme and WT cyt c, C15S, C18A, H19M, or H19A cyt c variants are shown in Fig. 7A. This region of the heme b spectrum is dominated by bands arising from totally symmetric skeletal vibrations that are characterized by in-plane motions of the porphyrin core atoms. The largest band in this range of the spectrum is νg, which is sensitive to the porphyrin π* electron density. The heme π* density is well correlated with the oxidation state of iron, except in its CO and NO complexes. The νg frequencies in Fig. 7A range from 1355 to 1359 cm⁻¹, consistent with the presence of ferrous heme in these complexes (29).

The bands near 1490 cm⁻¹ arise from ν3, a mode comprising significant Cβ-Cβ stretching and known to be particularly sensitive to the spin state of the iron center. This frequency is definitive for low spin (LS) ferrous hemes. Note that the spectra of the complexes comprising the H19A and H19M cyt c variants exhibit two ν3 bands, one attributable to hexacoordinate (6c) LS heme at 1490 cm⁻¹ and another at 1469 cm⁻¹, typical of pentacoordinate (5c) high spin (HS) ferrous heme. The UV-visible spectra in Fig. 2H are typical of a 6c-LS ferrous heme. Thus, the b-heme in HCCS is expected to exhibit one ν3 band near 1490 cm⁻¹. Even though evidence suggests that reaction of HCCS with H19A cyt c does not yield holocytochrome c, the RR spectrum makes it clear that interaction with this unproductive cyt c variant triggers scission of an iron-axial ligand bond in holocytochrome c. Interestingly, the relative ν3 intensities of the 5c-HS and 6c-LS hemes invert for the cyt c H19M mutant. This is attributed to an increase in the population of the 6c-LS heme due to a small extent of heme coordination by Met-19.

The next highest frequency band in HCCS-cyt c WT occurs at 1591 cm⁻¹. This is actually an envelope of overlapping bands that comprise contributions from the ν2 (~1597 cm⁻¹, A1g in D₄h) and ν19 (~1580 cm⁻¹, A2g in D₄h) modes. The assignments of these overlapping bands were disentangled by Q-band excitation, which selectively enhances Raman scattering by the non-totally symmetric, including A2g modes. A2g modes give rise to anomalously polarized bands. The inset of Fig. 7A shows the parallel and perpendicular polarized, Q-excited RR spectra.
of the HCCS-cyt c WT complex. These spectra clearly reveal three anomalously polarized bands at 1582, 1398, and 1313 cm\(^{-1}\), corresponding to the \(\nu_{1\alpha}, \nu_{2\alpha},\) and \(\nu_{21}\) assignments, respectively, in the Q-excited RR spectrum of cyt c (30). The depolarized band at 1538 cm\(^{-1}\) corresponds to \(\nu_{11},\) a \(B_{1g}\) mode, which also appears in the B-excited spectra at a frequency consistent with a 6c-LS heme. B-excited RR spectra of D\(_{th}\) metalloporphyrins do not typically contain a prominent \(\nu_{1\alpha}\) band because, due to its \(A_{2g}\) symmetry, its scattering is not enhanced. However, the prominence of \(\nu_{1\alpha}, \nu_{2\alpha},\) and \(\nu_{21}\) in the HCCS complexes examined here is consistent with a symmetry lowering due to an equilibrium out-of-plane distortion similar to that observed in holocyt c (30). Interestingly, the relative intensities of the \(\nu_{2\alpha}\) band is substantially greater in the C15S and C18A mutants. This band is small in the HCCS-WT cyt c complex, presumably due to only a residual population of uncross-linked vinyl substituents trapped in the complex with HCCS.

Low-frequency RR spectra of HCCS in complex with heme and the aforementioned cyt c constructs are shown in Figs. 7, B, and C. In heme b spectra, \(\nu_{2}\) gives rise to the most resonance enhanced band in this region of the B-excited RR spectra. That is not the case for cyt c. By contrast with heme b, the nearby \(\nu_{C_{as}-S}\) bands (see Scheme 1 for atom identities) are among the most strongly resonance enhanced bands in the B-excited RR spectrum of cyt c (30). The physical basis for this striking resonance enhancement is currently not well understood, although, given the extent to which both \(C_{as}-S\) bonds are oriented away from the mean porphyrin plane (31), it likely involves a combination of symmetry lowering and hyperconjugation of that bond into the \(a_{1u}\) (in \(D_{th}\)) porphyrinate HOMO (26). Based on the breadth and asymmetry of the RR feature at 681 cm\(^{-1}\) (Fig. 7B), and by analogy with the cyt c spectrum, that feature is an envelope of bands. Based on the positions of its shoulders, it comprises two bands assigned to \(\nu_{C_{as}-S}\) at \(\sim 681\) and \(\sim 690\) cm\(^{-1}\) and a third, relatively weak shoulder/band assigned to \(\nu_{2}\) at \(\sim 700\) cm\(^{-1}\). These frequencies and relative intensities are consistent with those of the corresponding modes in the B-excited spectrum of holocyt c, all of which give rise to polarized bands (30). In the spectra of HCCS-cyt c C15S and C18A, there is only one band in the \(\nu_{C_{as}-S}\) region at 676 cm\(^{-1}\), consistent with their inability to form thioether cross-links to both the 2- and 4-heme vinyl groups. The 676 cm\(^{-1}\) bands could arise from \(\nu_{2}\) in the non-fully cross-linked complexes. However, in the spectra of both Cys mutants, weak bands occur near 700
Mechanisms of Mitochondrial Cytochrome c Biogenesis

FIGURE 8. High and low frequency, B-excited RR spectra of ferric HCCS/cyt c complexes. A, high frequency; and B, low frequency window of the 413.1 nm excited RR spectra of ferric WT HCCS co-purified with WT cytochrome c (black), C15S cytochrome c (red), C18A cytochrome c (blue), H19M cytochrome c (magenta), and H19A cytochrome c (green). All complexes were in 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 0.02% dodecyl maltoside.

cm⁻¹, in the frequency range expected for νₛ, suggesting that symmetry lowering of the porphyrin π system is sufficient with either one or two cross-links to diminish its resonance enhancement in the B-excited RR spectra. Moreover, we suggest that the aforementioned resonance enhancement of νCa-S scattering indicates that, like cyt c, the single Ca-S bonds in these mutants are oriented significantly out of the mean porphyrin plane.

The non totally symmetric ν₁₅ bands at 747 cm⁻¹ (B₁ₓ in D₄h) are stronger than the ν₁ bands in these spectra. This has been attributed to vibronic coupling of Bₓ and Bₙ states thought to result from symmetry lowering in the cross-linked heme c (30). By contrast, the H19A and H19M mutants both exhibit strong ν₁ bands near 675 cm⁻¹ with I(ν₁):I(ν₄₈) ratios typical of heme b spectra (32). These features are consistent with minimal cross-linking between cyt c and the heme b in HCCS-heme-cyt c H19A and H19M.

In D₄h iron porphyrinates, ν₄₈ is a Raman-forbidden mode of Eₛ symmetry. In cyt c, it is activated by symmetry-lowering porphyrin distortions that give rise to puckering, in which the cross-linked edge of the porphyrin is folded about the meso carbon atom between the cross-linked pyrrole rings (see Scheme 1). This Cₛ distortion gives rise to bands near 640 cm⁻¹ in the spectra of the HCCS-heme-cyt c WT, C15S and C18A complexes (Fig. 7B). Interestingly, the single ν₄₈ frequency in the D₄h system is split into two frequencies in the WT (632 and 643 cm⁻¹) and C15S (631 and 647 cm⁻¹), consistent with the aforementioned Cₛ porphyrin distortion. By contrast, ν₄₈ is activated in HCCS-heme-cyt c C18A, but splitting is not detected. This suggests diminished puckering of its 2,4-β-pyrrole edge relative to that imposed in the C15S complex. This, we hypothesize, has implications for the role of the residues at positions 15 and 18 in the assembly mechanism (see below).

The 400 cm⁻¹ region of the spectra in Fig. 7C is revealing with regard to thioether cross-links, as the substituent bending modes (designated as δ) at β-pyrrole positions 2 and 4 give rise to bands near this frequency. In the B-excited RR spectra of heme b, there are typically two such δCₛ-Cₐₚₐₜₜ modes in the 415–420 cm⁻¹ range (32). However, the Cₛ-Sₐₚₐₜ modes at 394 and 401 cm⁻¹ (30, 33). In our spectrum of HCCS-cyt c WT, the δCₛ-Cₐₚₐₜ and δCₛ-Cₐₚₐₜ bands are centered around 400 and 414 cm⁻¹, respectively. Based on the B-excited RR spectra of cyt c (30, 33), we assign these bands to envelopes that comprise both bands from each type of substituent bending mode. The broadening that precedes resolution of both bands from each mode type is likely attributable to inhomogeneous broadening in the detergent-solubilized HCCS-cyt c complex. However, in Cys-15 and Cys-18 mutants complexes, it is clear that the frequencies of the vinyl and thioether substituents are different when they occur at the 2- and 4-β-pyrrole positions. Thus, the RR spectra of the HCCS-cyt c C15S and C18A mutants support the formation of single thioether cross-links between the heme and cyt c at the vinyl position alternate to the mutation.

The region of the spectra near 350 cm⁻¹ exhibits bands arising from modes having Fe-N porphyrine character. In the B-excited RR spectrum of a D₄h iron porphyrinate, this region contains a single strongly enhanced band near 345 cm⁻¹ corresponding to the totally symmetric Fe-N stretching mode, ν₂₀ (26, 27, 32). The non-totally symmetric Fe-N stretches are designated as ν₅₀ and, because of their noncentrosymmetric Eₛ sym-
Mechanisms of Mitochondrial Cytochrome c Biogenesis

OCTOBER 17, 2014 • VOLUME 289 • NUMBER 42
JOURNAL OF BIOLOGICAL CHEMISTRY 28805

metry, they are Raman forbidden. Protein bridging of the 2- and 4-β-pyrole carbons through the thioether bridges pucks the porphine ring, thereby lowering its symmetry to approximately C₅ (31). In this point group, ν₃₀ mode becomes Raman allowed and resonance enhanced in the B-excited RR spectrum. Accordingly, the spectrum of HCCS-cyt c WT in Fig. 7C exhibits two overlapping bands at 343 and 353 cm⁻¹, attributable to the ν₅ and ν₂₀ modes, respectively. Interestingly, these modes are differentially resonance enhanced in C15S and C18A mutant complexes. We attribute that enhancement pattern to differences in the porphyrin distortion between the C15S and C18A complexes. Specifically, substitution of the methyl side chain in the Ala variant for the longer ethylthiol side chain of Cys relaxes the steric constraints that the complex can impose on the porphyrin conformation. Although Ser-15 cannot be cross-linked to the 2-β-pyrole position, the activation of ν₃₀ and several out-of-plane modes (see below) in the RR spectrum of the C15S complex suggests that it is capable of imposing the puckered conformation of holocytc through steric interactions that mimic those of Cys-15 in HCCS-heme bcytc WT. Thus, the RR spectra of the WT, C15S, and C18A complexes suggest that the assembly mechanisms require the HCCS-heme bcytc complex to pucker the 2,4 edge of the porphine ring for release. This region of the C18A spectrum is dominated by the band at 343 cm⁻¹, the same frequency observed for ν₅ in the His-19 mutant complexes wherein the heme remains unattached (i.e. heme b).

Finally, in D₄ᵥ iron porphyrinates, ν₅₁ is an Eₚ mode and, therefore, Raman forbidden (26, 27). However, it is activated by the symmetry-lowering porphyrin distortions in the HCCS-heme-cyt c WT and C15S complexes, giving rise to RR bands at 306 and 300 cm⁻¹, respectively (Fig. 7C). The inactivity of ν₅₁ in the C18A mutant is further evidence that its complex cannot enforce significant heme puckering. The inactivity of ν₅₁ in H19A and H19M mutants is consistent with the lack of other distortion-sensitive bands and their lack of significant holocytc production.

Out-of-plane Porphyrin Deformation Modes—The out-of-plane deformation modes, γ₅ and γ₁₅ have A₂u and B₃u symmetry, respectively, in D₄ᵥ metalloporphyrins. As non-centrosymmetric u modes, they are both Raman forbidden. Thus, as expected, there are no bands attributable to these modes in the B-excited RR spectra of the HCCS-heme-cyt c C18A, H19A, or H19M. Consistent with their activation of other Raman forbidden modes (see below), Fig. 7B shows bands arising from γ₅ and γ₁₅ at 735 and 716 cm⁻¹ in the spectrum of the WT complex. The spectrum of the C15S complex only contains γ₁₅ at 714 cm⁻¹. The reason for this selectivity is not clear. However, that these modes are activated in the spectra of the WT and C15S complexes clearly show that they impose an equilibrium out-of-plane distortion that removes the iron porphyrinate center of symmetry.

The centrosymmetric γ₃₁ mode is doubly degenerate (Eₚ) in D₄ᵥ metalloporphyrins. As such, it is Raman allowed, but due to its out-of-plane coordinates, it is only poorly enhanced with π-π* excitation (27). The low RR intensities of γ₃₁ in the H19A and H19M spectra of Fig. 7C suggest that the hemes in these complexes exhibit only moderate out-of-plane distortion. By contrast, the γ₂₁ bands in the spectra of HCCS-heme-cyt c WT, C15S and C18A are noticeably more intense, suggesting that the complex is able to increase the out-of-plane distortion when the His-19 residue of cyt c is available to coordinate the heme. Moreover, the double degeneracy is lost in the WT complex, as evinced by the splitting of γ₁₅ into two bands at 549 and 564 cm⁻¹, clearly showing that the rotational symmetry of the heme has been lost. We take this as further evidence that the WT complex imposes C₂, puckering of the heme, similar to that seen in the crystal structure of holocytc (31).

Possible Fe-S₅₁ Mode in HCCS-Heme-Cytochrome c H19M—The ν₅ frequencies in Fig. 7A reveal that heme exists as a mixture of 5c-HS and 6c-LS Fe(II) in HCCS complexes with H19A and H19M. Assuming that the RR cross-sections for ν₅ are the same for these two mutant complexes, the H19M complex has a larger fraction of its heme in the 6c-LS state. One interpretation of this difference is that the thioether side chain of Met-19 is coordinated to the Fe(II) center in a small fraction of the complexes and that, by virtue of this coordination to cyt c, those complexes could proceed beyond step 2 of the proposed mechanism to the cross-linking and release steps.

The ⁵⁷Fe nuclear resonance vibrational spectrum of ferrous cyt c provides strong evidence for a band at 372 cm⁻¹ having significant Fe-S₅₁ stretching character (33). This out-of-plane mode is also present in the B-excited RR spectrum. Thus, it is reasonable to speculate that, if Met-19 were to coordinate to the heme iron in ferrous HCCS-heme-cyt c H19M, it would give rise to a RR band of similar frequency having Fe-S₅₁ stretching character. The spectrum of the H19M complex in Fig. 7C clearly exhibits a band in this Fe-S₅₁ stretching region at 385 cm⁻¹. Although it occurs at a frequency 12 cm⁻¹ higher than the band in cyt c, this frequency difference could be accounted for by differences in the trans effect of their His ligands and/or differences in the coupling of the Fe-S₅₁ bond stretch and porphyrin out-of-plane coordinates. This possible coordination is of mechanistic interest, as it could, within the mechanistic model emerging from this work, account for the ability of the H19M complex to generate small amounts of holocytc c. However, this region of the spectrum is also home to the propionate bending bands, δC=CHCC=O. Therefore, based on the data at hand, it is not possible to unambiguously assign the 385 cm⁻¹ band to a Fe-S₅₁-1 stretching mode. That assignment would require ⁳⁵S isotope labeling of the cyt c H19M.

DISCUSSION

The following conclusions concerning the mechanisms of HCCS function can be made from this study. The cysteines and histidine within the cyt c heme binding (attachment) site play more active roles in assembly than just as recognition determinants by the HCCS. Single Cys, His, and double Cys variants of cytochrome c are each still recognized by the HCCS and co-purify. A triple mutant (both Cys and His) does not co-purify. UV-visible and RR spectroscopy of purified complexes revealed that each trapped cyt c (C15S, C18A, H19A, and WT) showed very distinct spectroscopic profiles. To our knowledge, these are the first published RR spectra of single thioether-attached cytochromes. These data support the contention that such spectra reflect the active site of HCCS with distinct heme envi-
Mechanisms of Mitochondrial Cytochrome c Biogenesis

environments for each variant, and that His-19 is an axial ligand (in the complexes where His-19 is present). We suggest that the single cysteine substitutions, each with a single thioether formed at the HCCS active site, do not efficiently release from this complex because both thioethers are needed to alter the conformation of the heme to weaken binding by the HCCS enzyme (for release). The RR spectra of HCCS complexes with WT, C15S, and C18A cyts c indicate that the WT exhibits more puckering than C15S, and C15S exhibits more puckering than the C18A variant. This is consistent with the results that C15S is released (step 4) at ~8% WT levels and C18A at only 3% WT levels (12). We propose that puckering induced by spontaneous thioether bond formation reduces the interaction of heme with HCCS, leading to release (step 4).

As depicted in the model (Fig. 1), His-19 (of the CXXCH site) is critical for (i) providing the second axial ligand to the heme at the HCCS active site (step 2); (ii) positioning the adjacent thiols of CXXC in close proximity to the two vinyl groups of heme for attack (step 3); and (iii) once the two thioethers are formed (step 3), for pulling the heme away from the HCCS active site (and the His-154 axial ligand), thus releasing the holocytocrome c (step 4) for final folding. During this folding process cyt c Met-81 would replace the HCCS His-154 ligand. We discovered that methionine can partially replace the roles played by the His-19 residue, thus it is possible to biosynthesize a bis-methionine cyt c. We currently are optimizing the biosynthesis of this H19M, bis-met cyt c for future studies.

Acknowledgment—We thank Dr. Molly Sutherland for critically reading our manuscript and providing helpful suggestions.

REFERENCES

1. Nicholls, D. G., and Ferguson, S. (2013) Bioenergetics, 4th Ed., Academic Press
2. Dickerson, R. E., Takano, T., Eisenberg, D., Kallai, O. B., Samson, L., Cooper, A., and Margolish, E. (1971) Ferricytochrome c I. General features of the horse and bovine proteins at 2.8 Å resolution. J. Biol. Chem. 246, 1511–1535
3. Dumont, M. E., Ernst, J. F., Hampsey, D. M., and Sherman, F. (1987) Identification and sequence of the gene encoding cytochrome c heme lyase in the yeast Saccharomyces cerevisiae. EMBO J. 6, 235–241
4. Visco, C., Tanuchi, H., and Berlett, B. S. (1985) On the specificity of cytochrome c synthetase in recognition of the amino acid sequence of apocytochrome c. J. Biol. Chem. 260, 6133–6138
5. Zollner, A., Rödel, G., and Haid, A. (1992) Molecular cloning and characterization of the Saccharomyces cerevisiae CYT2 gene encoding cytochrome-c1-heme lyase. Eur. J. Biochem. 207, 1093–1100
6. Hamel, P., Corvest, V., Giegé, P., and Bonnard, G. (2009) Biochemical requirements for the maturation of mitochondrial c-type cytochromes. Biochim. Biophys. Acta 1793, 125–138
7. Bernard, D. G., Gabilly, S. T., Dujardin, G., Merchant, S., and Hamel, P. P. (2003) Overlapping specificities of the mitochondrial cytochrome c and c1 heme lyases. J. Biol. Chem. 278, 49732–49742
8. Liu, X., Kim, C. N., Yang, J., Jemmerson, R., and Wang, X. (1996) Induction of apoptotic program in cell-free extracts: requirement for dATP and cytochrome c. Cell 86, 147–157
9. Kiryu-Seo, S., Gamo, K., Tachibana, T., Tanaka, K., and Kiyama, H. (2006) Unique anti-apoptotic activity of EAAC1 in injured motor neurons. EMBO J. 25, 3411–3421
10. Schafer, L., Ballabio, A., and Zoghbi, H. Y. (1996) 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 34, 166–172
11. Kranz, R., Lill, R., Goldman, B., Bonnard, G., and Merchant, S. (1998) Molecular mechanisms of cytochrome c biogenesis: three distinct systems. Mol. Microbiol. 29, 383–396
12. San Francisco, B., Bretsnyder, E. C., and Kranz, R. G. (2013) Human mitochondrial holocytochrome c synthase’s heme binding, maturation determinants, and complex formation with cytochrome c. Proc. Natl. Acad. Sci. U.S.A. 110, E788–E797
13. Kranz, R. G., Richard-Fogal, C., Taylor, J. S., and Frawley, E. R. (2009) Cytochrome c biogenesis: mechanisms for covalent modifications and trafficking of heme and for heme-iron redox control. Microbiol. Mol. Biol. Rev. 73, 510–528
14. Allen, J. W. (2011) Cytochrome c biogenesis in mitochondria: systems III and V. FEBS J. 278, 4198–4216
15. Feissner, R. E., Richard-Fogal, C. L., Frawley, E. R., Loughman, J. A., Earley, K. W., and Kranz, R. G. (2006) Recombinant cytochromes c biogenesis systems I and II and analysis of haem delivery pathways in Escherichia coli. Mol. Microbiol. 60, 563–577
16. Feissner, R., Xiang, Y., and Kranz, R. G. (2003) Chemiluminescence-based methods to detect subpicomole levels of c-type cytochromes. Anal. Chem. 75, 90–94
17. Frawley, E. R., and Kranz, R. G. (2009) CcsBA is a cytochrome c synthetase that also functions in heme transport. Proc. Natl. Acad. Sci. U.S.A. 106, 10201–10206
18. Berry, E. A., and Trumpower, B. L. (1987) Simultaneous determination of hemes a, b, and c from pyridine hemochromogen spectra. Anal. Biochem. 161, 1–15
19. Kleingardner, J. G., and Bren, K. L. (2011) Comparing substrate specificity between cytochrome c maturation and cytochrome c heme lyase systems for cytochrome c biogenesis. Metalloids 3, 396–403
20. Verissimo, A. F., Sanders, J., Daldal, F., and Sanders, C. (2012) Engineering a prokaryotic apocytochrome c as an efficient substrate for Saccharomycenses cerevisiae cytochrome c heme lyase. Biochem. Biophys. Res. Commun. 424, 130–135
21. Tanaka, Y., Kuhota, I., Amachi, T., Yoshizumi, H., and Matsubara, H. (1990) Site-directed mutated human cytochrome c which retains heme c via only one thioether bond. J. Biochem. 108, 7–8
22. Roll, F. I., and Mauk, A. G. (2002) Spectroscopic properties of a mitochondrial cytochrome c with a single thioether bond to the heme prosthetic group. Biochemistry 41, 7811–7818
23. Arrick, D. (1994) Replacement of the proximal ligand of sperm whale myoglobin with free imidazole in the mutant His-93→Gly. Biochemistry 33, 6546–6554
24. San Francisco, B., Bretsnyder, E. C., Rodgers, K. R., and Kranz, R. G. (2011) Heme ligand identification and reox properties of the cytochrome c synthetase, CcmF. Biochemistry 50, 10974–10985
25. Sorrell, T., Martin, P., and Bowden, E. (1989) A novel, functional variant of cytochrome c replacement of the histidine ligand with arginine via site-directed mutagenesis. J. Am. Chem. Soc. 111, 766–767
26. Li, X. Y., Czernuszewicz, R. S., Kincaid, J. R., Stein, P., and Spiro, T. G. (1990) Consistent porphyrin force field: 2. nickel octaethylporphyrin skeletal and substituent mode assignments from nitrogen-15, meso-d4, and meso-16Raman and infrared isotope shifts. J. Phys. Chem. 94, 47–61
27. Li, X. Y., Czernuszewicz, R. S., Kincaid, J. R., Pe, Y. O., and Spiro, T. G. (1990) Consistent porphyrin force field: 1. normal-mode analysis for nickel porphyrin and nickel tetraphenylporphine from resonance Raman and infrared spectra and isotope shifts. J. Phys. Chem. 94, 31–47
28. Li, X. Y., Czernuszewicz, R. S., Kincaid, J. R., and Spiro, T. G. (1989) Consistent porphyrin force field: 3. out-of-plane modes in the resonance spectra of planar and ruffled nickel octaethylporphyrin. J. Am. Chem. Soc. 111, 7012–7023
29. Spiro, T. G., Czernuszewicz, R. S., and Li, X. Y. (1990) Metalloporphyrin structure and dynamics from resonance Raman spectroscopy. Coord. Chem. Rev. 100, 541–571
30. Hu, S., Morris, I. K., Singh, J. P., Smith, K. M., and Spiro, T. G. (1993)
Complete assignment of cytochrome c resonance Raman spectra via enzymic reconstitution with isotopically labeled hemes. *J. Am. Chem. Soc.* **115**, 12446–12458

31. Murphy, M. E., Nall, B. T., and Brayer, G. D. (1992) Structure determination and analysis of yeast iso-2-cytochrome c and a composite mutant protein. *J. Mol. Biol.* **227**, 160–176

32. Streit, B. R., Blanc, B., Lukat-Rodgers, G. S., Rodgers, K. R., and DuBois, J. L. (2010) How active-site protonation state influences the reactivity and ligation of the heme in chlorite dismutase. *J. Am. Chem. Soc.* **132**, 5711–5724

33. Leu, B. M., Ching, T. H., Zhao, J., Sturhahn, W., Alp, E. E., and Sage, J. T. (2009) Vibrational dynamics of iron in cytochrome c. *J. Phys. Chem. B* **113**, 2193–2200