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Review

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Structure, function, and assembly of heme centers in mitochondrial respiratory complexes $\stackrel{\bigstar}{\succ}$

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A R T I C L E I N F O

ABSTRACT

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Keywords: Heme Succinate dehydrogenase Cytochrome c oxidase Ubiquinol cytochrome c oxidoreductase Mitochondria Respiration The sequential flow of electrons in the respiratory chain, from a low reduction potential substrate to O_2 , is mediated by protein-bound redox cofactors. In mitochondria, hemes-together with flavin, iron-sulfur, and copper cofactors-mediate this multi-electron transfer. Hemes, in three different forms, are used as a protein-bound prosthetic group in succinate dehydrogenase (complex II), in bc_1 complex (complex III) and in cytochrome c oxidase (complex IV). The exact function of heme b in complex II is still unclear, and lags behind in operational detail that is available for the hemes of complex III and IV. The two b hemes of complex III participate in the unique bifurcation of electron flow from the oxidation of ubiquinol, while heme c of the cytochrome c subunit, Cyt1, transfers these electrons to the peripheral cytochrome c. The unique heme a_3 , with Cu_B, form a catalytic site in complex IV that binds and reduces molecular oxygen. In addition to providing catalytic and electron transfer operations, hemes also serve a critical role in the assembly of these respiratory complexes, which is just beginning to be understood. In the absence of heme, the assembly of complex II is impaired, especially in mammalian cells. In complex III, a covalent attachment of the heme to apo-Cyt1 is a prerequisite for the complete assembly of bc_1 , whereas in complex IV, heme *a* is required for the proper folding of the Cox 1 subunit and subsequent assembly. In this review, we provide further details of the aforementioned processes with respect to the hemes of the mitochondrial respiratory complexes. This article is part of a Special Issue entitled: Cell Biology of Metals

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1. Introduction

Insertion of a ferrous iron atom into the porphyrin macrocycle by the enzyme ferrochelatase creates heme, which is a common cofactor used for a multitude of biological reactions. Within a protein scaffold, heme can carry out O_2 transport [1], nitric oxide and carbon monoxide sensing [2], oxygenase reactions [3], enzyme activity regulation [4,5], and electron transport [6]. The latter function is central in the mechanism associated with the driving force in oxidative phosphorylation. In eukaryotic cells, seven hemes of differing architecture (hemes *b*, *c* and *a*) are embedded in the proteins of the electron transport chain (ETC), and they are reduced and oxidized in generally increasing reduction potentials to transfer four electrons ultimately to reduce O_2 (Fig. 1). These "cellular pigments," or cytochromes, give rise to spectral absorption bands from heme *b* of complex II (succinate dehydrogenase), hemes *b* of complex III (*bc*₁ complex), hemes *a* of complex IV (cytochrome *c* oxidase) and hemes *c* of complex III and the soluble electron

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mediator, cytochrome *c*. Complex I (NADH Dehydrogenase) contains flavin and iron–sulfur cofactors, but is devoid of hemes.

For efficient long-range electron transfer, and to generate a proton gradient across the inner membrane of mitochondria or plasma membrane of bacteria, nature has evolved highly specialized modifications to heme to fine tune the electron relay system. For instance, heme a is present only in terminal oxidases. It is synthesized from heme b (protoheme) -the product of ferrochelatase-by two sequential chemical modifications using two specialized enzymes: heme o synthase (HOS) and heme a synthase (HAS). HOS converts one of the vinyl carbon of the macrocycle into a hydrophobic hydroxyfarnesyl tail (heme o) followed by a HAS catalyzed conversion of one of the methyl carbon of the macrocycle to a formyl group (heme *a*). The latter methyl group oxidation results in a marked increase in the mid-point potential ($\sim +180$ mV) of heme a relative to heme o [7,8]. The farnesyl tail serves a structural role in stabilizing, via hydrophobic interactions, the helical structure of the Cox1 subunit of cytochrome c oxidase (CcO) [9]. These two heme aprosthetic groups are necessary for electron transfer and O₂ reduction activities in CcO.

Protein scaffolds are also used to provide a modification to the heme macrocycle. Proteins bind hemes via the combination of three interactions: (i) the interaction between an axial ligand and the heme iron; (ii) hydrophobic interactions between the protein and the porphyrin macrocycle; and (iii) polar interactions between the

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protein and the propionic acids of the macrocycle [7]. To strengthen this heme-protein interaction, heme *b* can be covalently attached to the protein backbone, which defines the heme as *c*-type. Proteins with *c*-type hemes (cytochromes *c*) contain a CxxCH heme-binding motif where two cysteine (rarely one cysteine) residues form thioether linkages to the vinyl carbons of the heme macrocycle, and the His residue binds to the axial position of the heme iron.

The above specialization is not necessarily to provide tuning of the redox potential of the heme as previously believed; the potential can more easily be tuned over a wide range using only the protein environment [10,11]. The *c*-type modification does add structural stability of the heme-protein complex over its cytochrome *b* globin counterpart [12]. In addition to being a cofactor in the soluble cytochrome *c* found in the inter-membrane space (IMS) of mitochondria, covalently bound heme is also a cofactor in the cytochrome *c*₁ subunit of the *bc*₁ complex. It is interesting to note that both the non-covalently linked *b* and *a* hemes of the respiratory chain reside in the relatively protected environments within the inner membrane (IM) and the matrix, whereas both *c* hemes of cytochrome *c*₁ and cytochrome *c* reside in the more oxidizing intermembrane space, analogous to the harsher environment of the periplasm in bacteria where a stable heme linkage might be advantageous.

In this review, we take a hemocentric view of the mitochondrial respiratory chain. We describe the heme cofactors from structural and functional perspectives, and also the assembly of heme cofactor centers within respiratory complexes II, III, and IV. We focus on the respiratory complexes from the yeast *Saccharomyces cerevisiae* with information from bacterial and mammalian systems when appropriate for comparisons and when yeast information is lacking.

2. The heme moiety of succinate dehydrogenase (SDH)

2.1. Overview

Succinate dehydrogenase (SDH, complex II), also known as succinate:quinone oxidoreductase in bacterial systems (SQR), is a heterotetrameric, integral membrane–protein complex (~120 kDa) that catalyzes the FAD-dependent, two-electron oxidation of succinate to fumarate, coupled with the reduction of ubiquinone to ubiquinol [13–16]. It is one of the eight enzymes of the citric acid cycle. It is also part of the aerobic respiratory chain, as it transfers reducing equivalents to the bc_1 complex in the form of quinol. SDH is the only enzyme that couples the Kreb's cycle to the respiratory electron transport chain, although unlike the other respiratory complexes, there is no proton transfer across the membrane coupled with its activity. The closely related menaquinol:fumarate oxidoreductase (QFR) catalyzes the reverse reaction in bacteria during anaerobic respiration, and due to the structural and sequence similarities between SQR and QFR, both enzymes are believed to have evolved from a common ancestor [15,17].

The two electrons abstracted from the dehydrogenation of succinate are channeled sequentially into the ETC, from the flavin adenine dinucleotide (FAD) to the membrane-entrapped ubiquinone (UQ), through the cofactors [2Fe–2S], [4Fe–4S], [3Fe–4S] and possibly heme *b*. This long-range, near-linear electron conduit extends over 40 Å from the soluble catalytic domain of SDH to the membraneanchoring domain of the enzyme [18]. The participation of heme *b* as part of the electron transfer chain has not been clearly established, so the functional significance of heme within SDH is unclear (see Section 2.4).

Although a few exceptions exist, SDH is in general comprised of four distinct polypeptides designated Sdh1–4 in yeast (SDHA-D in bacteria and humans). Sdh1 is a large ~70-kDa flavoprotein (Fp) with a mono/dinucleotide-binding Rossmann fold [19] that contains a buried, covalently attached FAD via a histidine residue. The three distinct iron–sulfur clusters in the ~27-kDa Sdh2 act as an electron wire connecting the FAD to the ubiquinone-binding site. The Sdh1– Sdh2 catalytic dimer is oriented exclusively on the matrix side of the IM in eukaryotes and cytosolic side of the plasma membrane in bacteria. Two hydrophobic membrane-spanning subunits (Sdh3 and Sdh4) anchor the catalytic dimer to the membrane and, due to their relevance to heme, are discussed in detail below.



Fig. 1. Distribution of hemes *b*, *c*, and *a*, with respective midpoint potentials, in respiratory complexes II, III, and IV in the mitochondria. Note the general trend of increasing redox potential from the *b* heme of Complex II to the *a*₃ heme of Complex IV, the site of the terminal electron acceptor and O₂ reduction. Other cofactors in the complexes are grayed for clarity: Complex II; FAD, [2Fe–2S], [4Fe–4S], [3Fe–4S]: Complex III; Rieske 2Fe–2S: Complex IV; binuclear Cu_A, Cu_B. Complex III is shown as a dimer with cytochrome *c* docked to one of the subunit. References used for midpoint potentials: Complex II [21]; Complex III [124]; Complex IV [125]. The figure was generated using PDB accession numbers: 1YQ3, 3CX5 and 2CC.

2.2. Presence of heme within the SDH transmembrane anchor domain

The small membrane-spanning domains, Sdh3 (SdhC, ~15 kDa) and Sdh4 (SdhD, ~14 kDa), together form a classical six-helix bundle. SDH crystal structures from Escherichia coli, porcine, and avian have confirmed the presence of a six-coordinate bis-His heme b located at the inter-subunit interface to form what is referred to as a cytochrome *b* domain. Heme propionate groups interact with nearby charged Arg and His residues [18,20] with further stabilization by hydrophobic interactions between the porphyrin group and four of the six helices [20]. The heme lies spatially close to the UQ-binding cavity located near the matrix-lipid interface comprised of Sdh2, Sdh3 and Sdh4 [18] and proximal to the [3Fe-4S] center (UQp). A second UQ site, distal to the 3Fe-4S cluster (Uqd), has been suspected in mitochondrial SDH [21]. The crystal structure of porcine SDH provided further evidence of this second site based on the presence of a Q site inhibitor, and that this second site is located close to the IMS side of the IM [20]. This structure places the heme in between the two UQbinding sites and poses interesting speculative scenarios regarding the electron flux in the transmembrane (TM) domain. For instance, the two electrons from the oxidation of succinate could flow sequentially to the Op site, and when ubiquinone reduction becomes rate limiting for electron flux, the excess electrons could then be transferred to the Qd site mediated by heme b. This heme pathway may assist in the coordination of succinate and UQ turnover rates.

The superfamily of SQR, and the related QFR proteins, can be classified into five subfamilies (type A to E) based on variations in the membrane-anchoring subunits [15,21]. A single group associated with Archaea (Type E) lacks the typical Sdh3–Sdh4 association to the membrane and likely lacks heme. QFR enzymes from the Type D group also lack heme, although they contain the two distinct hydrophobic domains in which heme would reside. However, all SQR proteins that contain the distinct membrane anchors contain one to two hemes *b* [15]. Thus, the presence of the heme between the two TM domains is highly conserved, suggesting functional importance. Curiously, though, investigations into functionally characterizing this heme have brought into question the physiological importance of this redox center and whether the heme is required for assembly of SDH [22–24].

2.3. The Physical and Thermodynamic Barriers to Electron Transfer to Heme b

The chain of FAD-[2Fe-2S]-[4Fe-4S]-[3Fe-4S]-UO are all positioned within a relatively short edge-to-edge distance (10-14 Å) between each pair of cofactors that allows electrons to tunnel rapidly between them [25]. Heme *b* lies curiously outside this linear chain; the UQ is positioned between the [3Fe-4S] cluster and the heme, resulting in what appears to be a branching of the electronic path at the [3Fe-4S] cluster (Fig. 2). Furthermore, the edge to edge distance between the [3Fe–4S] cluster and heme b (11.4 Å) is greater than the distance to UQ (7.6 Å) [18,20]. Although this spatial position of heme b does not necessarily indicate directional specificity, the notion that the heme is not involved is further substantiated by the redox potentials of the three cofactors in question. The potential of heme b (-185 mV), is considerably lower than that of the [3Fe-4S] cluster (+60 mV) and of ubiquinone reduction (+113 mV) that would indicate an uphill electron transfer [21]. Thus, both in structural and thermodynamic terms, a sound, albeit theoretical, argument can be made that the heme may not be part of the electronic pathway from FAD to UQ.

A caveat to the above argument is the presumption that each redox center is discrete and operates independently of neighboring redox centers. However, with close edge to edge distances, deviations from Nernstian behavior might be likely and that allosteric electronic interactions between the redox centers could exist [26,27]. Thus,

under physiological conditions, the reduction of one redox center could influence the potential of the neighboring center to a more favorable value for reduction. Additionally, and as mentioned in the previous section, the presence of a second distal UQ binding site places the heme between the two UQ-binding sites. This raises the possibility that the heme could function to deliver electrons to the second Qd site.

2.4. The functional role of heme in SDH

Much of the investigations into the function of the heme in SDH have utilized *E. coli* SQR as a model system due to its high protein expression levels and easy genetic manipulations. Comparison of the crystal structures of *E. coli* [18] and mitochondrial SQRs [20] showed that the heme-binding His residues are conserved, lending validation to use of the *E. coli* model system. An exception to this conservation is found in *S. cerevisiae*, where one of the His ligands is replaced by a Tyr, which has led to an interesting debate regarding the presence of the heme in yeast.

The literature regarding the functional aspect of the heme in SDH spans three decades. While true that the heme is not involved in catalysis of succinate or fumarate, the exact nature of its function in electron transfer is still unclear. Heme might have a structural role in stabilizing the two TM subunits; however, the QFR from *E. coli* lacks heme with no effect on its stability [28]. Some SQRs and QFRs contain two hemes of high and low potentials, reminiscent of the bc_1 complex hemes b_H and b_L hemes involved in trans-membrane electron transfer (see Section 3). Heme could be involved in the prevention of reactive oxygen species (ROS) formation during electron transfer from FAD to UQ by acting as a capacitor in times of high electron flux [18]. The presence of the heme center may prevent ROS formation with reverse electron flow from ubiquinol, minimizing the level of semiquinone.

It is clear that SDH contains a bound heme moiety; thus, the major question relates to the function of the heme center. Below, we highlight some studies focused on the functional aspects of the heme in SDH from bacteria, yeast, and mammals.

2.4.1. Effect of heme on the assembly and activity of SDH in bacteria

Initial hints of coupling between heme and the assembly of SDH was described using heme-deficient *Bacillus subtilis* strains where abrogation of heme synthesis resulted in a decrease in membrane-bound SDH [29]. Parallel to this decrease, Sdh1 (presumed to be associated with Sdh2) was found in the cytosolic fraction in an increased amount. This led to the conclusion that the cytochrome *b* subunits cannot anchor the Sdh1–Sdh2 complex in the absence of heme.

Corroborating these early observations, *E. coli* cells that lack the gene for either of the two TM subunits form only the catalytic Sdh1–Sdh2 heterodimer and its succinate oxidase activity is found only in the cytoplasmic fraction [30,31]. Expression of all four subunits in a heme-deficient, ferrochelatase mutant results in the same phenotype where the catalytic SQR was found only in the cytoplasmic fraction, suggesting heme is required for the proper insertion of SQR into the membranes. The two TM subunits could not be found in the membranes when heme was not present [31]. When heme is presented to cells lacking ferrochelatase, SQR is assembled and active within the membrane.

Subsequent studies, conducted to analyze the role of heme in SDH, have employed the generation of site-directed mutants of the hemebinding ligands [22–24,32,33]. Anaerobic expression of SQR *E. coli* histidyl mutants, SDHC H84L or SDHD H71Q, from a plasmid showed that heme was retained in an altered six-coordinated environment, or as a penta-coordinated high-spin heme, respectively, as determined by EPR [33]. The enzyme was shown to be catalytically active, but with lowered activities and lowered substrate affinity. Contrary to these results, the same mutants when expressed aerobically showed



Fig. 2. Structural and thermodynamic barriers to electron transfer from the 3Fe–4S cluster to heme *b* during quinone reduction in complex II. The transfer of an electron from the 3Fe–4S cluster (yellow and red spheres) to a lower potential heme *b* (yellow stick) would involve an "uphill" transfer, whereas a transfer to the higher potential quinone (green stick) would be more favorable thermodynamically. Furthermore, electron transfer to heme *b* involves a longer distance relative to the distance to the quinone. Subunit 2; yellow: Subunit 3; purple: Subunit 4; green. PDB accession number: 1YQ3 was used to generate the figure.

that the heme was lost in SDH, but the enzyme assembled and was catalytically active [32].

Substitution of the same histidyl ligands to tyrosine residues (SDHC H84Y or SDHD H71Y), either as a single or double mutant, appears to cause a loss of the heme in SDH in E. coli cells grown under a microaerobic condition. The most convincing case was demonstrated using enriched, overexpressed SQR mutants in E. coli where the loss of the cofactor was confirmed via optical and EPR spectra of the solubilized membranes [23,24]. Cells harboring either of these mutations grew in succinate-supplemented medium with a doubling time similar to WT cells. According to the authors, the loss of the heme did not cause an assembly defect, based partly on the fact that all four subunits were visible by SDS-PAGE analysis. The mutant enzyme was compromised in catalytic activity and substrate affinity as measured by quinone reduction. The substitution of the His ligand with a Cys residue induces a penta-coordinated high-spin heme that can bind CO, indicating that the Cys residue is unable to provide the sixth coordination site to heme b [24]. This result has implications to the yeast SDH, which is believed by some to bind heme using a Cys residue [34].

2.4.2. Effect of heme on the assembly and activity of SDH in eukaryotes

A phylogenetic analysis of SDH from *S. cerevisiae* places the enzyme in the type C subgroup of SQR/FRD superfamily of proteins based on the sequence of either of the catalytic subunits [15]. All type C enzymes possess two transmembrane subunits with a single inter-subunit heme *b*. Thus, the prediction based on this *in silico* analysis is that SDH in yeast is a single heme-containing enzyme. However, it has been suggested that yeast SDH may lack heme altogether, perhaps giving way to evolutionary pressure to dispense the heme from a possible lack of need [24].

Before any SQR/FRD structural information was available, biochemical evidence indicated the presence of a SDH-specific heme in a ratio of ~1 mol of heme per mol of FAD in yeast membranes [34]. The heme was termed "fumarate-oxidizable heme" and corresponds to an absorbance peak at 562 nm that appears in a difference spectrum after the addition of fumarate to dithionite-reduced mitochondrial membranes. The 562 nm heme peak is not present in mutants lacking either Sdh3 or Sdh4. The reliability of using the fumarate-oxidizable spectrum as an indicator of a SDH-associated heme has been questioned; however, due to contributions from the more abundant cytochromes *b* from complex III having an absorption at 563–564 nm [24]. The absence of the heme 562 nm peak in a Sdh3 or Sdh4 deletion; however, appears to be suggestive of a non-complex III dependent peak.

Mutation of either of the identified residues to alanines (Sdh3 H106A or Sdh4 C78A) causes a decrease in 562 nm heme content in mitochondrial membranes as measured by fumarate-oxidizable difference absorbance spectra [34]. It also led to a shift in the λ max from 562 to 560 nm. Both results suggest partial loss of the heme; the remaining heme existed in an altered environment. The steady-state level of assembled SDH and thus total SDH activity was reduced in both mutants. The stability of the SDH complex may be decreased from the loss of the heme. These results mirror those seen with the E. coli system: the amount of assembled enzyme decreases by about half, with parallel decreases in specific activity. A subsequent follow-up yeast study showed that mutation of both Sdh3 H106A and Sdh4 C78A results in a complete absence of the cytochrome b 562 nm peak in the fumarate-oxidizable absorption spectrum of the mitochondrial membranes [22]. Covalent FAD levels decreased ~30% and catalytic activity of the membranes decreased ~53%

In mammalian cells, mutations harboring SDHC H127A and SDHC H127Y were constructed in Sdh3 to mimic the mutations mentioned above made in yeast and in E. coli, respectively [35]. However, unlike the effects in yeast and in E. coli, a change in the heme-binding ligand in SDHC led to the conclusion that heme is indispensable for SDH assembly in mammalian cells. For both SDHC H127A and SDHC H127Y transient reconstitutions in HeLa cells with downregulated endogenous SDHC, steady-state levels of SDHC were significantly decreased. Concomitant decreases in enzyme activities of about 50% were also observed [35]. Stable reconstitution of the same SDHC mutants in SDHC negative cells caused an even more dramatic effect with a near complete loss of SDHC and SDHD steady-state protein levels and near complete loss of SDH activities. These results would suggest that either SDH assembly was completely impaired, or that the assembled complex is rapidly degraded resulting from destabilization in the absence of the heme.

2.5. Dual function of Sdh3

The heme in yeast SDH (or its absence) has become even more intriguing recently-the Sdh3-Sdh4 heme-containing domain has been identified in another, separate mitochondrial protein complex. In S. cerevisiae, the Sdh3 subunit forms a heterodimer with Tim18 as part of the TIM22 channel-forming, protein translocase complex [36]. This translocase functions to import nuclear-encoded carrier family proteins destined to the mitochondria [37,38]. Sdh3 and Tim18 likely form a membrane anchor complex very similar to the Sdh3/Sdh4 membrane anchor in SDH. Using structural prediction based on pig and chicken complex II structures as templates, the overall fold of Tim18 shares an identical three-transmembrane helical bundle fold to that of Sdh4, and the predicted Sdh4-Tim18 structures can be superimposed with relatively small deviations. Remarkably, the quinone-binding site of Sdh3-Sdh4 is conserved in Sdh3-Tim18, both in terms of structure and the residues involved in forming the quinone ligands [24,36]. The atypical tyrosine residue in yeast Sdh4 that replaces the conserved heme-binding His residue is also present in Tim18, suggesting that this Tyr residue could be critical for function both in Sdh4 and in Tim18. One major unanswered question is whether the Sdh3-Tim18 complex contains a heme at the interface of the heterodimer utilizing this Tyr residue, and if so, whether this heme is merely a structural moiety or has an additional functional role.

3. Hemes of cytochrome *bc*₁ complex

3.1. Overview

The ubiquinol:cytochrome c oxidoreductase (cytochrome bc_1 , complex III) is a central component of the respiratory and photosynthetic electron transport chains involved in oxidative phosphorylation. In eukaryotes, the complex exists as a dimer, embedded in the IM of mitochondria. In many bacteria, bc_1 complexes are present in the plasma membrane, and the bc_1 complex equivalent, the b_{6f} complex, is located in the thylakoid membrane of cyanobacteria and plant chloroplasts. The function of the bc_1 complex is to mediate transfer of electrons to the diffusible soluble cytochrome c via the oxidation of membrane-localized quinol, which can be generated by SDH, as described above. This redox-dependent reaction is coupled to translocation of protons across the lipid bilayer in which the complex resides.

All bc_1 complexes contain three protein subunits with redox prosthetic groups known as the core subunits: a di-heme cytochrome b (Cob) containing both the high-potential b_H (b562) and the lower-potential b_L (b565); the c-type heme c_1 associated with the Cyt1 subunit; and an iron–sulfur protein (ISP, Rip1) with a Rieske-type 2Fe–2S cluster (Fig. 3). Bacterial bc_1 complexes usually contain only these core subunits, and at most, a single supernumerary (containing no redox cofactors) subunit, whereas the mitochondrial enzymes contain up to eight supernumerary subunits per monomer. The supernumerary subunits are not required for either electron flow or proton translocation by the bc_1 complexes. In accordance with the endosymbiotic theory of mitochondrial origin, the catalytic core of each bc_1 monomer in eukaryotes is comprised of the same essential redox cofactor-containing subunits with a high degree of conservation between kingdoms.

The redox protein Cob is the only bc_1 subunit encoded by the yeast mitochondrial genome. It contains eight TM helices containing two heme b moieties and serves as the foundational protein in bc_1 biogenesis. The other subunits are encoded by the nuclear genome and must be imported into the mitochondria. Both the Cyt1 and ISP catalytic subunits are anchored in the IM by a single TM domain and contain a soluble globular domain containing its heme c_1 and 2Fe–2S and prosthetic groups, respectively, in the IMS. The ISP protein is intertwined between adjacent monomers of the complex, with its globular domain in one unit and its TM helix in the adjacent unit. Thus, electron transfer can only occur when the complex is assembled as a dimer. Furthermore, movement of the globular catalytic domain in ISP is essential to the electron transfer to cytochrome c_1 in the catalytic mechanism. The dimeric *bc*₁ complex forms higher-order supercomplexes with CcO (Section 4) in yeast and with both CcO and the NADH dehydrogenase (complex I) in higher eukaryotes.

3.2. Organization and function of bc₁ complex redox sites

3.2.1. Structure of bc_1 complex heme sites

High-resolution crystal structures of the bovine, chicken and yeast bc_1 complexes identify the redox-active sites within the enzyme [39–42]. Each bc_1 monomer has three 6-coordinate, low-spin heme centers. The midpoint reduction potentials for heme $b_{\rm H}$, heme $b_{\rm L}$, and heme c_1 are ~ + 120 mV, ~ - 30 mV, and ~ + 240 mV, respectively [43], although there is slight variation between species and strains (Figs. 1 and 3).

The di-heme cytochrome *b* polypeptide forms the two quinonebinding sites. According to the crystal structures, the two planar *b*type hemes are rotated approximately 45° to one another, but are positioned perpendicular to the IM within a four-helix bundle of the cytochrome *b* subunit. Earlier spectroscopic and mutagenesis [44] studies determined that two His residues in each of the second (helix B) and fourth (helix D) TM helices bind the hemes. These His residues are invariant in over 800 sequences of *b*-type cytochromes, including cytochrome b_6 of the b_6f complex [45]. The distance between the heme iron atoms and the ligating His varies from 2.05 to 2.08 Å [39–42]. The distance between the iron centers of the *b* hemes in the same monomer is approximately 21 Å [40–42]. Interestingly, this same distance is also observed for the b_L hemes in opposite monomers, implying that electron transfer may be possible between them [46]. The low-potential heme b_L is located close to the IMS side of the IM, but protected from solvent by the cytochrome c_1 subunit. Conversely, the high-potential heme b_H is located near the surface of Cob in a solvent-filled cavity accessible from the matrix. In this location, heme b_H can receive electrons from b_L to drive the Q cycle.

The IMS-localized domain of the cytochrome c_1 subunit (Cyt1) contains a single heme c_1 moiety that is covalently attached to the polypeptide by two thioether bonds to Cyt1 cysteine residues [40] (a rare one thioether bond Cyt1 exists [47]). Cyt1 is larger and differs in structural elements from other *c*-type cytochromes in addition to having a TM segment [48]. Like most apo-cytochromes *c*, a CxxCH motif provides the sulfhydryls for the two thioether bonds and the imidazole nitrogen of histidine serves as the fifth axial ligand to the heme iron. Methionine is typically the sixth axial iron ligand.

3.2.2. Catalytic mechanism of the bc_1 complex

Details of the bc_1 catalytic mechanism can be found in many excellent reviews [49–51]. Here, we provide a general overview while highlighting hemes' roles. A central function of the two hemes, b_H and b_L is to provide a bifurcation pathway to the two electrons abstracted from the oxidation of quinol. The third heme of bc_1 , heme c_1 , accepts electrons from the Rieske 2Fe–2S cluster and mediates them to the soluble cytochrome c. Specifics are outlined below.

The bc_1 complex contains two quinone reaction centers located on opposite sides within the IM [52] (Fig. 3). Ubiquinol binds at the Q_o , or the *P* site of the complex. After deprotonation, one electron is released to reduce sequentially the Rieske 2Fe–2S cluster, heme c_1 and then heme *c* of the soluble cytochrome *c*. The second electron is passed to the low-potential heme b_L and then to the high-potential heme b_H which is then used to reduce the quinone at the Q_i , or *N* site, forming a semiquinone. Another cycle of quinol oxidation at the Q_o site



Fig. 3. Arrangement of the hemes and the Rieske iron–sulfur clusters in the bc_1 dimeric complex. For clarity, the protein backbone is in transparent grey and only the cofactors corresponding to a monomeric unit are labeled. The axial ligands to the heme iron are highlighted along with respective residue numbers and subunits (PDB ID: 3CX5 numbering). The axial ligands to heme b_L are His183 and His82 (not shown) of Cob. The quinone analogue inhibitor stigmatellin (Stg) is also shown and denotes the *p*-side quinone-binding site that is located between the low potential *b* heme (Heme b_L) and the Rieske iron–sulfur cluster. The *n*-side quinone-binding site (not shown) lies close to the high potential *b* heme (Heme b_H).

provides the second electron necessary for the reduction of the semiquinone at the Q_i site from the first reaction sequence. The resulting quinol re-enters the Q cycle at the Q_o site. Thus, the net product of the Q cycle is the translocation of four protons to the IMS, the uptake of two protons from the mitochondrial matrix, and the reduction of two molecules of cytochrome *c*.

While the overall reaction mechanism remains largely undisputed, the bifurcated oxidation of ubiquinol is proposed to occur by two different mechanisms. In the sequential mechanism, the electron is first passed to the high-potential Rieske protein. This reaction results in the remaining electron, now present as an unstable semiquinone (Q•), to be passed to b_L and then to b_H . However, this historic mechanism lacks the experimental evidence to support the existence of a semiquinone intermediate at the Qo center. Trumpower and colleagues proposed a refined model in which the oxidation of ubiquinol occurs by a concerted mechanism in which electrons are simultaneously donated to the Rieske protein and heme b_L [53]. Cytochrome b_L immediately reduces cytochrome b_H , which then reduces ubiquinone to the semiquinone at the Q_i site. The electron transfer from b_L to b_H also results in a protein conformational change that facilitates movement of the Rieske Fe/S protein between cytochromes b and c_1 [54].

3.3. Formation and maturation of bc₁ redox sites

The individual maturation of cofactor-containing subunits requires several deliberate steps, including cofactor delivery to the appropriate cellular compartment, manipulation and/or preservation of the apoprotein in a conformation amenable to cofactor insertion, and ligation of the cofactor to the specific functional groups of the subunit polypeptide. An additional complication is that all known *c*type holocytochromes are located in a different cellular compartment than the site of either apo-polypeptide or heme synthesis [55]. It is also unclear which side of the plasma membrane/inner membrane hemylation takes place to form cytochromes *b*. Additionally, little is known about the mechanism of *b*-type cytochrome maturation because assembly defects have severe cellular phenotypes [56].

3.3.1. Di-heme cytochrome b maturation

In prokaryotes, mutagenesis of the ligating histidine residues in cytochrome *b* prevents cofactor insertion and results in a nonfunctional enzyme that impairs bc_1 assembly [56]. The non-covalent chemical nature and lack of known specific assembly factors has led to the suggestion that heme is spontaneously inserted into the apoprotein. This assumption can be supported by *in vitro* experiments where heme is able to bind synthetic peptides mimicking cytochrome *b* helices [57]. Thus, the model for bacterial bc_1 complex assembly assumes that the *b* hemes are inserted either very soon, after, or concurrent, with the folding of the cytochrome *b* polypeptide into the membrane.

Maturation of cytochrome *b* is the nucleating factor in eukaryotic bc₁ complex biogenesis (Fig. 4). COB pre-mRNA is processed by the matrix protein Cbp2 and the mitochondrial proteins Cbs1 and Cbs2, which activate translation via interaction with the 5' untranslated region of COB mRNA and the mitochondrial ribosome [58]. Cbp6 was also identified as a translational activator of COB mRNA, but was more recently found to form a complex with the matrix assembly protein Cbp3 at the ribosomal exit tunnel, where the two proteins interact with the newly synthesized Cob. The Cob-Cbp6-Cbp3 complex recruits the additional IMS assembly factor Cbp4 after dissociation with the ribosome [59]. An early assembly intermediate containing Cob was identified by native gel electrophoresis in yeast lacking important bc_1 subunits [60,61]. This intermediate also contained two small bc1 subunits, Qcr7 and Qcr8. Although it remains unclear whether the newly synthesized Cob associated with Cbp3, Cbp6 and Cbp4 is a distinct complex from the Cob/Qcr7/Qcr8 assembly intermediate, it is likely that the initial assembly intermediate contains each of these assembly factors. This putative Cob-containing complex with Cbp3, Cbp4, Cbp6, Qcr7 and Qcr8 may exist to stabilize Cob for population of the two heme centers.

The mechanism of Cob hemylation is unknown. It remains unclear whether the reaction occurs from the matrix or the IMS side of the IM. Heme synthesis occurs on the matrix side of the IM by ferrochelatase. The two heme moieties associated with Cob lie within a four-helix bundle. If Cob hemylation occurs within the assembly intermediate containing Qcr7, Qcr8, Cbp3, Cbp4 and Cbp6, the Qcr7 subunit and the N-terminus of Qcr8 are packed against the matrix-facing loops of Cob, blocking access to the four-helix bundle interior. In addition, Cbp3 and Cbp6 are matrix-facing subunits that may also block access to Cob. In contrast, the IMS side of the Cob intermediate would be devoid of steric constraints by Qcr8. Cbp4 is projected to have a globular domain facing the IMS, but Cbp4 is not conserved through eukaryotes. Thus, hemylation may occur through the IMS surface of Cob. No information exists on how heme is translocated across the IM, vet that is an essential step, as heme is used in other cellular compartments.

Hemylation of Cob is likely required for subsequent maturation of the Cob/Qcr7/Qcr8 complex through the addition of subunits Cor1, Cor2, Cyt1 and Qcr9 [61,62]. The late maturation step includes the addition of 2Fe–2S-containing Rip1 and Qcr10. Since Rip1 contains the essential 2Fe–2S center that mediates electron transfer to the cyto-chrome c_1 subunit Cyt1, the late core intermediate is devoid of function. The addition of Rip1 and Qcr10 enables association with CcO, forming the supercomplex in yeast [62,63].

3.3.2. Cytochrome c_1 heme maturation

In prokaryotes, the Sec translocon delivers cytochrome c_1 to the periplasm where the covalent attachment of heme c to apo-cytochrome by the system I machinery occurs early in bacterial bc_1 complex biogenesis [64]. Deletion, mutation, or removal of the transmembrane anchor of the cytochrome c_1 gene or mutation of the heme binding site of cytochrome c_1 protein prevents the addition of either cytochrome b or Rieske protein subunits [65–67] to the complex. Furthermore, removal of the Cterminal membrane anchor in R. sphaeroides causes retention of detectable amounts of the soluble holo-protein in the periplasm [67], suggesting that heme c_1 insertion occurs independently of Cyt1 membrane insertion and that a functional holo-Cyt1 is required for assembly and association of the remaining bc_1 complex subunits.

Cyt1 is the only bc_1 TM subunit oriented with its *C*-terminus directed towards the matrix. Therefore, proper localization and topogenesis requires a bipartite targeting signal sequence in Cyt1 to direct its intra-mitochondrial sorting. Following inner membrane insertion of the C-terminal tail, the heme c_1 cofactor is inserted into the new IMS-localized N-terminal domain of Cyt1 [68,69]. The final step in Cyt1 maturation is cleavage by the Imp2 signal peptidase to remove the matrix and N-terminal TM portions of Cyt1, resulting in mature holo-Cyt1 (Fig. 4). Positive heme staining on SDS-PAGE of the intermediate form of Cyt1 is evidence that heme attachment occurs prior to Imp2 processing. Furthermore, in the absence of Imp2, the intermediate holo-cytochrome c_1 persists [70].

In yeast, the covalent attachment of heme to apo-Cyt1 is mediated by either of the two heme lyases in the IMS, CCHL and CC₁HL [70]. In mammals, the activities of both CCHL and CC₁HL are possessed by the HCCS heme synthetase, which functions to assemble both cytochrome c_1 and the mobile IMS protein cytochrome c. Both CCHL and HCCS can functionally substitute for CC₁HL in yeast, although the converse is not true [70]. Therefore, it remains possible that CCHL and CC₁HL are actually part of a larger complex that is required for covalent bond formation in cytochrome c-type proteins, and that the so-called lyases are not the catalytic component of this complex.

The fungal CC₁HL, designated Cyt2, is a small protein of 216 residues in *S. cerevisiae*. The *N*-terminal segments of CC₁HL orthologs exhibit considerable length variation, but contain dual CP motifs that



Fig. 4. Hemylation of cytochrome *b* and cytochrome c_1 polypeptides in eukaryotic bc_1 complex. Translation of COB mRNA initiates bc_1 complex biogenesis (1a). Cob forms a ternary complex with the Cbp3/Cbp4/Cbp6 proteins independent of the mitochondrial ribosome (2a). Following transport of newly synthesized heme across the IM (3a), this is a possible point at which heme is inserted into the apo-cytochrome *b* from the IMS side of the inner membrane. This insertion could occur prior to, in conjunction with, or post Qcr7 and Qcr8 additions (4a). Transport across the IM would not be required if the product heme from ferrochelatase is deposited into the IM (see Fig. 6a). Hemylation of Cob could also occur directly from the matrix side. Apo-cytochrome c_1 is synthesized in the typosite of newly synthesized heme across the IM, the heme lyase, Cyt2, participates in the covalent attachment of heme c_1 to apocytochrome c_1 (3b). The final step in holocytochrome c_1 maturation is cleavage by Imp2 to remove the *N*-terminal membrane anchor, resulting in an N_{out}-C_{in} topology (4b). PDB accession number: 3CX5 was used to generate the figure.

may transiently bind hemes [71]. It is unclear whether Cyt2 or any CCHL protein catalyzes the thioether bond formation directly. An alternative scenario is that Cyt2 is a chaperone that stabilizes a conformation of Cyt1, allowing spontaneous thioether bond formation.

The IMS protein Cyc2 has been identified both in a screen for mutants deficient in hemylated cytochrome *c* and also isolated as a multicopy suppressor in cells lacking CC₁HL, suggesting a role in the maturation of mitochondrial *c*-type cytochromes [72]. Cyc2 has a non-covalently bound FAD cofactor with a proposed role in the redox modulation of the heme lyase reaction. Cyc2 is essential for hemylation of Cyt1 by CCHL, especially when the CxxCH (CAPCH) heme-binding motif of Cyt1 is mutated to affect the redox chemistry of the cysteinyl thiols [73]. However, Cyc2 is not required for the hemylation of Cyt1 by its cognate CC₁HL lyase. This observation suggests that a different candidate reductase than Cyc2 may be necessary for Cyt1 maturation.

Cyt1 along with Cor1, Cor2 and Qcr9 are added to the early core assembly intermediate (Cob, Qcr7, Qcr8) to form the late core assembly intermediate lacking only Rip1 and Qcr10. Hemylation of Cyt1 likely occurs prior to this assembly step, since it appears to precede Imp2 proteolytic processing (Fig. 4). No information is available regarding whether hemylation of Cob and Cyt1 is coupled. Further research is needed to define the steps and protein components necessary for these hemylation reactions.

4. Heme *a* of cytochrome *c* oxidase

4.1. Overview

Eukaryotic CcO is the terminal enzyme of the mitochondrial electron transport chain. A member of the heme-copper oxidase superfamily, this enzyme catalyzes the reduction of molecular oxygen to water concurrently with the oxidation of ferrocytochrome *c*. These redox reactions, like that in complex III, are coupled to the translocation of protons across the inner mitochondrial membrane contributing to the formation of the electrochemical proton gradient utilized to generate ATP.

In contrast to its 3–4 subunit bacterial counterparts, eukaryotic CcOs consist of 11 (yeast) to 13 (mammals) subunits; nevertheless, the catalytic core of the enzyme formed by the three largest proteins, Cox1, Cox2 and Cox3, shows a significant degree of conservation throughout evolution [74]. Three core subunits are encoded by mito-chondrial DNA, while the additional subunits are products of the nuclear genome [75,76].

Structures of CcO enzymes have been elucidated from bovine heart mitochondria as well as from bacterial counterparts *Paracoccus denitrificans* and *Thermus thermophilus* [9,77–80]. Cox1 is the largest and most conserved of the core subunits. It is a highly hydrophobic protein with 12 TM helices, and it contributes to both proton pumping and oxygen reduction reactions. The second catalytic subunit Cox2 has only 2 TM helices. Studies both in bacteria and eukaryotes suggest that Cox3 is likely important for stability of the catalytic core and modulates the proton pumping pathways in Cox1 [81]. The additional nuclear-encoded subunits are important for both assembly and function of the enzyme [75,76].

4.2. Organization and function of CcO redox sites

4.2.1. Structure of CcO redox sites

Redox sites of CcO are represented by the copper ions and the *a*-type heme cofactors that together form very unique cofactor sites found only in terminal oxidases (Fig. 5). The Cox2 subunit contains the binuclear Cu_A center located within the IMS-exposed *C*-terminal domain of Cox2. The other redox cofactors are present in Cox1. First, the low-spin six-coordinate heme *a* is ligated by two axial histidyl ligands. The fully extended farnesyl group of this heme is inside an α -helical bundle formed by helices 1, 11, and 12 of Cox1 [77,82]. The second heme molecule exists within a heterobimetallic center consisting of copper (designated Cu_B) and heme *a*. This heme, designated *a*₃, is five-coordinate high-spin, with a single axial ligand.

The Cu_B site resides 13 Å below the IM surface, and the heme a_3 is localized in a cavity with its farnesyl tail located at the interface of the

Cox1 and Cox2 subunits. Consistently, the association of the Cox1 and Cox2 subunits has been reported to be an important factor for proper coordination of heme a_3 in mammalian CcO [83]. The cavity is partially solvent-accessible from the IMS side, and Cox2 docking in the course of assembly appears to occlude the cavity [9,77]. Both hemes have their farnesyl and vinyl groups extending toward the mitochondrial matrix side, while hydrophilic propionate moieties are facing the IMS side.

4.2.2. Function of CcO redox sites

The CcO redox sites in combination with hydrogen bonds and the peptide backbone form an intricate molecular wire that allows shuttling of the electrons through the enzyme [84]. The electrons from the reduced cytochrome *c* enter CcO through the Cu_A center located near the docking site of Cox2 and Cyc1. The Cu_A site is located 19 Å away from the heme *a* prosthetic group. Electron transfer between the Cu_A and heme *a* does not involve proton movement [85]. Electrons in heme *a* are subsequently passed to the heterobimetallic heme *a*₃:Cu_B center where oxygen reduction occurs. For more detailed reviews on CcO catalysis see [85,86].

4.3. Formation and maturation of CcO redox sites

As mentioned in Section 1, heme *a* is generated by the sequential modification of the protoheme, the product of ferrochelatase (Figs. 6 and 7a). In the first step, a 17-hydroxyethylfarnesyl group is added to the C2 vinyl side chain of heme *b* catalyzed by a farnesyltransferase-type enzyme, generally referred to as heme *o* synthase (HOS). In many bacterial oxidases, this intermediate heme *o* serves as a terminal prosthetic group. Unlike in bacteria, only trace amounts of heme *o* are found in normal mitochondria, but detectable levels are observed in CcO assembly mutants [87,88].

In the subsequent step, the C8 methyl group of heme *o* is converted to a formyl group. Based on studies in bacteria, it has been postulated that conversion occurs by sequential oxidation of the methyl group through a short-lived C8-alcohol intermediate, sometimes referred to as heme *i* or heme o_x [89,90]. This reaction is performed by heme *a* synthase (HAS). Oxidation of the C8 methyl group has been proposed to proceed via two mono-oxygenase reactions [89]. However, this mechanism has been challenged by O₂ isotope-labeling studies demonstrating no significant incorporation of the label into either heme *a* nor heme *i* [91]. The same study suggested that water might be a donor of the oxygen atom in this reaction.

4.3.1. Heme o synthase

The heme *o* synthase (HOS) is designated Cox10 [92]. It is a large membrane protein that in yeast assembles into a multimeric complex of ~300 kDa believed to be a homo-oligomer [93]. The stability of the Cox10 oligomer appears to depend on the presence of newly synthesized Cox1 and its early assembly intermediates. This connection of heme *a* biosynthesis and CcO biogenesis may serve regulatory purposes to minimize excess and toxic levels of highly reactive heme *a*. Cox10-catalyzed conversion of protoheme appears to be a rate-limiting step in heme *a* formation, given its ~8-fold dearth over Cox15 in yeast mitochondria [94]. It is therefore likely that regulation of Cox10 abundance/activity is the way to modulate the heme *a* biosynthetic pathway.

An N196K gain-of-function mutation in Cox10 was identified as a dominant suppressor in cells lacking the CcO assembly factor Coa2. These studies established that Coa2 functions in concert with Cox10 in the hemylation of Cox1 (see Section 4.4.2). While Cox10 exists only as the monomer in *coa2* Δ cells and, apparently, is largely inactive, the N196K mutant Cox10 forms a stable oligomer, and the enzyme is active as the cells respire normally [93]. Cox10 oligomerization appears to be a key feature of HOS enzymatic activity.



Fig. 5. Arrangement of the hemes *a* and $a_3:Cu_B$ and Cu_A in CcO. The binuclear Cu_A center is located in Cox2 subunit (transparent green) and is the entrance site for electrons from reduced cytochrome *c*. Electrons are subsequently passed to the low-spin, *bis*-His heme *a* and then to the heterobimetallic heme $a_3:Cu_B$ center in Cox1 (transparent grey) where O_2 reduction occurs. The axial ligands to the heme iron are highlighted along with respective residue numbers and subunits (PDB ID: 2OCC numbering).

4.3.2. Heme a synthase

Heme *a* synthase was first described in *B. subtilis* [95–97]. Further studies identified eukaryotic orthologs commonly designated Cox15 [98,99]. Purified CtaA has been shown to contain sub-stoichiometric amounts of both hemes *b* and *a*. This led to a conclusion that HAS is a heme *b*-containing enzyme utilizing the cofactor at the active site, akin to other oxygenases. Bound heme *a* was interpreted as an unreleased reaction product [97]. A bioinformatic approach helped to identify proteins acting in concert with Cox15. In *Schizosaccharomyces pombe*, HAS exists as a part of a natural fusion protein, consisting of Cox15 and a Yah1-like ferredoxin extension [100,101]. Further studies confirmed the involvement of Yah1 as well as ferredoxin reductase Arh1 in heme *a* biosynthesis [88].

The catalytic domain of Cox15 is projected to exist on the IMS side of the IM. Since the catalytic domain of Cox10 resides on the matrix side of the IM, an unresolved question concerns how heme o is translocated to the IMS side of the IM for Cox15-mediated oxidation. Electrons for this step are likely passed through the heme b in Cox15 from ferredoxin in the matrix.

Similar to HOS, heme *a* synthases are polytopic membraneembedded proteins with up to eight predicted TM segments [90,98]. Studies in yeast demonstrated that Cox15 forms a high molecular weight complex [93,98], but its composition is unknown. Brown et al. overexpressed *B. subtilis* CtaB and CtaA, as well as *R. sphaeroides* Cox10 and Cox15 in *E. coli* and showed interaction of HOS and HAS in both cases [91]. This observation led to the hypothesis that heme *o* is channeled directly from HOS to HAS. Studies in yeast, however, failed to demonstrate interaction of Cox10 and Cox15. Moreover, the deletion of *COX15* does not affect the stability of the Cox10 complex and vice versa [93] (and our unpublished results). Unlike Cox10, the Cox15 high-mass complex is not responsive to perturbations of Cox1 synthesis or maturation.

Yeast lacking Cox15 contain no heme a, but show low levels of heme o, suggesting that the two enzymes are not intricately linked [87]. Likewise, fibroblasts isolated from patients with fatal infantile hypertrophic cardiomyopathy associated with mutations in Cox15 are characterized by reduced heme a levels, but heme o content is elevated [102]. It should be noted, however, that overexpression of



Fig. 6. Chemical modifications of heme *b* to heme *a* via heme *o* catalyzed by the enzymes Cox10 (Heme *o* synthase, HOS) and Cox15 (Heme a synthase, HAS). In mitochondria, heme *o* is considered to be an intermediate and does not serve as a cofactor; where as in bacteria, heme *o* can functionally replace heme *a* in its terminal oxidase (e.g., cytbo₃). Ferredoxin Yah1 and adrenodoxin Arh1 supply electrons for Cox15-mediated heme *o* to heme *a* conversion.

COX10 in Cox15-deficient yeast cells does not result in the increase of heme *o* levels. Based on this observation, Barros and Tzagoloff suggested that there is positive regulation of HOS function by either Cox15 or the product of the Cox15-catalyzed reaction.

4.4. Maturation of heme a-containing reactive sites within CcO

Hemylation of Cox1 is one of the key events in CcO biogenesis. Heme *a* is required for stability and folding of the Cox1 subunit [92,103,104]. This is consistent with structural data indicating that heme *a* is not only a reactive site but also a "structural unit" linking together distant helical bundles of Cox1. In some bacteria, such as *R. sphaeroides*, CcO assembly proceeds normally even in the absence of heme *a* [105]. Based on these observations, it has been assumed that hemylation of Cox1 occurs either co-translationally or upon its incorporation into the lipid bilayer [106,107].

A more recent study indicated that Cox1 hemylation is a posttranslational event [108]. In yeast, CcO biogenesis initiates with Cox1 synthesis on mitochondrial ribosomes tethered to the IM by IMassociated translational activators Pet309 and Mss51 (Fig. 7b). Mss51 has a second function in translational elongation of Cox1, which is performed within a high-molecular-weight complex consisting of Mss51, chaperone-like factors Cox14 and Coa3/Cox25, and mHsp70 chaperone Ssc1, which represents, along with newly synthesized Cox1, an early CcO assembly intermediate [75,76]. Cox1 maturation proceeds to downstream assembly complexes containing assembly factors designated Coa1, Coa2 and Shy1 (SURF1 in humans) [108–111]. Neither the Mss51- nor Coa1-containing Cox1 intermediates have the redox sites



Fig. 7. (A) Synthesis of heme *a* from protoporphyrin IX (PPIX) for hemylation of CcO in the mitochondria. PPIX (product of PPIX oxidase, not shown) likely enters ferrochelatase (shown as a dimer) from the IM. The product heme *b* exits either directly to the IM, or to the matrix, where it is utilized for hemylation of complex II and complex III. A sub population of heme *b* is converted to heme *a* via Cox10 and Cox15 within the IM. The exit path of heme *a* from Cox15 is unknown. As drawn, the heme exits on the matrix side of the IM to be used for CcO assembly. (B) Possible route of hemylation of Cox1 subunit in yeast CcO. Translation of COX1 mRNA commences CcO biogenesis (a). Newly synthesized Cox1 forms a stable complex with Mss51, Cox14, Coa3 and mitochondrial Hsp70 chaperone, Ssc1, independent of the mitochondrial ribosome (b). Subsequently, Coa1 appears to mediate progression of Cox1 translation/assembly (c). With the help of Shy1 and Coa2, heme *a* is incorporated into maturing Cox1 (d) to form the stable Cox1–Cox5a–Cox6 S2 complex (e). PDB accession number: 2OCC was used to generate the figure.

populated; removal of either Cox10 or Cox15 (or other molecules involved in cofactor synthesis or incorporation) has no effect on the stability of either complex. Likewise, these intermediates are not affected in *cox1* strains with mutated heme *a* axial ligands [108].

The formation of both the heme *a* and the heme a_3 -Cu_B sites appears to occur within or adjacent to the Shy1/SURF1-containing Cox1 assembly intermediate. Studies with fibroblasts from patients with mutations in Cox10 and Cox15 reveal the absence of the partially assembled Cox1 intermediate [102,112,113]. Similarly, the lack of each protein in yeast affects the Shy1 associated Cox1 assembly intermediate [108]. These observations are supported by the genetic interactions in yeast between Cox10, Coa1, Shy1 and Coa2 [110,111]. CcO assembly blocked at either Cox2 maturation or addition of the Cu_B moiety results in accumulation of a transient Cox1 pro-oxidant intermediate. The nature of such a pro-oxidant is not fully understood, but it appears to correlate with the presence of a reactive five-coordinate heme a_3 [114]. The pro-oxidant heme a_3 :Cox1 intermediate is absent in cells lacking Shy1 or Coa1 [111], another observation supporting the postulate about the heme *a* maturation point.

There are a few lines of evidence that suggest the heme *a* site may be formed earlier than the heme a_3 center. First, CcO isolated from *R. sphaeroides* or *P. denitrificans* cells lacking SURF1 orthologs is deficient in heme a_3 but not heme *a* [115,116]. Second, a stalled Cox1 assembly intermediate containing a subset of nuclearencoded subunits is observed in the fibroblasts from SURF1, but not Cox10- or Cox15-deficient patients [102,112,113]. It is plausible that heme *a* insertion may be necessary for formation/stabilization of the intermediate. Likewise, studies on the assembly of *E. coli* bo_3 oxidase, where heme *b* is analogous to the heme *a* of CcO, demonstrated that formation of the heme *b* site is critical for further maturation of the enzyme [117].

4.4.1. Shy1/Surf1 proteins

Heme *a* can spontaneously self-insert into maquettes, chemically engineered four-helix bundles mimicking the ones of Cox1 [118]. However, questions exist regarding the speed and efficacy of such insertion, which become critical aspects *in vivo*, where unfolded hydrophobic proteins are quickly shielded or removed by quality control systems to prevent aggregation and/or deleterious side effects [119]. It is therefore plausible that dedicated factors exist to facilitate heme *a* insertion.

SURF1/Shy1 proteins have been implicated in heme a site formation. Although the exact role of the protein remains nebulous, P. denitrificans SURF1 isoforms were shown to bind heme a in a 1:1 stoichiometry when co-expressed along with heme a biosynthetic enzymes in E. coli [120]. The same study identified a conserved histidine residue important for heme binding and implicated SURF1 as a heme a insertase. In Thermacea bacteria, the ba3-type CcO biogenesis is independent of a SURF1 protein, but an unrelated CbaX factor is required for heme *a*³ site formation [121]. The CbaX factor lacks any histidine residues, and no heme binding studies were conducted. The heme-insertase scenario may not hold true for eukaryotic Shy1/SURF1; mutation of the conserved histidine residues in yeast Shy1 has only minor or no effect on respiration [122]. Consistently, yeast cells lacking Shy1, as well as SURF1-deficient patient cell lines retain substantial respiratory activity. Therefore, it remains unclear whether Shy1/SURF1 simply acts as a chaperone stabilizing the maturing Cox1 protein or whether it has a more direct role in heme a_3 insertion.

4.4.2. Coa2

Genetic studies in yeast identified another molecule, designated Coa2, critical for Cox1 hemylation. Coa2 has been identified as a high-copy suppressor of the respiratory defect in $coa1\Delta$ and $shy1\Delta$ deletion strains and has been shown to transiently interact with Shy1 [111].

Subsequent genetic studies found that Coa2 functions downstream of Mss51 and Coa1 [93]. Cells lacking Coa2 exhibit a facile degradation of Cox1 that appears to arise from impaired hemylation and subsequent misfolding of the molecule [93,123]. The respiratory defect of *coa2* Δ cells is suppressed by the dominant N196K gain-of-function mutation in Cox10 [93]. The Cox10 high-mass complex is severely attenuated in *coa2* Δ cells. The suppressive effect of the mutation is restricted to Coa2-deficient cells and appears to depend on restoration of the Cox10 oligomeric state and the abundance of the oligomer. In addition, attenuation of Oma1 protease substantially restores respiration in *coa2* Δ cells [93,123]. The absence of the Oma1 protease appears to increase the time for the inefficient Cox1 hemylation process in *coa2* Δ cells.

Similar to Shy1/SURF1, Coa2 does not appear to be a dedicated heme chaperone; substitutions or removal of the residues that potentially could constitute a heme-binding motif have no effect on respiratory function (our unpublished results). Coa2 is important in coupling Cox1 synthesis to Cox10 oligomerization and in addition appears to have a role in the formation of the low spin heme *a* site in Cox1. It remains to be determined whether formation of the heme *a* site occurs in a similar fashion in human mitochondria. No robust mammalian homolog of Coa2 has been identified so far.

5. Concluding remarks and perspective

Hemes are integral components of the respiratory complexes II, III, and IV that perform multi-electron transport and catalysis. The functional significance of the heme in complex II remains unclear, as does the relevance of its second quinone-binding site. An intriguing observation that the SDH subunit Sdh3 exists in a second complex with Tim18 creates the possibility that heme has a role in the TIM22 translocase for mitochondrial import of carrier proteins. Future studies will need to resolve whether the hemes in SDH and the Sdh3/Tim18 complex have only a structural role or participate in an electron transfer reaction.

Important biological questions persist regarding the maturation of heme centers in complex III and complex IV. Hemylation of the subunit Cob of complex III, for example, is likely required for subsequent nucleation biogenesis of additional subunits. It will be important to resolve at which stage of Cob maturation the heme is inserted, and whether additional proteins function in the hemylation reaction. Covalent association of heme with cytochrome c and c_1 occurs in the IMS, yet information is lacking on how heme is translocated from the site of its formation on the matrix-facing surface of the IM back to the IMS. The translocation step is likely coupled to heme presentation to the proteins involved in covalent heme addition. Complex IV biogenesis is closely linked to heme site maturation. Significant questions persist on the mechanism of heme a insertion into Cox1 from the IMS side of the IM as well as the translocation of heme o from the matrix side of the IM to Cox15. The heme *b* in Cox15 is likely a key cofactor for the electron transfer from ferredoxin in the matrix to the Cox15 reaction center. A final key question remains on whether bioavailable heme b pools exist within the mitochondria or IMS for hemylation reactions. Although much information is known for heme reaction mechanisms, the biological movement of heme for use in hemylation reactions remains uncharacterized.

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