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Cytochrome *c* Oxidase Biogenesis: New levels of Regulation

Flavia Fontanesi[1], **Ileana C. Soto**[2], and **Antoni Barrientos**[1,2,*]

1*Department of Neurology, The John T. MacDonald Foundation Center for Medical Genetics. University of Miami Miller School of Medicine. Miami, FL*

2*Department of Biochemistry & Molecular Biology. The John T. MacDonald Foundation Center for Medical Genetics. University of Miami Miller School of Medicine. Miami, FL*

Summary

Eukaryotic cytochrome c oxidase (COX), the last enzyme of the mitochondrial respiratory chain, is a multimeric enzyme of dual genetic origin, whose assembly is a complicated and highly regulated process. COX displays a concerted accumulation of its constitutive subunits. Data obtained from studies performed with yeast mutants indicate that most catalytic core unassembled subunits are post-translationally degraded. Recent data obtained in the yeast Saccharomyces cerevisiae have revealed another contribution to the stoichiometric accumulation of subunits during COX biogenesis targeting subunit 1 or Cox1p. Cox1p is a mitochondrially encoded catalytic subunit of COX which acts as a seed around which the full complex is assembled. A regulatory mechanism exists by which Cox1p synthesis is controlled by the availability of its assembly partners. The unique properties of this regulatory mechanism offer a means to catalyze multiple-subunit assembly. New levels of COX biogenesis regulation have been recently proposed. For example, COX assembly and stability of the fully assembled enzyme depend on the presence in the mitochondrial compartments of two partners of the oxidative phosphorylation system, the mobile electron carrier cytochrome c and the mitochondrial ATPase. The different mechanisms of regulation of COX assembly are reviewed and discussed.

Keywords

Mitochondria; cytochrome oxidase; cytochrome *c*; F_1F_0-ATPase; Cox1p translational regulation

1. Cytochrome *C* Oxidase Assembly is a Highly Regulated Process

Cytochrome *c* oxidase (COX) or complex IV of the mitochondrial respiratory chain plays a fundamental role in energy production of aerobic cells. This multimeric enzyme of the inner mitochondrial membrane catalyzes the last step of respiration, the transfer of electrons from cytochrome *c* to molecular oxygen. By coupling electron transfer with protons translocation from the mitochondrial matrix to the intermembrane space, COX also contributes to the storage of energy in the form of an electrochemical gradient that will be used by the oxidative phosphorylation system for the synthesis of ATP. COX central role in aerobic metabolism is highlighted by its participation in respiratory control. When phosphorylated in a cAMP-dependent fashion, COX is converted into the rate limiting step of respiration, being inhibited at high intramitochondrial ATP/ADP ratio (reviewed in (1)).

*Address correspondence to: Antoni Barrientos, Dep. of Neurology and Biochemistry & Molecular Biology; The John T. Macdonald Center for Medical Genetics; Universtiy of Miami. Miller School of Medicine; 1600 NW 10th Ave.; RMSB # 2067; Miami, FL-33136; Phone: (305) 243 86 83; FAX: (305) 243 39 14; E. mail: abarrientos@med.miami.edu.

Eukaryotic COX is formed by 11-13 subunits (11 in the yeast *Saccharomyces cerevisiae* and 13 in *Homo sapiens*) of dual genetic origin. Subunits 1, 2 and 3 are large, highly hydrophobic, transmembrane proteins encoded in the mitochondrial genome. They form the catalytic core of the enzyme and contain metal prosthetic groups. Copper and heme A, a unique heme compound found exclusively in COX, form three redox centers: a Cu_A center in subunit 2 and a heme *a* and a Cu_B-heme a_3 binuclear center in subunit 1. The nomenclature of the subunits varies from yeast to mammals and it is clarified in Table 1. The remaining small subunits that surround the core of the enzyme are encoded in the nuclear genome. They are necessary for the assembly/stability of the holo-enzyme and for its dimerization (Table 1). They are also involved in the modulation of the catalytic activity and in the protection of the core from reactive oxygen species (ROS). Some of the nuclear encoded subunits have evolved isoforms able to confer different kinetic properties to the enzyme. For example, subunit Cox5p in the yeast *S. cerevisiae*, exists in two isoforms, Cox5ap and Cox5bp, which are expressed according to the oxygen availability. In humans, tissue specific isoforms have been reported for four nuclear encoded subunits, COX4 (homolog of yeast Cox5p), COX6a, COX6b and COX7a (2,3).

Overall, COX is a ductile enzyme, whose activity can be modulated according to the energetic requirement of the cell through isoform subunits composition and physiologically controlled phosphorylation. Although COX structure was resolved in 1996 (4) and an impressive amount of experimental data have shed light into the organization and catalytic properties of the enzyme, many questions of fundamental bioenergetic interest still remain uncovered (reviewed in (5)). COX assembly is a fascinating process, which can be described as a sequential and ordinate addition of subunits and co-factors to an initial seed consisting of Cox1p. COX assembly represents a multistep progression through discrete short-term intermediates requiring a plethora of more than 30 diverse assistant factors, a complex coordinated event that we have just begun to elucidate. In addition to its biological significance, the increasing interest to disclose the process of COX assembly derives from biomedical statistics showing that COX deficiency is the most frequent cause of mitochondrial encephalomyopathies, a heterogeneous group of human disorders characterized by alteration of aerobic energy production (OXPHOS defects). Notably, all the cases of Mendelian inherited COX deficiencies, for which the genetic cause is already known, are the consequence of COX assembly defects. These deficiencies are associated with mutations in nuclear genes encoding COX assembly factors (*SCO1* and *SCO2*, necessary for copper insertion into COX2; *COX10* and *COX15*, necessary for heme *a* biosynthesis; *SURF1*, involved in an early step of assembly and *LRP130*, homologue of the yeast *COX1* translational activator Pet309p) (reviewed in (6,7)). The non-structural factors accomplish diverse functions at all the levels of the assembly process. These roles include transcription and mRNA maturation, translation of COX mitochondrial genes, as well as import into mitochondria of nuclear encoded subunits and insertion of transmembrane subunits into the inner mitochondrial membrane. Essential additional roles involve heme A biosynthesis, copper homeostasis and insertion into the apoenzyme and formation of assembly intermediates. All these functions are interconnected and co-regulated (reviewed in (8)). Furthermore, an additional level of regulation has begun to emerge in the last few years, which connects COX assembly with other players of the OXPHOS system, like cytochrome *c* and ATP synthase (Fig. 1).

The aim of this review is to summarize the new emerging data regarding COX assembly, specifically concerning the several levels of its regulation.

2. Concerted Accumulation of Cox Subunits and Formation of Subassembly Intermediates

2.1 Regulation of Cox1p translation

COX assembly is characterized by a concerted accumulation of its constitutive subunits. Data obtained mainly from studies performed with yeast mutants indicate that most unassembled subunits, particularly the ones forming the catalytic core, are post translationally degraded (9,10). In particular, non-matured or unassembled newly synthesized subunit 1 and the other catalytic core subunits that do not proceed to COX holoenzyme assembly are targeted to degradation by the AAA ATP-dependent proteases of the inner mitochondrial membrane (reviewed in (11)). Active degradation will avoid the accumulation of unassembled proteins that could have a tendency to aggregate and disturb membrane homeostasis. It will also limit the accumulation of partially matured core subunits that, as recently proposed for yeast subunit 1, could contribute to the production of unstable pro-oxidant intermediates (12).

Recently, another contribution to the stoichiometric accumulation of subunits during COX biogenesis in the yeast *Saccharomyces cerevisiae* has been reported. It consists of a regulatory mechanism by which the synthesis of subunit 1 is regulated by the availability of its assembly partners (13-16). Decreased Cox1p synthesis relative to other mitochondrial translation products had been previously reported as a secondary observation in yeast *pet111* mutants that have a lesion in a translational activator of *COX2* mRNA (17) and in *cox7* mutants, lacking the nuclear-encoded subunit 7 (18). This phenotype could not be accounted for by a defect in the translation system because the mutations were not in proteins related to this mitochondrial activity. Translation of *COX1* in *S. cerevisiae* is under the control of the *MSS51* and *PET309* gene products (Fig.2) that are also involved in maturation of the *COX1* mRNAs (19,20). A direct connection between Cox1p expression regulation and COX holoenzyme assembly came from studies in which mutations in *MSS51* or overexpression of the wild type gene were found able to suppress the COX assembly defect of null mutant of *shy1* by enhancing synthesis of Cox1p (13). Shy1p is a COX assembly factor that functions in maturation and/or assembly of Cox1p (13,15,21,22). The precise role of Shy1p, still unknown, is of considerable interest because mutations in its human homologue, SURF1, are responsible for most diagnosed cases of Leigh's syndrome presenting a COX deficiency (23,24). Cox1p synthesis is decreased in most COX assembly mutants, including *shy1* mutants, but it is restored to normal levels by *mss51* suppressors of *shy1* or by mutations in *COX14* (14), which codes for another COX assembly factor (25). Like other translational activators, Mss51p acts on the 5′-UTR of its cognate mRNA molecule, to promote translation (15,16), binding that could be necessary for optimal initiation of translation by Pet309p (16). However, *mss51* mutants, unlike *pet309* mutants, can not be suppressed by changes in the 5′-UTR of *COX1* mRNA (14,15). In addition, Mss51p acts on a target in the protein coding sequence of *COX1* mRNA that could be necessary to promote elongation (15). Mss51p and newly synthesized Cox1p form a transient complex (14,15) that is stabilized by Cox14p (14). These interactions have been postulated to down-regulate Cox1p synthesis when COX assembly is impaired (14). According to this model, the release of Mss51p from the ternary complex and its availability for Cox1p synthesis occur at a downstream step in the assembly pathway, most likely catalyzed by Shy1p (14,26) (Fig. 2).

Despite the already large number of factors known to be involved in COX assembly, new factors continue to be discovered today. Coa1p was recently described as a new player in the subunit 1 synthesis regulatory loop (26,27) (Fig.2). Coa1p is a protein associated with the inner mitochondrial membrane where it is part of the high molecular weight complex containing Cox14p, Mss51p and newly synthesized Cox1p (26,27). The interaction Cox14p-Mss51p is unaffected in a *Δcoa1* mutant, while, in the absence of Cox14p, the binding of Coa1p-Mss51p is disrupted, suggesting that the interaction of Coa1p with the Cox1p-Cox14p-Mss51p complex

is through Cox14p (27). Shy1p is not part of the Cox1p-Cox14p-Mss51p-Coa1p complex (27), but it has been shown to co-precipitate with Coa1p (26,27), suggesting that these two factors probably interact once Mss51p has disengaged from the complex (27). As for *cox14* mutants, mutations in *coa1* do not affect the synthesis but the accumulation of Cox1p in the holoenzyme, suggesting that Coa1p also plays a role in the feedback regulation of Cox1p expression (26,27). The COX assembly defect in a strain carrying a null allele of *coa1* can be suppressed by overexpression of Mss51p and Cox10p, the heme *a* farnesyl transferase required for biosynthesis of heme *a* (28). Both suppressors can act synergistically when the two proteins are co-expressed, linking Coa1p to both translational regulation and maturation of Cox1p (27). Pierrel and colleagues have speculated that Coa1p could stabilize the Cox1p-Cox14p-Mss51p complex until Shy1p interacts with Coa1p in a step involving heme A insertion into Cox1p and further progression in the assembly process (27). While Cox1p maturation certainly occurs within these initial Cox1p complexes, the precise role of each factor remains to be elucidated.

At present, it is not clear if Cox1p translation in other organisms is also subject to regulation by downstream events. In mammals, as well as in yeast, mitochondrial DNA encoded subunits are fast degraded when not assembled into the holoenzyme. However, no extensive data are available regarding the rate of Cox1p synthesis in mammalian COX assembly mutants. Among the few cases reported, COX subunit 1 synthesis was shown slightly reduced by 20% of wild type levels in fibroblasts from a *cox10* knockout mouse (29). Mitochondrial genes in higher eukaryotes do not have 5′untranslated region and their expression does not seem to depend on gene-specific translational activators. In addition, mammalian homologues of Mss51p, Cox14p and Coa1p have not been identified to date. This does not necessarily exclude the possibility that they may exist in mammalian and other genomes but have not yet been recognized because of their smaller size and/or divergent sequences. For example, the products of yeast *PET309* and human *LRPPRC* (*LRP130*), responsible for the Canadian form of Leigh syndrome (30), display very weak sequence similarity, even though they both bind to mitochondrial RNAs and are essential for COX expression. It is also possible that translational factors such as Pet309p and Mss51p could have more than one function (i.e. mRNA metabolism, membrane insertion of the newly synthesized protein), only some of which are conserved across different organisms. We expect that understanding the nature and players involved in the mechanism regulating COX subunit 1 biogenesis and assembly in yeast will help to identify their functional homologues in humans.

2.2 Formation of assembly intermediates

COX assembly was modeled a decade ago by Nitjmans and colleagues as a linear process consisting of the sequential incorporation of COX subunits to an initial seed formed by subunit 1. Data supporting COX subunit 1 biogenesis as the first step of COX assembly came from analyses by Blue-native electrophoresis of human cells treated with cycloheximide, a specific inhibitor of cytoplasmic translation. They showed the existence of Cox1p as either unassembled or in subassemblies (31). The same COX assembly intermediates were observed when the cells were treated for short times with doxycycline, a specific inhibitor of mitochondrial translation, which reduces the level of COX subunits available for assembly (31). In these experiments, intermediates containing the other mitochondrial DNA encoded core subunits 2 and 3, but not COX1, were not detected (31). These results suggested that COX1 alone constitutes the first assembly intermediate.

Subunit 1-containing intermediates were also subsequently detected in COX deficient human and mouse cells (29,32-34) and in yeast cells carrying *cox2* mutations (35), suggesting that COX assembly is largely conserved from lower to higher eukaryotes.

According to the model proposed by Nitjmans and coworkers, the addition of subunits COX4 and COX5a (yeast Cox5ap and Cox6p, respectively) to the first assembly intermediate (S1) formed by COX1, results in the progression to the second assembly intermediate (S2). These nuclear DNA-encoded subunits are in direct contact with the core of the mature enzyme. COX5a caps the matrix side of COX1, while COX4 interacts with COX2 through its C-terminal domain and COX1 through its single transmembrane domain (4). Interestingly, three assembly intermediates containing COX1 but not of the other COX subunits were detected in *SURF1* and *SCO2* as well as in *COX10* mutant fibroblasts from human patients (33,34). These COX1 containing intermediates could correspond to COX1 maturation intermediates. (Fig.2). Insertion of heme A into COX1 occurs before the addition of subunits 4 and 5a (yeast subunits 5 and 6), as suggested by the accumulation of the COX1-COX4-COX5a intermediate in *SCO1* and *SCO2* mutant fibroblasts (33,34), but not in *COX10* and *COX15* deficient cells (33,36,37). These findings suggest that the presence of heme A in COX1 might stabilize its binding to COX4 and COX5a. In addition, the heme *a* and heme a_3 – Cu_B catalytic centers in subunit 1 are buried into the transmembrane portion of the protein. The binuclear center in subunit 1 is coordinated by histidine residues in helices VI, VII and X while heme *a* is coordinated by histidine residues in helices II and X (4). Because the helices involved must be adjacent to coordinate heme, it is plausible that heme *a* insertion could play a role in COX subunit 1 folding. Although the precise moment of subunit 1 biogenesis when the metal prosthetic groups are inserted into the complex remains to be determined, this clearly seems to be an early event in COX assembly and probably occurs in either a co-translational or a co-membrane insertion manner. After the formation of the COX1-COX4-COX5a subassembly, the COX assembly process continues with the formation of the third proposed intermediate (S3) by the addition to S2 of most of the remaining subunits with the exception being COX6a and COX7a/b (yeast subunits 10 and 7) that are finally added to complete the holoenzyme (31,33).

Recently, Stiburek and coworkers used COX deficient mitochondria from different tissues of patients with mutations in the COX assembly factors *SCO2* and *SURF1* to identify eight different COX assembly intermediates/unassembled subunits, six of which were detectable also in wild type cells (34). The analysis of these intermediates provided novel information to refine the COX assembly model previously proposed. First, it appears that mammalian COX4 and COX5a subunits form a dimer before their incorporation into S1. Noticeably, the equivalent yeast Cox5p-Cox6p dimer was also detected in a COX mutant in which assembly is compromised in the latter stages of the process (38). This result is consistent with the observation that in yeast the presence of subunit 6 is required for subunit 5 stability (39). Second, an additional intermediate seems to exist between S2 and S3, consisting of at least mammalian subunits COX1-COX2-COX4-COX5a (34). It remained unanswered whether COX3 is also present in this intermediate because an antibody against subunit 3 was not used in this study. In a different report, however, COX3 was not detected in the S2 intermediate in human fibroblasts in which copper metallation of COX2 is impaired (33). In yeast, an assembly intermediate formed by Cox1p-Cox3p-Cox5ap-Cox6p, and not Cox4p, Cox6ap, Cox7p, Cox7ap and Cox8p, was detected in two *cox2* point mutant strains (35), suggesting that Cox2p is not necessary for the incorporation of Cox3p in the subassembly intermediate in yeast.

COX2 may associate with COX1-COX4-COX5a intermediate upon its copper metallation by the specific SCO1-SCO2 copper chaperones, as suggested by the observation that *SCO1* and *SCO2* mutant fibroblasts from patients with COX deficiency accumulate the COX1-COX4-COX5a intermediate (33,34). Significant experimental evidence suggests a possible involvement of the mammalian COX assembly factor *SURF1*, the homologue of the yeast *SHY1*, in facilitating the COX1-COX4-COX5a interaction. In both organisms, human and yeast, *SURF1*/*shy1* mutant cells retain some residual COX activity of approximately 15% of wild-type cells, indicating that, though strongly compromised, the COX assembly process is

still maintained in these cells. The precise function of SURF1/Shy1p in COX assembly is currently unknown. However, a large body of information has been gathered by studying spontaneous suppressor mutations and high copy suppressors of yeast *shy1* mutants, which have indicated that SURF1/Shy1p plays a role in the formation of an early COX assembly intermediate containing subunit 1. As mentioned above, yeast strains carrying mutated and null alleles of *shy1* spontaneously revert to a respiratory competent phenotype by mutations in *MSS51* which increase the rate of Cox1p synthesis (13). The Mss51p mediated suppression is enhanced by both, co-overexpression of *COX10* (27) and co-overexpression of the COX subunits Cox5p and Cox6p, the latter being partners of Cox1p in an early assembly intermediate mentioned above (22). In this context, we have recently reported that overexpression of *HAP4* suppresses the respiratory deficient phenotype of yeast *shy1* mutant strains. Hap4p is the catalytic subunit of the CCAAT binding site transcriptional activator Hap2p,3p,4p,5p complex, which globally activates transcription of nuclear genes involved in mitochondrial respiration during transition from fermentation to respiration. The *shy1* suppression by *HAP4* is mediated by a specific increase in the expression of subunits Cox5p and Cox6p (22). Interestingly, overexpression of *NF-YA*, the catalytic subunit of the human NF-Y transcriptional activator complex homologue of the yeast HAP complex, is able to increase the mitochondrial COX in *SURF1* deficient fibroblasts through a mechanism that remains to be characterized (22).

The presence of *SURF1* orthologues in terminal oxidase operons of several prokaryotes (40) in which COX contains the evolutionary conserved core subunits (Cox1p, Cox2p, Cox3p) but not the core surrounding subunits such as Cox5p and Cox6p, suggests a role of SURF1/Shy1p in forming the catalytic core of the enzyme. Studies in *Rhodobacter sphaeroides*, suggest that bacterial SURF1 is required for either insertion of heme A at the a_3 center or stabilization of the a_3-Cu_B binuclear center in COX1 (21). A function of SURF1/Shy1p in maturation of the a_3 center would imply the existence of a second chaperone specific for the heme A of the cytochrome *a* center. Smith and coworkers proposed that SURF1 could perform this role indirectly by interacting with subunits 1 or 2 to facilitate the formation of the heme a_3-Cu_B center. This would be in agreement with a previous observation suggesting an interaction of SURF1 with COX2 in human cultured cells (41). No interaction of yeast Shy1p was detected with either newly synthesized Cox1p or Cox2p (14). Perhaps such an interaction could occur only after the Cox1p-Cox5p-Cox6p subassembly is formed and Cox2p has been matured by insertion of copper at the Cu_A site. This possibility is strongly supported by the fact that the COX1-COX4-COX5a (yeast Cox1p-Cox5p-Cox6p) sub-complex accumulates in fibroblasts from *SURF1* patients (33) as in fibroblasts from *SCO1* and *SCO2* patients (33,34) but not in heme A deficient fibroblasts of patients with lesions in *COX10* or *COX15* (33) (37). SCO1 and SCO2 are required for the formation of Cu_A (42) and hence maturation of subunit 2. In turn, the association of metallated COX2 with COX1-COX4-COX5a could affect the stability of the Cu_B-hem a_3 center by capping the heme-insertion channel proposed to be formed in the COX1-COX4-COX5a subassembly (43). As mentioned above, SURF1/Shy1p could play a role in facilitating this interaction. The results showing suppression of yeast *shy1* mutants by increasing the amount of Cox1p-Cox5p-Cox6p (22) support this possibility, although a direct role of Shy1p on Cox1p maturation can not be excluded. Further support of the role of Shy1p as an assembly factor was recently reported by showing that Shy1p promotes COX biogenesis through association with different protein modules, potential COX assembly intermediates containing Cox1p and Cox5p among other proteins (26). In addition, Shy1p, together with Cox14p, were also found to interact with partially and fully assembled forms of COX associated with complex III of the mitochondrial respiratory chain. Based on the analysis of several high molecular weight complexes containing Shy1p and Cox14p, it has been proposed that these two COX assembly factors could act beyond the early stages of COX assembly. They could act in the late stages of the process when the COX subunits necessary for the interactions with complex III have been already incorporated (26). Shy1p and Cox14p would accompany

partially assembled forms of COX, maintaining their competency for the incorporation of additional subunits, during the process of COX biogenesis (26).

Although most studies on COX assembly focus on the early steps leading to the assembly of the catalytic core subunits, some data are starting to emerge concerning the late steps of assembly in which addition of nuclear encoded subunits lead to complete the formation of the functional holoenzyme. The roles of Shy1p and Cox14p seem to affect these late steps as explained above. In addition, a subassembly intermediate formed by Cox7p-Cox7ap was detected in yeast *cox2* mutants (35). Similarly, a high molecular weight complex containing Cox7p-Cox7ap-Cox8p in association with the chaperone Pet100p was observed in yeast wild-type cells (38). However, the complete composition of this complex still remains to be elucidated. The finding of subassembly complexes exclusively containing nuclear DNA-encoded subunits (35,38) can explain their stability in comparison with the mitochondrial DNA-encoded subunits, which are quickly degraded when the assembly is compromised. This observation also supports the possibility that some nuclear encoded subunits exist in an unassembled pool in the inner mitochondrial membrane ready to enter the assembly process when required.

In summary, COX assembly is a progressive process in which the holoenzyme is built around a seed formed by COX subunit 1, which biogenesis, as part of the formation of the catalytic core, constitutes the principal regulatory step as depicted in Fig. 2.

Cox Assembly Regulation by the Integrity of Cox Functional Partners in the Mitochondrial Membranes

COX assembly has been shown to be sensitive to the protein composition of the mitochondrial membranes. As discussed below, the presence of both the electron carrier cytochrome *c* and the F_1F_0-ATPase, are essential for proper COX assembly and stability. Although this double requirement has been observed and studied by several research groups, the mechanisms involved remain to be fully understood.

3.1. Cytochrome *c* is required for COX assembly

In *S. cerevisiae*, cytochrome *c* occurs in two isoforms (iso-1 and iso-2) encoded by nuclear genes *CYC1* and *CYC7* (44,45), respectively. *CYC1* expression is regulated by oxygen and accounts for 95% of cytochrome *c* present in mitochondria in normoxic conditions (46). The 5% of iso-2-cytochrome *c* expressed in null mutants of *CYC1* is enough to support respiratory growth albeit showing a reduced rate compared to wild type cells (47). Interestingly, the double null mutant Δ*cyc1*Δ*cyc7* strain does not only present a complete absence of cytochrome *c* and as a consequence is unable to respire, but also shows an additional deficiency in COX (48). This phenotype is also observed in mutants of *CYC3*, a gene encoding for the cytochrome *c* heme lyase required for the covalent attachment of heme to apocytochrome *c* (49). Cytochrome *c* mutants have the characteristics of *bona fide* COX assembly mutants, including lower levels of heme aa_3 and a drastic reduction in the steady state levels of COX mitochondrial subunits Cox1p, Cox2p and Cox3p. In COX assembly mutants, the incorporation of these subunits into the complex is aborted, and the turnover for their degradation increases. In addition, like in other COX mutants, failure of COX assembly in cytochrome *c* mutants results in down regulation of Cox1p synthesis (50) through the same mechanism involving Mss51p and Cox14p (14) as described above.

The role of cytochrome *c* in COX assembly is not well understood. Oxidized cytochrome *c* can accept electrons from cytochrome *c*1 of the *bc*1 complex, from cytochrome b_2 of lactate dehydrogenase and also from the sulfhydryl oxidase Erv1p (51,52). The main function of

reduced cytochrome *c* is to donate electrons to COX. Conceivably, cytochrome *c* could also promote an oxidation or reduction event essential for COX assembly. For example, cytochrome *c* could be involved in heme A biosynthesis, a possibility that has been already excluded (53). The recent discovery of an interaction of cytochrome *c* with Erv1p could suggest an interesting possibility involving the import of COX assembly chaperons. The import and folding into the mitochondrial intermembrane space of several copper chaperons required for COX assembly is mediated through the recently described Mia40p pathway (54). These proteins contain a characteristic twin CX_9C motif, as in Cox17p, Cox19p and Cox23p, critical for their import. After passage through the mitochondrial outer membrane TOM channel, these proteins are covalently trapped by Mia40p *via* disulfide bridges. Mia40p also contains cysteine residues, which are oxidized by the Erv1p, functioning as a disulfide relay system that catalyzes the import of proteins into the IMS by an oxidative folding mechanism (54). How Erv1p itself is oxidized to become competent for new rounds of Mia40p oxidation was initially unclear. Recently, it has been reported a connection between the disulfide relay import system and the mitochondrial respiratory chain consisting of electron transfer from Erv1p to molecular oxygen via interaction with cytochrome *c* (51,52). Cytochrome *c* efficiently oxidizes Mia40p in oxygen-limiting conditions (52). Although oxidized cytochrome *c* facilitates Mia40p oxidation, it was found non-essential in normoxic conditions suggesting the existence of additional electron acceptors for oxidizing reduced Erv1p (52). These results weaken the possibility that cytochrome *c* prevents COX assembly by blocking the import of its copper chaperones.

Cytochrome *c* could be required for COX assembly in a structural capacity. For example, its interaction with a COX intermediate could be necessary for some step in the assembly pathway. To distinguish between a functional and structural requirement of cytochrome *c* in COX assembly, Barrientos and colleagues transformed a Δ*cyc1*Δ*cyc7* double null mutant with a *cyc1* mutant gene that was previously reported to express stable but catalytically inactive cytochrome *c* (50). The mutant allele, *cyc1–166*, expresses nonfunctional and thermolabile iso-1-cytochrome *c* and has a serine replacement of the tryptophan at position 65 (W65S), which corresponds to the invariant tryptophan residue found in cytochromes *c* from all eukaryotic species (55). Analyses of the transformant strains showed a recovery of the COX assembly defect of the double null mutant. The heme *a* levels of the transformant strain were increased to about 50% of wild type, and the steady state levels of Cox1p, Cox2p and Cox3p were close to normal (50). These results support the hypothesis of cytochrome *c* playing a structural role in COX assembly because the sole presence of its inactive form was able to rescue the respiratory deficient phenotype of the double null mutant. Interestingly, COX assembly does not seem to depend on stoichiometric concentration of cytochrome *c*. This possibility is supported by the fact that although the molar concentration of cytochrome *c* in mitochondria of wild type yeast was estimated to be approximately the same as that of COX, iso-2-cytochrome *c*, which concentration in a *cyc1* mutant is only 12% of the total amount of cytochrome *c* in wild type yeast, it is able to support the expression of 70% of normal amounts of COX (50). Furthermore, the apo-cytochrome *c* (heme less protein) cannot substitute for the mature cytochrome, suggesting that the function of cytochrome *c* probably depends on a properly folded protein (50). In addition to its requirement for assembly, the results obtained with the thermolabile W65S mutant exposed to 37 °C indicated that the main role of cytochrome *c* is in assembly but that it also contributes toward the stability of the enzyme (50). Although the current literature is consistent with a structural role for cytochrome *c* in COX assembly, further studies are required to fully understand the mechanism involved.

3.2. COX assembly and stability requires an intact functional ATP synthase

Most of cellular ATP of eukaryote cells is synthesized by the F_1F_0-ATPase complex of the mitochondrial inner membrane during oxidative phosphorylation. Mitochondrial F_1F_0-ATP

synthase or ATPase is formed by 15–18 distinct subunits. It is composed of two functionally and physically coupled portions, the membrane-embedded F_0 sector to which the hydrophilic F_1 sector is attached from the matrix side. In *S. cerevisiae*, the F_0 sector is formed by eight subunits (reviewed in (56,57)). An oligomer of Atp9p forms a ring-like structure that rotates in the membrane bilayer, and together with Atp6p, drives proton translocation across the inner membrane. Six other subunits (Atp8p, Atp4p, the oligomycin sensitivity-conferring protein and subunits d, f, and h) form the stator arm connecting F_0 to F_1. Two other subunits (e and g) are required for ATPase dimerization. The γ, δ and ε subunits of F_1 are part of a central stalk linking it to the Atp9p ring. The rotation of this stalk within the static catalytic F_1 hexamer formed by three α and three β subunits is required for ATP synthesis. The remaining seven F_1F_0-ATPase subunits function in the regulation or oligomerization of the yeast complex (57).

Although the mitochondrial ATPase is functionally coupled to the mitochondrial respiratory chain (MRC) activity through oxidative phosphorylation, no physical interaction of ATPase with MRC enzymes has been reported to date. In tightly coupled mitochondria, the oxygen consumption rate and ATP synthesis activity depend on each other, which results in decreased respiratory activity in many yeast ATPase defective mutants. Short regulatory responses involving COX have been described both in yeast and mammals, including for example allosteric ATP inhibition of COX activity at high ATP/ADP ratios as mentioned above (reviewed in (58)). However, data accumulated over the last twenty years in different organisms suggest the existence of long term regulations in which COX biogenesis and stability are modulated in response to the ATPase activity.

Mutations in several ATPase structural genes in *S. cerevisiae*, including *ATP4* (59), *ATP7* (60), *ATP8* (61) and *ATP9* (62) have been reported to severely affect ATPase assembly and/or function and to produce a pleiotropic effect on COX biogenesis. However, ATPase mutations affect mtDNA stability producing a large amount of “petite” (respiratory deficient) cells, making it difficult to conveniently assess to which degree and how COX assembly is specifically affected in these mutants. In some cases, it was suggested that the COX biogenesis defect was the result of a decrease in Cox1p synthesis (59,60,63), while in others protein synthesis was normal and the COX defect was suggested to be caused by a block in heme insertion or synthesis (61). Similarly, ATPase mutants of *Schizosaccharomyces pombe*, with altered α or β subunits of the F_1 portion, also exhibit pleiotropy on COX biogenesis, associated with a reduction on synthesis of the mitochondrial DNA encoded protein subunits of the complex (64). Recently, the role of ATPase on COX assembly has been explored in a *S. cerevisiae* strain engineered to stably maintain its mtDNA under selection even in the absence of ATPase (63). The authors took advantage of an approach developed by Fox and coworkers (65), based on the insertion of the non-respiratory nuclear gene *ARG8* into the mtDNA of a strain carrying a null allele of *arg8*. In this system, cells growing in the absence of arginine receive selective pressure to maintain their mtDNA. Analysis of the phenotype resulting from an *atp6* deletion in this context clearly showed that the absence of this subunit, the last to be incorporated into the ATPase during its assembly, did not compromise the assembly of the rest of the complex (63). As expected, the ATPase activity in this mutant decreased by ~30% of the wild type, indicating that the F_1 portion was expressed and functional in the absence of Atp6p, but was not inhibited by oligomycin, a specific inhibitor of the F_1F_0 complex proton channel. The lack of Atp6p resulted in a selective decrease in COX accumulation to residual amounts barely detectable. The COX accumulation defect was associated to a decreased synthesis of Cox1p, which probably resulted from a down-regulation of Cox1p translation in the absence of COX assembly through a mechanism involving Mss51p and Cox14p, as previously described for classical COX mutants, as well as for cytochrome *c* mutants, as mentioned above. We have explored these issues in *S. cerevisiae* strains developed by our group in which Atp6p assembly into the ATPase complex is prevented by a mutation in its

specific chaperone Atp10p. In *atp10* mutants, the Cox1p synthesis defect was restored by either deleting *COX14* or by integrating a second copy *of MSS51* into the nuclear genome (unpublished results), two of the hallmarks of the Cox1p translational regulatory loop previously described (14). Thus, Cox1p synthesis down-regulation is in this context a consequence rather than a cause of COX assembly defects.

The mechanism by which the F_1F_0-ATPase affects COX biogenesis remains unknown. Rak and colleagues have proposed that the very poor COX synthesis is a vital requirement allowing ATPase mutants to import glycolytic ATP into mitochondria by decreasing the membrane potential. This hypothesis is supported by the fact that COX biogenesis is not altered in uncoupled ATP synthase mutants, missing either subunits δ (66) or ε (67), where maintenance of a membrane potential is severely compromised by significant proton leaks through the F_0. While this is a very interesting hypothesis, it remains to be elucidated the COX biogenesis step/s that would be primarily affected by an accumulation of protons in the intermembrane space, e.g. *atp6* or *atp10* mutants, provoking a failure to assemble COX.

Recently, several research groups have explored structural constraints to explain the COX deficiency observed in ATPase mutants. In addition to its well established function on ATP generation, the F_1F_0-ATP synthase has a role in determining the ultra-structure of mitochondria (68-70), which depends on its ability to form dimeric and higher oligomeric super-complexes (68-71). It has been recently reported that mutants of the dimer-specific subunits e and g, which destabilize dimeric and oligomeric F_1F_0-ATP synthase super complexes, have a decreased mitochondrial membrane potential. The enzymatic activities of F_1F_0-ATPase as well as the tubular mitochondrial morphology were not affected by the absence of subunits e and g. In these mutants, COX activity was affected to a minor extent (retain ~80% of wild type COX activity), indicating that ATPase dimerization and oligomerization are not essential for COX assembly (72). To explain the loss of membrane potential, the authors proposed a role for the super-complexes of the F_1F_0-ATPase in organizing microdomains within the inner membrane, ensuring optimal bioenergetic competence of mitochondria, which when disrupted would affect the overall flux through the respiratory chain and result in a lower membrane potential (72). By contrast, in a more recent study, the mitochondrial membrane potential in yeast mutant lacking either subunit g or e was not found compromised (73). In this study, it was shown that COX activity is actually decreased down to 55% and 75% of wild type levels in strains missing subunits e and g, respectively, while the amount of enzyme appears normal (73). However, the presence of ATPase subunits e and g was reported necessary to maintain the correct organizational state of the super-complex formed by complexes bc_1 and COX, which stoichiometry was found altered in the mutant strains (73).

Studies in *S. cerevisiae* mutants that are unable to form the F_1 $\alpha_3\beta_3$ oligomer, either because the α or the β subunit is missing or because the cells are deficient for proteins that mediate F_1 assembly (*e.g.* Atp11p, Atp12p, or Fmc1p), have pleiotropic effects on several MRC enzymes (74). In these mutants, mitochondria were severely deficient not only for COX but also, at a lower extent, for Complex III or bc_1 complex (74). These mutants were devoid of mitochondrial cristae, supporting the idea that the F_1F_0-ATPase is important for biogenesis of the mitochondrial inner membrane, but it remained unclear whether the MRC deficiency resulted from the altered mitochondrial ultra-structure. Finally, the mutants accumulated inclusion bodies containing unassembled F_1 α and/or β subunits, an aspect that was considered necessary to prevent the formation of incomplete F_1F_0 assemblies that could passively transport protons across the inner mitochondrial membrane, thus preventing a total dissipation of the mitochondrial membrane potential, which could be catastrophic for the cell (74). However, the maintenance of a too high membrane potential could also be deleterious by limiting glycolytic ATP import as mentioned above, and the cells would react by blocking the assembly of respiratory enzymes, most significantly, COX.

Although the mechanism underlying the COX biogenesis defect in ATPase mutants is not fully understood, it seems to be conserved from yeast to higher eukaryotes. In human, mtDNA and nDNA mutations affecting ATPase function result in rare devastating encephalomyopathies. In several cases reported, the ATPase deficiency was accompanied by a pleiotropic decrease in COX activity specifically or as part of a more general MRC alteration. NARP (neuropathy, ataxia and retinitis pigmentosa) and MILS (maternally inherited Leigh's syndrome) are mitochondrial disorders associated with mutations in *MTATP6*. For example, a T9,185C mutation in the mitochondrial gene *ATP6* was reported to produce MILS (75). Biochemical analyses in the patients showed that they presented a significant COX activity decline in skeletal muscle (75). Analysis of the NARP mutation T8,993G in cybrids cell lines also showed a MRC deficiency, including a COX defect, which interestingly was alleviated by supplementing the growth media with antioxidants (76). The antioxidant treatment was justified because in these cell lines there was an increase of reactive oxygen species that could be responsible for the MRC damage (76). However, the possibility remained that the antioxidants could act by directly introducing reducing equivalents into the mitochondrial matrix, thereby reducing its pH, which would affect the regulation of mitochondrial respiratory chain enzymes (76). Although most ATPase deficiencies have been attributed to mutations in *MTATP6*, it has been recently reported a case of ATPase deficiency in the human homologue of the nDNA encoded assembly gene *ATP12*. As in yeast mutants of the homologue gene (74), the patient presented a pleiotropic COX defect in skeletal muscle (77).

4. Concluding Remarks

Biogenesis of a functional cytochrome *c* oxidase complex depends on the expression of all the structural and more than two dozen COX-specific assembly genes. The latter impinges on all aspects of the biogenesis process. The assembly of COX, made of subunits of dual genetic origin is a complicated and highly regulated process. The multiple levels of regulation described to date involve the availability of subunits and assembly factors regulated at the transcriptional and translational levels, availability of co-factors, protein import into mitochondria and membrane insertion, and coordination of sequential or simultaneous steps of the process (reviewed in (8)).

New levels of regulation have been recently reported and were reviewed here: 1- Translation of subunit 1 in the yeast *S. cerevisiae* is contingent with the availability of its assembly partners, thereby acting as a negative feedback loop that paces Cox1p translation to its utilization during assembly of the complex. In the case of a membrane complex such as COX, this form of negative regulation may prevent non-productive aggregation of unassembled hydrophobic membrane proteins by restricting their steady-state concentration. This mechanism could also play a crucial role in facilitating multimeric protein sequential assembly. 2- The arrangement of the mitochondrial chain and oxidative phosphorylation system in the inner mitochondrial membrane plays a crucial role on the stability and assembly of COX. The physical presence of cytochrome *c* is essential for COX assembly. A fully assembled and functional ATP synthase is also required for maximal levels of COX biogenesis. The discovery of the exact mechanisms involved are expected to shed light into new levels of mitochondrial membrane organization depending on physical and functional constraints.

Finally, in addition of the biological and biochemical interest, the full understanding of the regulation behind COX assembly will also enhance our understanding of aerobic energy production regulation, a process essential for cell performance and survival in normal and disease conditions.

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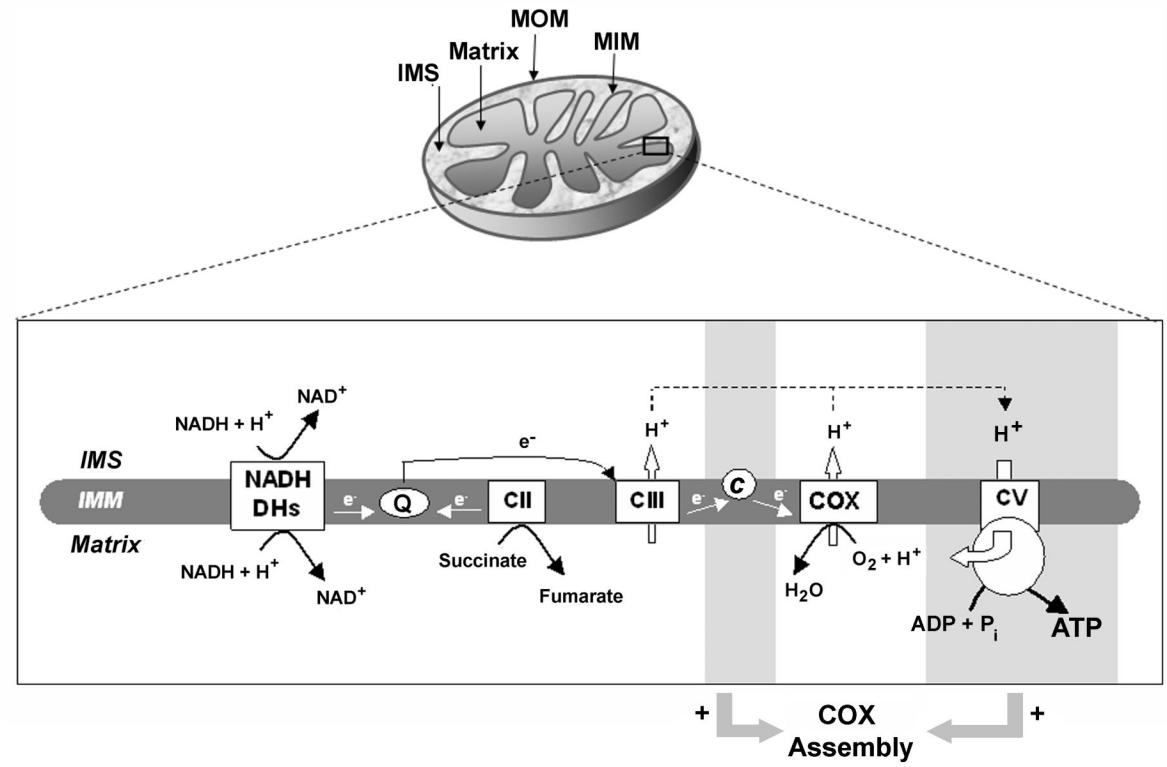

Figure 1.

Cytochrome *c* oxidase assembly depends on the presence in mitochondria of cytochrome *c* and fully assembled ATPase in the yeast *Saccharomyces cerevisiae.* (A) Schematic representation of mitochondria, the mitochondrial respiratory chain and the oxidative phosphorylation system. Mitochondrial ultrastructural analysis reveals a double membrane, outer (MOM) and inner (MIM), which delimit two compartments, the intermembrane space (IMS) and the matrix. The inner membrane form invaginations called cristae, where the enzymes forming the respiratory chain and OXPHOS system are located. The respiratory chain (MRC) is formed in yeast by a series of NADH dehydrogenases acting as the complex I of higher eukaryotes, complex II or succinate dehydrogenase, complex III or bc_1 complex, and complex IV or cytochrome *c* oxidase (COX), together with the electron carriers ubiquinone (Q) and cytochrome *c*. All the elements forming the MRC act in concert to transfer electrons (e^-) from reducing equivalents (NADH and FADH) to molecular oxygen, in a process coupled to the formation of a proton gradient in the intermembrane space that is used by the ATPase to drive the synthesis of ATP. The assembly of the different elements of the MRC and OXPHOS system is interconnected. For example, as discussed in the text, the presence of both cytochrome *c* and fully assembled ATPase (both marked with grey boxes), is required (+) for COX assembly. They affect COX assembly through mechanisms that remain to be defined.

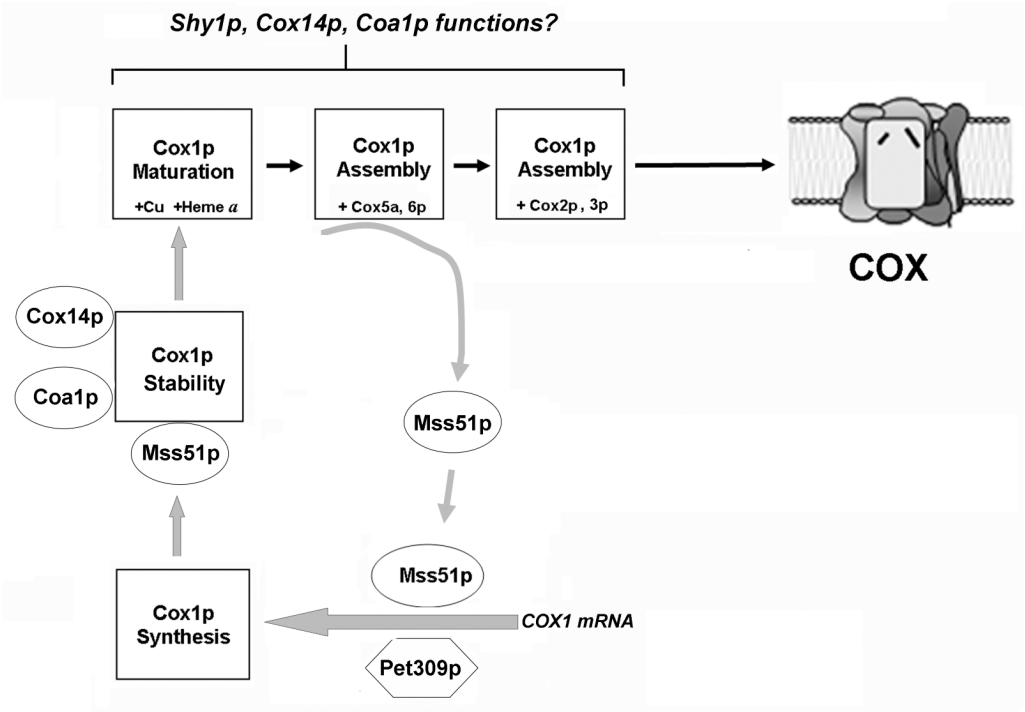

Figure 2.

Early steps in COX biogenesis in the yeast *Saccharomyces cerevisiae* connect synthesis, maturation and assembly of Cox1p through a regulatory loop involving the translational factor Mss51p and other proteins required for COX assembly. See explanation in the text.

Table 1

Homologue COX subunits in yeast and mammals.

YEAST		MAMMALS		REQUIREMENT FOR COX*	
GENE	**PROTEIN**	**GENE**	**PROTEIN**	**ASSEMBLY**	**ACTIVITY**
Catalytic core (mtDNA encoded subunits)					
COX1	Cox1p	*MTCOXI*	COX1	+	+
COX2	Cox2p	*MTCOXII*	COX2	+	+
COX3	Cox3p	*MTCOXIII*	COX3	+	+
Core protective shield (nDNA encoded subunits)					
COX4	Cox4p	*COXVb*	COX5b	+	+
COX5	Cox5p	*COXIV*	COX4	+	+
COX6	Cox6p	*COXVa*	COX5a	+	+
COX7	Cox7p	*COXVIIa*	COX7a	+	+
COX9	Cox7ap	*COXVIc*	COX6c	+	+
COX8	Cox8p	*COXVIIc*	COX7c	+	+
COX12	Cox9p	*COXVIb*	COX6b	-	-
COX13	Cox10p	*COXVIa*	COX6a	-	-
/	/	*COXVIIb*	COX7b	+	+
/	/	*COXVIII*	COX8	+	+

* Catalytic activity or assembly of the yeast enzyme.

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