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Redox and Reactive Oxygen Species Regulation of Mitochondrial Cytochrome *c* Oxidase Biogenesis

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Abstract

Significance: Cytochrome *c* oxidase (COX), the last enzyme of the mitochondrial respiratory chain, is the major oxygen consumer enzyme in the cell. COX biogenesis involves several redox-regulated steps. The process is highly regulated to prevent the formation of pro-oxidant intermediates. **Recent Advances:** Regulation of COX assembly involves several reactive oxygen species and redox-regulated steps. These include: (i) Intricate redox-controlled machineries coordinate the expression of COX isoenzymes depending on the environmental oxygen concentration. (ii) COX is a heme A-copper metalloenzyme. COX copper metallation involves the copper chaperone Cox17 and several other recently described cysteine-rich proteins, which are oxidatively folded in the mitochondrial intermembrane space. Copper transfer to COX subunits 1 and 2 requires concomitant transfer of redox power. (iii) To avoid the accumulation of reactive assembly intermediates, COX is regulated at the translational level to minimize synthesis of the heme A-containing Cox1 subunit when assembly is impaired. **Critical Issues:** An increasing number of regulatory pathways converge to facilitate efficient COX assembly, thus preventing oxidative stress. **Future Directions:** Here we will review on the redox-regulated COX biogenesis steps and will discuss their physiological relevance. Forthcoming insights into the precise regulation of mitochondrial COX biogenesis in normal and stress conditions will likely open future perspectives for understanding mitochondrial redox regulation and prevention of oxidative stress. *Antioxid. Redox Signal.* 19, 1940–1952.

Introduction

REDUCTION–OXIDATION (REDOX) CHEMISTRY, involving the transfer of electrons and hydrogen atoms, is central to energy conversion in cellular respiration, which generates reactive oxygen species (ROS) as inevitable by-products. In eukaryotes, the respiratory enzymatic machinery is housed within mitochondria. The mitochondrial respiratory chain (MRC) consists of four enzymatic complexes (complexes I to IV) and two mobile electron carriers (cytochrome *c* [cyt *c*] and coenzyme Q) that act in concert to transfer electrons from reducing equivalents to molecular oxygen (O₂). During respiration, approximately 1%–2% O₂ is converted into superoxide anion (O₂^{•−}) due to electron leak at complexes I (NADH-ubiquinone oxidoreductase) and III (ubiquinol cyt *c* reductase), the main natural producers of mitochondrial ROS [reviewed in Refs. (39, 59, 83)]. In some organisms, such as the yeast *Saccharomyces cerevisiae*, complex I is substituted by several single subunit NADH dehydrogenases, such as NDI1, which does not produce detectable ROS levels (90, 106), leaving complex III as the major ROS producer.

The focus of this review is on the terminal MRC enzyme cytochrome *c* oxidase (COX, complex IV), a heterooligomeric heme A-copper oxidase that catalyzes the reduction of O₂ by ferrocycytochrome *c*. Although COX is one of the major oxygen-consuming enzymes of the cell, ROS generation during O₂ reduction is negligible. The enzymatic catalytic core is formed by three mitochondrial DNA-encoded subunits that enclose three metal centers (Fig. 1A). Cox1 contains a low-spin heme *a* site and a binuclear center formed by a high-spin heme *a*₃ and a copper atom (Cu_B). Cox2, which interacts with cyt *c*, contains a mixed valence dinuclear copper center (Cu_A). Oxygen reduction requires four electrons, which enter COX through the Cu_A site, are transferred to heme *a*, and subsequently to the binuclear active site, where O₂ is bound and reduced. The reduction of O₂ to water proceeds *via* one electron at a time, and thus involves several successive reduction steps. The COX catalytic cycle has been recently reviewed in detail (104). Importantly, in the MRC, COX retains all partially reduced oxygen intermediates until full reduction is achieved (104), which avoids ROS generation. In COX, electron transfer is coupled to proton pumping across

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FIG. 1. Cytochrome c oxidase (COX): metal cofactors and function. (A) Metal cofactors in COX catalytic subunits 1 and 2. The catalytic core of COX contains copper and iron prosthetic groups. Two copper atoms bound to subunit 2 constitute the Cu_A site. In subunit 1, there are two redox centers: a low-spin heme a group, and a binuclear center formed by a third copper atom, Cu_B , associated with the high-spin heme a_3 group. The two heme planes are both essentially perpendicular to the mitochondrial membrane plane (not depicted here, but see panel B). Ligand-bound structures of bovine COX subunits 1 and 2 were obtained from the protein data bank (PDB) website (www.rcsb.org/pdb/home/home.do) and modified to prepare the figure. The YASARA molecular-graphics, -modeling, and -simulation program, developed by Elmer Krieger, was used to generate a cartoon model of the different proteins. **(B)** COX functions as an electron-driven proton pump. The Cu_A site in subunit 2 is the primary acceptor of electrons from ferrocyanide c . Electrons are subsequently transferred to the low-spin heme a group located in subunit 1. And from there to the binuclear Cu_B -heme a_3 center of subunit 1, where oxygen binds and is sequentially reduced to water. Electron transfer to dioxygen is coupled to proton pumping across the inner mitochondrial membrane that contributes generating a gradient that is used by the F_1F_0 -ATPase to synthesize adenosine-5'-triphosphate (ATP). In addition, one substrate proton per electron (not depicted here) is delivered into the binuclear site to form water.

the inner mitochondrial membrane (Fig. 1B), a function that may be modulated by the core subunit Cox3, thus contributing to establish the proton gradient required to synthesize adenosine-5'-triphosphate (ATP). In addition to the core subunits that are conserved in the prokaryotic enzyme, mitochondrial COX contains 8 (yeast) to 10 (mammals) nuclear-encoded subunits. They play roles in regulating COX assembly and function and are believed to act as a shield to protect the catalytic core (31).

Investigations in recent years have shed light into the sophistication surrounding COX biogenesis, disclosing several redox-controlled or redox-dependent processes and regulatory mechanisms to minimize oxidative damage. Besides its structural subunits, COX assembly requires an extensive and growing number of ancillary factors, which act at all the steps of the pathway (31). The assembly process is thought to be linear, consisting on the successive incorporation of subunits to membrane-inserted Cox1. Recent data have suggested that an additional alternative pathway may exist to incorporate COX subunits and subunits of other MRC complexes directly into a macromolecular structure to form supercomplexes or respirasomes (72). COX is known to display intrinsic heterogeneity concerning its subunit composition. Cox5 exists in two oxygen-regulated isoforms, and two newly identified COX-associated proteins, Rcf1 and Rcf2, are required for growth in hypoxia and for oligomerization of a subclass of COX complexes into respirasomes (18, 95, 101).

Here, we will discuss recent data on COX biogenesis steps and regulatory processes involving ROS signaling to regulate COX subunit composition, mitochondrial oxidative folding, and redox regulation of copper delivery to COX as well as translational control to prevent formation of pro-oxidant

intermediates (Fig. 2). We focus on the processes described in the yeast, *S. cerevisiae*, with information from prokaryotes and higher eukaryotes when appropriate.

Redox Control of COX Isoenzyme Assembly

Oxygen-regulated COX isoenzymes involving subunit Cox5/COX4

COX plays an important role in MRC regulation to adapt to changing environmental oxygen levels through a mechanism involving a nuclear-encoded subunit, termed Cox5 in yeast and COX4 in mammals (31). Structurally, Cox5/COX4 have interacting surfaces with two core subunits, Cox1 and Cox2 affecting COX redox centers and the stability of the holoenzyme (31). Both Cox5 and COX4 exist in two interchangeable isoforms termed Cox5a/b and COX4-1/2, respectively, whose expression is regulated by oxygen (52). Cox5a/COX4-1 are expressed in normoxia (above $0.5 \mu M/1\%$ of O_2), whereas Cox5b/COX4-2 are expressed under hypoxia (below $0.5 \mu M/1\%$ of O_2) (12, 34). In yeast, cyt c also exists in two oxygen-regulated isoforms: normoxic iso-1-cyt (CYC1) and hypoxic iso-2-cyt (CYC7), which act synergistically with the Cox5 isoforms (2, 102). Cox5 isoforms have differential effects on the COX enzymatic activity. The hypoxic isoforms modify an internal step in electron transport between heme a and the binuclear reaction center, which occurs three to four times faster than in the normoxic COX isoenzyme, thus altering the enzyme maximal turnover number (TN_{max}), but not its K_m (2). As a result, by regulating the proportion of each isoform assembled into the holoenzyme, the catalytic efficiency of COX is modulated adjusting the MRC electron transfer rate to adapt to changes in the environmental oxygen tension (44).

FIG. 2. Reactive oxygen species (ROS) and redox control mechanisms involved in COX biogenesis. General chaperones and RNA-specific translational activators (not depicted here) are required for synthesis of the mtDNA-encoded subunits forming the COX catalytic core. Following their insertion into the inner membrane (MIM), Cox1 and Cox2 are matured by addition of metal cofactors. At some point, substrate-specific chaperones bind Cox1, Cox2, and Cox3 to maintain them in an assembly-competent state. Following Cox1 maturation, the nuclear DNA-encoded Cox5 and 6 subunits are added to Cox1 before incorporation of the other core subunits and the rest of the accessory subunits to form the holoenzyme. The ROS and redox control mechanisms involved in COX biogenesis are noted in start-shape boxes.

Transcriptional regulation of COX5a/COX5b isoforms in yeast

In yeast, the oxygen regulation of all nuclear COX genes is mediated by heme that acts as an intracellular effector of the environmental signal through two heme-dependent transcriptional activators. Hap1 arbitrates the oxygen regulation of aerobic genes and its activity is modulated by heme binding (107). The Hap complex (Hap2/3/4/5) mediates regulation in response to two environmental stimuli, oxygen concentration and carbon source availability (33). How heme modulates the Hap complex activity remains unclear. In normoxia, the expression of nuclear COX genes, including *COX5a*, is induced by the Hap2/3/4/5 complex, while *COX5b* is repressed by the transcriptional repressor encoded by *ROX1*, whose expression is induced by Hap1 (23) (Fig. 3A). A second poorly characterized repressor, the oxygen-independent *ORD1*, specifically contributes to *COX5b* aerobic repression (60). Instead, in hypoxia, heme is poorly synthesized and neither the Hap2/3/4/5 complex can activate its target COX genes nor *Rox1* represses *COX5b* expression (97) (Fig. 3A).

Role of hypoxic COX in redox signaling in yeast

COX can act as a nitric oxide (NO) reductase using nitrite as a substrate (15). In normoxia, oxygen binds COX with a higher affinity than nitrite. However, in hypoxia, the NO reductase activity is enhanced, thus producing NO (15). The recent discovery that in hypoxic conditions Cox5b-containing hypoxic COX produces a higher amount of NO than the normoxic

enzyme, further emphasizes the role of COX in hypoxic signaling, and suggests a positive feedback mechanism since NO serves as an additional signal to activate expression of several hypoxic genes, including *COX5b* and *CYC7* (15).

Regulation of mammalian COXIV-1/COXIV-2

In mammals, COX4-1 and COX4-2 are respectively encoded in the genes *COXIV-1* and *COXIV-2*, which expression are also inversely regulated by oxygen concentrations (Fig. 3B). Additionally, *COXIV-2* expression varies depending on the development stage and tissue (52). For example, a high expression level for *COXIV-2* mRNA is paradoxically observed in highly oxygenated adult lung and trachea of human and rat. Highly efficient hypoxic COX could minimize electron leakage and contribute to protect these tissues from oxidative damage (52). The hypoxia-inducing factor 1 (HIF-1) complex, a transcriptional activator that functions as a master regulator of oxygen homeostasis in all metazoan species, regulates COX subunit isoform switch (34). HIF-1 is a heterodimeric complex composed of a constitutively expressed HIF-1 β subunit and an O₂-regulated HIF-1 α or HIF-2 α subunit. HIF-1 activity is inhibited by prolyl (PHDs) and asparaginyl (factor inhibiting HIF-1 [FIH-1]) hydroxylases, which use O₂ as a substrate to hydroxylate HIF-1 α and HIF-2 α . In hypoxic cells, hydroxylation is inhibited, leading to HIF-1-mediated transcriptional activation of *COXIV-2* and the *LON* gene, which encodes a protease required for COX4-1 degradation (34), thus facilitating the COX4-1 to COX4-2 subunit switch. However, promoter analysis have revealed that an

The structural and functional organization of the respiratory chain has been a matter of debate over more than 50 years (17, 37). Around a decade ago, the groups of Schagger (86, 88) and Stuart (22) produced new evidence of stoichiometric assemblies of individual complexes in yeast and in mammalian mitochondria, and suggested an organization of the MRC in supercomplexes or respirasomes. It is currently accepted that both individual enzymes and supercomplexes probably co-exist. This plasticity model suggests that OXPHOS complexes

A

YEAST

Normoxia: O_2 leads to **HEME**. HEME promotes **Hap2/3/4/5** and **Hap1**. **Hap1** promotes **ROX1** (which inhibits **Ord1**) and **CYC1**. **Ord1** inhibits **COX5b**. **CYC1** promotes **Cox5a** (95%) and **Cox5b** (5%). **CYC7** promotes **Cox5b** (5%). The final complex is **COX** (Cox5a 95%, Cox5b 5%), which uses $O_2 + 4H^+ \rightarrow 2H_2O$. Iso-1 cyc is associated with Cox5a, and Iso-2 cyc is associated with Cox5b.

Hypoxia: O_2 leads to **HEME**. HEME inhibits **Hap2/3/4/5** and **Hap1**. **Hap1** promotes **ROX1** (which inhibits **Ord1**) and **CYC1**. **Ord1** inhibits **COX5b**. **CYC1** promotes **Cox5a** (95%) and **Cox5b** (5%). **CYC7** promotes **Cox5b** (5%). The final complex is **COX** (Cox5a 95%, Cox5b 5%), which uses $O_2 + 4H^+ \rightarrow 2H_2O$. **NO** inhibits **COX**. **NO** is produced from $NO_2 + 2H^+ \rightarrow NO + H_2O$. Iso-1 cyc is associated with Cox5a, and Iso-2 cyc is associated with Cox5b.

B

MAMMALS

Normoxia: **FIH1** and **PHD1** hydroxylate **HIF1α** at **p300**, leading to **Degradation**. **HIF1α** and **HIF-β** form **HIF-1α**, which promotes **COX4-1** and **COXIV-2**. **COX4-1** promotes **COX** (COX4-1, COX4-2). **COXIV-2** promotes **COX** (COX4-1, COX4-2). **LON** inhibits **COX4-1**.

Hypoxia: **PHD1/FIH-1** are inhibited, leading to **HIF1α** stabilization. **HIF1α** undergoes **Nuclear translocation** and forms **HIF-1α** with **HIF-β**. **HIF-1α** promotes **COX4-1** and **COXIV-2**. **COX4-1** promotes **COX** (COX4-1, COX4-2). **COXIV-2** promotes **COX** (COX4-1, COX4-2). **LON** inhibits **COX4-1**.

(CI to CIV) switch from freely moving to fixed structures and vice-versa to adapt to changes in cellular metabolism (1, 43). Accordingly, the coexistence of the respirasome unit, composed in mammals of at least CI + CIII + CIV, together with the intermediate supercomplexes CI + CIII and CIII + CIV and free CIII and CIV has been widely described (1, 3, 22, 29, 40, 85–88, 105). The functional role of the respirasomes in cellular bioenergetics remains unclear, but they have been hypothesized to offer structural and functional advantages to the system, such as the prevention of destabilization and degradation of the OXPHOS complexes, the enhancement of electron transport efficiency through substrate channeling, or the decrease of electron or proton leakages (63). Recent data support a role in controlling the formation of reactive oxygen intermediates (18, 101).

In addition to their structural subunits, the biogenesis of OXPHOS complexes requires a large number of ancillary proteins, generally termed assembly factors. While most factors involved in the assembly of complex I have been discovered in human patients carrying disease-causing versions (100), most factors involved in the assembly of complexes III and IV have been discovered in the yeast *S. cerevisiae* (31, 93, 94). In *S. cerevisiae*, COX assembly factors (such as Shy1, Cox14, or Cox25/Coa3) have been found associated with structural subunits of both complexes (30, 71) in what probably represent CIII-CIV supercomplex assembly intermediates. However, the first respirasome-specific assembly/stability factors, the conserved Rcf1 and Rcf2, were identified only recently. Rcf1 is a member of the conserved hypoxia-induced gene 1 (Hig1) protein family (18, 95, 101). Three independent groups have reported that yeast Rcf1 is important for growth in hypoxia and the assembly of the peripheral COX subunits, Cox12 and Cox13. Additionally, Rcf1 interacts independently with CIII and CIV although the interaction with COX seems to be stronger and occurs through Cox3, predicted to be at the CIII-CIV interface. Both Rcf1 and Rcf2 interact with CIII-CIV supercomplexes, but they do not copurify. This has suggested that Rcf1 promotes the oligomerization/stability of only a subclass of CIV monomers that do not contain Rcf2 on CIII₂-CIV₂ supercomplexes (18, 95, 101). These studies have suggested that CIV displays intrinsic heterogeneity with regard to its subunit composition and that distinct forms of respirasomes can be formed by CIV variants (101). Noteworthy, the impairment of CIII₂-CIV₂ supercomplexes in the yeast *rcf1* null strain was found associated with an increase in ROS generation and sensitivity to oxidative stress (18, 101), suggesting the supercomplexes somehow minimize electron leakage.

The reciprocal effects between ROS and supercomplexes remain to be fully understood. Notably, recent studies on mouse complex III cells knocked out for the Rieske iron-sulfur protein (RISP) subunit have suggested that ROS produce a deleterious effect on the stability of CI, CIV, and supercomplexes, at least in the context of defective OXPHOS (27). In support of that conclusion, treatment of the mutant cells with an SOD mimetic compound (MnTBAP) was shown to partially stabilize CI and supercomplexes (27).

Redox Control of Copper Delivery to COX

Redox environment within mitochondria

Mitochondria are the major ROS-generating cellular organelle, and therefore redox regulation within the mitochondria

is essential for several processes, including COX biogenesis (39). While the redox environment of the cytosol (−286 mV) and mitochondrial matrix (−296 mV) is reducing, the intermembrane space (IMS) is more oxidizing (−255 mV) (51). Consistently, the reduced glutathione/oxidized glutathione (GSH/GSSH) ratio, one of the major redox regulatory systems in the cell, was estimated to be 250:1 in the IMS (compared to 900:1 in the matrix or 3000:1 in the cytosol) (51). It remains unclear though, whether regulation of the IMS redox environment is really contributed by GSH. In human cells, glutaredoxin 1 (GRx1), which catalyzes reversible protein glutathionylation, specifically localizes to IMS (76). However, in yeast, the IMS does not contain Glr1, the GSSH reductase that regenerates GSH from GSSH (75) and redox deregulation induced by unbalancing the GSH/GSSH ratio in the cytosol and the matrix was reported to not influence the IMS redox environment (51). This conclusion has been partially challenged in a very recent article, which has convincingly demonstrated that the glutathione pools of the IMS and cytosol are dynamically interconnected *via* porins, while no communication was observed between the IMS and matrix glutathione pools (58). By modulating redox pathways in the cytosol and IMS, the authors found that the cytosolic glutathione reductase system is the major determinant of the glutathione redox potential in the IMS, thus explaining why they find it similar to the glutathione redox potential in the cytosol (58).

Although the precise mechanisms involved in the IMS redox regulation remain to be fully characterized, this compartment harbors several redox pathways, including a pathway for oxidative protein folding, and redox chemistry important for COX biogenesis.

Mitochondrial import of COX copper chaperones through an oxidative folding pathway

Because the vast majority of mitochondrial proteins are encoded in nuclear genes, the cells have devised several protein transport mechanisms for delivery to the correct submitochondrial compartments. A small fraction of IMS proteins use a conserved disulfide relay system that involves the redox-regulated import receptor Mia40 and the sulfhydryl oxidase Erv1 (Fig. 4) [reviewed in Refs. (26, 41, 92)].

The Mia40/Erv1 system promotes the oxidative folding of newly imported cysteine-rich small precursor proteins, thus ensuring their retention within mitochondria. Most import substrates contain either twin CX₃C (73) or CX₉C (16) structural motifs, which upon oxidation form two disulfide bonds. Focusing on the twin CX₉C proteins, they are mostly required for COX biogenesis (66). In the current model, Mia40 substrates are synthesized in the cytosol and cross the outer mitochondrial membrane in an unfolded state through the translocase of the outer membrane complex. The IMS localized Mia40 traps the substrate proteins by forming an intermolecular disulfide bond. Subsequently, it introduces disulfide bonds in the substrate to promote their native conformation. Upon substrate release, Erv1 reoxidizes Mia40 and delivers electrons to cyt *c* for further delivery to COX, thus connecting oxidative folding to respiration. The IMS redox environment probably influences the import by the Mia40 pathway [reviewed in Refs. (26, 41, 92)]. Supporting this possibility, glutathione increases the *in vitro* and *in organello*

FIG. 4. Mechanism of oxidative folding in the mitochondrial IMS. The mitochondrial IMS harbors the Mia40 pathway that promotes the oxidative folding of newly imported cysteine-rich small precursor proteins, thus ensuring their retention within mitochondria. Some import substrates contain twin CX₉C structural motifs, which upon oxidation form two disulfide bonds. Several twin CX₉C proteins are required for COX assembly. The oxidative pathway consists of at least two enzymes, the redox-regulated receptor Mia40 and the sulphhydryl oxidase Erv1. In the current model, Mia40 traps the substrate proteins by forming a mixed disulfide bond with them and also introduces disulfide bonds to promote their native conformation. Subsequently, Erv1 re-oxidizes Mia40 and delivers electrons to cytochrome *c*, thus connecting oxidative folding to respiration. Yeast Mia40 is anchored to the inner mitochondrial membrane, while its human homologue is a soluble protein. A soluble Mia40 protein is depicted here for simplification.

Mia40-dependent oxidation of Cox19, a CX₉C substrate, by counteracting the formation of long lived Mia40 substrate intermediates (10) and has been shown to contribute to the partially reduced redox state of the Mia40 *in vivo* (58).

In addition to Mia40, thirteen conserved small CX₉C proteins are found in the IMS (66). At least ten of these proteins are known to play roles relevant to COX assembly/stability (66). With a couple of exceptions (Cox17 and Som1), their exact functions are largely uncharacterized. However, several CX₉C proteins have been proposed to function on copper and/or redox transfer within the IMS for ultimate delivery to COX (47).

Redox-regulated copper transfer to COX

The metallation of the Cu_A and Cu_B centers in COX uses copper from a matrix-located pool (21). By unknown mechanisms, copper is transferred to the IMS and reaches Cox17, a

hydrophobic CX₉C protein containing two additional cysteines proximal to the CX₉C motif forming a CC Cu(I) binding site (77). Cox17 transfers copper ions to two metallochaperones, Sco1 and Cox11 that facilitate copper insertion into the Cox2-Cu_A and Cox1-Cu_B sites, respectively (Fig. 5) (13, 35, 42, 49, 65). Cox11 and Sco1 are anchored to the mitochondrial inner membrane through a transmembrane α -helix and expose the copper-binding site in the IMS, where copper transfer occurs [(11, 56, 81) and reviewed in (21, 47, 84)].

The Cu_B site is formed by one copper ion coordinated by three histidine ligands and in close proximity to the heme *a*₃ moiety (67). The soluble C-terminal domain of Cox11 forms a dimer that coordinates one Cu(I) per monomer. The two Cu(I) ions in the dimer exist in a binuclear cluster, coordinated by three conserved cysteine residues (13). The mechanism by which Cox11 transfers copper to the Cu_B site remains to be disclosed.

The Cu_A dinuclear center in Cox2 is coordinated by a CxExCGx₂Hx₂M motif and exists as a [Cu(II)/Cu(I)] complex. A soluble truncated Sco1 variant binds copper donated from Cox17 *in vitro* (14). NMR studies suggested that human COX17 transfers reducing potential and Cu(I) to enable SCO1 metallation (4). Sco1 directly interacts with Cox2 (65) and has

FIG. 5. Copper delivery to COX involves redox chemistry. The mitochondrial matrix contains a pool of copper, bound to a nonproteinaceous ligand, used for metallation of COX and mitochondrial superoxide dismutase 1 (mt-Sod1). By an unknown mechanism, copper ions are transferred across the MIM to the intermembrane space (IMS), where they bind Ccs1, the mt-Sod1 copper chaperone and Cox17, a COX copper chaperone. Ccs1 forms an intermolecular disulfide bond with Sod1 to introduce copper and a disulfide bond into Sod1. The hypothetical role of the several CX₉C proteins (Cmc1/2, Cox19, and Cox23) in regulating copper transfer toward Cox17 is depicted. Cox17 transfers copper to Cox11, the metallochaperone for the Cu_B site in Cox1. Cox17 also transfers copper and redox power to Sco1, the metallochaperone for the Cu_A site in Cox2. OM, outer membrane. Arrows indicate copper transfer. Question marks indicate hypothetical steps.

an essential Cx_3C metal-binding motif, which is able to bind both Cu(I) and Cu(II) (50). It remains to be elucidated whether Sco1 mediates the transfer of both different valent ions or, alternatively if two Cu(I) are inserted in Cox2 by Sco1 and the active site is successively oxidized. The structural similarity of Sco1 with disulfide reductases has suggested that it could be involved in the reduction of cysteines in the Cox2 copper-binding site (19), facilitating copper incorporation (4).

In mammals, Sco1 has two homologues, SCO1 and SCO2, which play independent, cooperative roles in COX biogenesis (62). In the current model, after COX2 metallation by SCO2- and SCO1-dependent simultaneous or sequential copper insertion, SCO2 reoxidizes SCO1 cysteines, a reaction that allows resetting both proteins for further rounds of COX2 biogenesis (62). While both Sco proteins are required for copper transfer to COX2, SCO2 is additionally necessary for COX2 synthesis (62), which serves to coordinate COX2 availability and formation of the Cu_A site during COX biogenesis.

Is there a role for CX_9C proteins on copper and/or redox transfer?

Studies on the large number of CX_9C IMS proteins have suggested that at least a subset could be involved in copper traffic toward Cox17 (47) (Fig. 5). These proteins include Cox19, Cox23, Cmc1, and Cmc2 (47, 66, 84, 94). Supporting this possibility, Cox19 and Cmc1 were shown to bind copper *in vitro* (46, 82), Cmc2 to interact with Cmc1 (48), and the respiratory defect of Δcox23 and Δcmc1 cells to be partially

restored by exogenous copper although Δcox23 cells require concomitant overexpression of Cox17 (7). Current speculation have suggested a daisy chain transfer mechanism in which copper would be successively transferred from the matrix across the inner membrane by an uncharacterized transporter to the membrane-bound Cmc1/Cmc2 and successively to the soluble Cox19, Cox23, and ultimately to Cox17 (47). Such a mechanism would be expected to involve transfer of both reducing potential and Cu(I) to generate reduced Cu(I)-bound proteins. Alternatively, CX_9C proteins could play a role in copper transfer toward Cox17 by modulating the local IMS redox environment. Information concerning the *in vivo* copper-binding capacity and redox state of these proteins seems imperative to attain an understanding of their functions.

Copper distribution to COX and mitochondrial Sod1

The matrix copper pool used for COX metallation is also the copper source for the IMS-localized portion of superoxide dismutase 1 (mt-Sod1) (21). The active enzyme is a homodimer that has one intramolecular disulfide bond, one copper and one zinc atom bound per monomer. Sod1 activation requires the specific copper chaperone Ccs1 (Fig. 5), which forms an intermolecular disulfide bond with Sod1 to introduce a copper atom and a disulfide bond into Sod1 (21).

Interestingly, in *S. cerevisiae*, Δcmc1 and Δcmc2 cells (48) and most COX mutants have wild-type levels of mt-Sod1, but with a significantly higher activity, while Δccs1 cells have a significantly increased functional COX (our unpublished

FIG. 6. COX assembly-feedback regulation of COX1 mRNA translation limits the formation of pro-oxidant intermediates. Following Cox1 synthesis, the COX1 mRNA translational activator Mss51 forms a Cox1-stabilization and preassembly complex with the newly synthesized protein. Other chaperones involved are omitted for simplicity. The complex is disrupted when Cox1 is matured by insertion of its metal prosthetic groups and/or interacts with other COX subunits. Released Mss51 is recycled to perform its role in translation. During maturation, Cox1 form short-living pro-oxidant intermediates that contain heme A and does not accumulate when COX assembly proceed normally. When there is no need for assembling further COX or the case of COX assembly defects, Mss51 is trapped in the complex with Cox1 and is not available for new rounds of Cox1 synthesis. In this way, the formation of pro-oxidant intermediates and potential ROS damage is minimized.

results). These observations suggest a partition of the copper pool between the delivery pathways to COX and mt-Sod1. A balance between copper transfer to COX and Sod1 could control the intricate equilibrium between efficient respiration, ROS damage, and necessary signaling and protection against oxidative stress. However, similar to *ccs1* null mutant cells, it has been reported that a *Δsod1* strain shows increased COX biogenesis and mitochondrial mass, which has been proposed to result, at least, in part, from a ROS-dependent Hap2/3/4 complex activation (89).

Strategies to Regulate COX Biogenesis and Minimize Accumulation of Pro-Oxidant COX Assembly Intermediates

Pro-oxidant intermediates are formed during COX assembly

COX biogenesis is extensively regulated to facilitate the formation of the metal centers, while minimizing the formation of reactive assembly intermediates (Fig. 6). COX assembly starts with the synthesis, membrane insertion, and stabilization of the mitochondrially encoded COX subunits (Fig. 2) (94). Briefly, subunit mRNA-specific translational factors activate translation and coordinate the accumulation of the three core subunits (Cox1, Cox2, and Cox3). Following their membrane insertion by the Oxa1 machinery, subunit-specific chaperones (Mss51, Cox14, and Cox25/Coa3 for Cox1, and Cox20, Cox18, Mss2, and Pnt1 for Cox2), stabilize the core subunits and maintain them in an assembly-competent state (Fig. 2). Starting with the Cox1 biogenesis line, Mss51 handles the Cox1-preassembly complex over to conserved assembly chaperones, such as Coa1 and Shy1 (5, 71, 80). This process is concurrent with the maturation of Cox1 by incorporation of its metal prosthetic groups or the formation of the first assembly intermediates, involving Cox1 + Cox5 + Cox6 (Figs. 2 and 6). Metallated-Cox2 and Cox3 are subsequently incorporated to the growing subassembly,

followed by the rest of nuclear-encoded subunits to form the monomeric COX holoenzyme (45, 74) (Fig. 2).

The formation of the metal prosthetic groups within Cox1 are essential during the COX assembly process and will be more extensively described here because they bear the risk of producing highly reactive intermediates.

While Cox1 hemylation is essential for COX assembly, little is known about the players and mechanism that coordinate heme A synthesis and insertion into Cox1. Heme A biosynthesis from protoheme (heme B) involves at least two reactions (Fig. 7). The first reaction is catalyzed by the farnesyl-transferase Cox10 (98), and involves the formation of a heme O intermediate that is converted to heme A in a reaction catalyzed by Cox15, which functionally cooperates in an electron transport chain with ferredoxin Yah1 and the ferredoxin reductase Arh1 (6). Heme A insertion into Cox1 does not occur cotranslationally. Rather, the two heme A sites form downstream of Mss51- and Coa1-containing Cox1 preassembly and stabilization intermediates (53). In contrast, the Mss51-free, Shy1-containing Cox1 assembly intermediate is perturbed in the absence of heme A, thus suggesting that the incorporation of heme A into Cox1 occurs within this subassembly (53).

Cox1 copper metallation was initially envisioned to occur cotranslationally due to the positioning of the Cu_B site, deeply buried below the inner mitochondrial membrane. Such a hypothesis was supported by studies that show a small portion of Cox11 can interact with mitochondrial ribosomes (56). However, more recent investigations have established that the Cu_B site formation does not actually occur while newly synthesized Cox1 is interacting with Mss51, but near or at the Shy1-containing Cox1 assembly intermediate, perhaps, simultaneously to the incorporation of the heme a₃ moiety into the heterobimetallic center (53).

It remains unclear in what specific order the Cu_B-heme a₃ center is metallated, although studies in a *Rhodobacter sphaeroides Δcox11* strain have shown that the heme a₃ moiety can be delivered to this site in the absence of copper (42).

FIG. 7. COX assembly-feedback regulation of heme A biosynthesis limits the formation of pro-oxidant intermediates. A COX assembly intermediate yet to be identified positively regulates the conversion of heme O to heme A catalyzed by Cox15 in cooperation with the ferredoxin (Yah1)/ferredoxin reductase (Arh1) electron transfer system. On a second level of regulation described in the text, Cox15 regulates the conversion of heme B to heme O catalyzed by Cox10. Pacing heme A biosynthesis to its insertion into Cox1 serves to prevent the formation of heme A-containing reactive assembly intermediates.

Studies in yeast have revealed that null *cox11* mutants, as well as *sco1* mutants, have increased sensitivity to hydrogen peroxide (54). This sensitivity has been attributed to the presence of a highly reactive Cox1-heme a_3 intermediate that accumulates when the assembly pathway is blocked subsequent to Cox1 maturation (54, 57). Insertion of heme a/a_3 in Cox1 occurs before the addition of Cox2 during COX assembly (53), perhaps, through a partially accessible channel on the IMS side of Cox1 that is closed upon Cox2 incorporation (57). Hydrogen peroxide sensitivity is enhanced in strains lacking Cox2, but can be suppressed by overexpression of Cox11 and Sco1 (54). This suggested that these proteins can cap the Cox1 intermediate, thereby precluding peroxide access to the heme a_3 catalytic center (57). Particularly, although a direct Cox11–Cox1 interaction has not been detected, it has been proposed that Cox11 may bind and stabilize Cox1 through transient interactions in a conformer of Cox1 that has less solvent accessibility to the heme a_3 site (57). During normal COX assembly, incorporation of Cox2 would resolve the potentially deleterious Cox1 intermediate.

Noteworthy, the amount of the Shy1-containing Cox1 intermediate found markedly attenuated in Δcox11 cells (53), which could serve to prevent its potential deleterious effects. The model of a pro-oxidant Cox1-containing intermediate is supported by the identification of *AFG1*, coding for a protease involved in the degradation of mitochondrial-encoded COX subunits, as a multicopy suppressor of the peroxide-deficient phenotype of Δcox11 cells (50).

Additional factors seem to modulate hydrogen peroxide sensitivity in Δcox11 cells. Cox11 has been suggested to have a peroxidase function. This was supported by mutagenesis studies that successfully identified point mutations in Cox11 that specifically affected either COX assembly or hydrogen peroxide sensitivity (99). Additionally, the absence of either Cox11 or Sco1, limits peroxide degradation (99). However, while overexpression of *COX11* partially complemented the peroxide sensitivity of a *sco1* null mutant, the overexpression of *SCO1* in Δcox11 cells did not (99), indicating that Cox11 and Sco1 seem to play not exact, but related roles in peroxide metabolism. Interestingly, studies in Δsco1 and Δcox11 *petite* strains in which Cox1 is absent showed different effects when exposed to peroxide stress. Unlike Δsco1 cells, whose peroxide tolerance is restored in the absence of Cox1, the Δcox11 *petite* strain remained sensitive to peroxides (99). Collectively, these observations indicate that although the formation of pro-oxidant Cox1-heme a_3 intermediates play a role in peroxide sensitivity, it is not the only factor contributing to the peroxide sensitivity in the absence of Cox11.

Beyond these important details, the cells have developed several strategies to minimize the accumulation of reactive assembly intermediates. Importantly, these very same strategies serve to regulate the concerted accumulation of COX subunits in yeast mitochondria.

Protein quality control mechanisms regulate COX biogenesis and minimize accumulation of pro-oxidant COX assembly intermediates

Most unassembled highly hydrophobic COX core subunits are very efficiently post-translationally degraded by the ATP-dependent AAA proteases of the inner mitochondrial membrane (61). Additional proteases specifically prevent the accu-

mulation of immature Cox1 selectively, such as the conserved metallopeptidase Oma1 that degrades Cox1 in absence of the COX chaperone Coa2 (55). Active degradation will avoid the accumulation of unassembled proteins that could have a tendency to aggregate and disturb membrane homeostasis or to form pro-oxidant species as discussed earlier.

Translational regulation coordinates Cox1 biogenesis and COX assembly and minimizes the accumulation of pro-oxidant COX assembly intermediates

Hierarchically, the assembly line of Cox1 determines the rate of COX assembly. Its relevance is highlighted by the existence of at least two regulatory mechanisms pacing Cox1 synthesis and hemylation to its assembly into COX.

On a first mechanism, Cox1 translation is regulated by the availability of its assembly partners (5, 69, 94) (Fig. 6). This particular type of assembly-controlled translational regulation was initially described for the chloroplast's cytochrome *b₆f* of the green unicellular alga *Chlamydomonas reinhardtii* (20) and termed control by epistasis of synthesis (CES). A distinctive characteristic of these translational autoregulatory systems is the role of ternary factors, mRNA-specific translational activators, whose availability is regulated by specific gene products (20). In the case of yeast Cox1, the ternary factor is Mss51. Briefly, Cox1 synthesis requires two translational activators, Pet309 (68) and Mss51 (24). While both interact with the *COX1* mRNA 5'-UTR, Mss51 plays additional roles in coordinating Cox1 synthesis and assembly (Fig. 6). During and after synthesis, Cox1 is bound to Mss51 in a complex stabilized by two small COX-specific chaperones, Cox14 (5) and Cox25/Coa3 (30, 70), and the mitochondrial Hsp70 chaperone Ssc1 (32). When Cox1 proceeds in the assembly process within Coa1 and Shy1-containing complexes, Mss51 is released, binds Ssc1, and is available for a new round of Cox1 synthesis (32, 69). Pro-oxidant intermediates formed during Cox1 maturation and assembly are short-lived when COX assembly proceeds normally. However, in physiological situations when COX assembly needs to be slowed down, or when COX assembly is impaired, Mss51 remains trapped into the Cox1-containing complex and largely unavailable for its role in translation (5, 30, 32, 70, 71, 78–80, 91). In this way, the coordination between Cox1 synthesis and the following assembly steps could serve to minimize the lifespan of pro-oxidant assembly intermediates (Fig. 6). Whether the *COX1* translational regulatory system is conserved in higher eukaryotes remains unknown. However, human homologues of Mss51, Cox14, and Cox25/Coa3 have been recently identified (96), and so far, the human homologue of Cox14 (C12orf62) has been suggested to coordinate the early steps of COX assembly with the synthesis of COX1 (103).

On a second mechanism, heme A biosynthesis is regulated by downstream events in the COX assembly process (8) to coordinate synthesis to its incorporation into Cox1 (Fig. 7). In most yeast COX mutants, there is a dramatic decrease on heme A steady-state levels. However, by overexpression of *COX15* alone or in combination with the ferredoxin *YAH1*, heme A levels are significantly increased in these mutants, including some in which Cox1 is not synthesized (8). This suggested a feedback regulation of the heme A synthesis when the COX assembly process is stalled (8). In addition to low heme A synthesis, COX mutants also accumulate heme O,

indicating that this molecule is stable. heme O levels were very low in Δcox15 cells, a phenotype that was not rescued by the overexpression of *COX10*, thus suggesting that the first step of the heme A biosynthesis pathway is positively regulated by Cox15 (8). It remains unknown whether increased heme A levels by *COX15* overexpression in the absence of COX assembly bypasses Cox1 synthesis downregulation. If that was the case, we could envision a direct connection between *COX1* translation and heme A biosynthesis regulations (Fig. 7). However, Δcox14 cells, whose Cox1 synthesis is not down-regulated due to the instability of the Mss51-Cox1 early intermediate (5), have heme A and heme O levels similar to other COX mutants (8), thus suggesting that an increase in Cox1 synthesis does not promote heme A biosynthesis. The future identification of the COX assembly intermediate that operate in the regulation of heme A biosynthesis will allow better understanding of the mechanisms that control COX biogenesis to prevent the formation of pro-oxidant intermediates.

Concluding Remarks

Recent investigations have helped defining a substantial network of ROS- and redox-dependent or -regulated processes that operate in the assembly of functional and efficient COX. To further our understanding of these processes, future studies will need to elucidate: (i) the ROS-induced mechanisms of COX subunit isoform switch in yeast and mammals and its impact in cellular physiology; (ii) the effects of ROS in COX assembly into supercomplexes; (iii) how the redox environment in the IMS modulates the function of the Mia40/Erv1 import and oxidative folding pathway and whether the small CX₉C proteins play a role in redox control in the IMS; (iv) how copper is transported from the matrix to the IMS and subsequently to Cox17, whether it involves the CX₉C proteins, and how the redox environment regulates the process; and (v) how COX assembly-dependent regulation of Cox1 synthesis and heme A biosynthesis are precisely coordinated to prevent the formation/accumulation of pro-oxidant assembly intermediates.

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Abbreviations Used

ATP = adenosine-5'-triphosphate
 CI to CIV = mitochondrial respiratory chain complexes I to IV
 COX = cytochrome *c* oxidase
 cyt *c* = cytochrome *c*
 FIH-1 = factor inhibiting HIF-1
 GRx1 = glutaredoxin 1
 GSH = reduced glutathione
 GSSH = oxidized glutathione
 HIF-1 = hypoxia inducing factor-1
 Hig1 = hypoxia inducing gene 1
 HRE = HIF-1 responsive element
 IMS = intermembrane space
 MIM = inner membrane
 MRC = mitochondrial respiratory chain
 mt-Sod1 = mitochondrial superoxide dismutase 1
 NO = nitric oxide
 OM = outer membrane
 PHD = prolyl hydroxylases
 RISP = Rieske iron-sulfur protein
 ROS = reactive oxygen species
 TNmax = maximal turnover number
 TOM = translocase of the outer membrane
 UTR = untranslated region