Biogenesis of Eukaryotic Cytochrome c Oxidase

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Summary

Eukaryotic cytochrome c oxidase (CcO), the terminal component of the mitochondrial electron transport chain is a heterooligomeric complex that belongs to the superfamily of heme-copper containing terminal oxidases. The enzyme, composed of both mitochondrially and nuclear encoded subunits, is embedded in the inner mitochondrial membrane, where it catalyzes the transfer of electrons form reduced cytochrome c to dioxygen, coupling this reaction with vectorial proton pumping across the inner membrane. Due to the complexity of the enzyme, the biogenesis of CcO involves a multiplicity of steps, carried out by a number of highly specific gene products. These include mainly proteins that mediate the delivery and insertion of copper ions, synthesis and incorporation of heme moieties and membrane-insertion and topogenesis of constituent protein subunits. Isolated CcO deficiency represents one of the most frequently recognized causes of respiratory chain defects in humans, associated with severe, often fatal clinical phenotype. Here we review recent advancements in the understanding of this intricate process, with a focus on mammalian enzyme.

Key words

Cytochrome c oxidase • Assembly • Heme a • Copper • Surf1 • OXA1L

Introduction

Eukaryotic cytochrome c oxidase (CcO) is the terminal multicomponent enzyme of the energy-transducing mitochondrial electron transport chain (Capaldi 1990). It belongs to the superfamily of heme-copper containing terminal oxidases, characterized by the presence of histidine ligands to two heme groups and to a CuB copper ion (Michel et al. 1998). The mitochondrial enzyme, an aa3-type terminal oxidase, catalyzes the sequential transfer of electrons from reduced cytochrome c to dioxygen, coupling this reaction with electrogenic proton pumping across the inner mitochondrial membrane. Eukaryotic CcO is a heterooligomeric complex composed of 7 (Dictyostelium discoideum), 11 (Saccharomyces cerevisiae) and 13 (mammals) protein subunits embedded in the protein-rich, highly convoluted inner mitochondrial membrane. The core of the enzyme is composed of three mitochondrially encoded subunits that exhibit high evolutionary conservation. Unlike prokaryotic enzymes, mitochondrial CcO consists of additional small peripheral subunits, encoded by the nuclear genome and synthesized in cytoplasm (Taanman 1997, Ludwig et al. 2001). The redox-active heme and copper cofactors, directly involved in electron transfer, are coordinated by the mitochondrially encoded subunits Cox1 and Cox2 (Tsukihara et al. 1995).

The biogenesis of eukaryotic CcO complex is complicated by its subcellular location, dual genetic origin of constituent subunits, the hydrophobic nature of most of them, and mainly by a number of prosthetic groups required for function, including two heme a moieties, three copper ions, and zinc, magnesium and sodium ions (Carr and Winge 2003). Consequently, a
number of specific gene products have evolved to accommodate such complex requirements. Although some of these factors act in a general manner and participate also in the biogenesis of other respiratory chain complexes, studies on yeast have identified over thirty accessory factors essential exclusively for proper biogenesis of the eukaryotic enzyme, while a number of them were shown to have human homologues (Khalimonchuk and Rödel 2005, Herrmann and Funes 2005). Isolated CeO deficiency represents one of the most commonly recognized causes of respiratory chain defects in humans associated with a wide spectrum of clinical phenotypes (Shoubridge 2001a, Böh m et al. 2005). Pedigree studies suggest that the majority of genetic defects associated with fatal infantile CeO deficiency are of nuclear origin and inherited as autosomal recessive traits. To date, autosomal recessive mutations in six nuclear encoded factors (SURF1, SCO1, SCO2, COX10, COX15, LRPPRC) required for the assembly of functional CeO complex have been identified in humans (Shoubridge 2001a, 2001b, Barrientos et al. 2002, Pecina et al. 2004). In this review we summarize current knowledge pertinent to the eukaryotic CeO biogenesis, with a special focus on mammalian enzyme whenever possible.

1. CeO structure and function

Mammalian CeO is a heterooligomeric complex of approximately 200 kDa composed of thirteen structural subunits encoded by both the mitochondrial and nuclear genes (Capaldi 1990, Taanman 1997). The enzyme is embedded in the inner mitochondrial membrane, with one part extending 37 Å into the intermembrane space (IMS) and an opposite part protruding 32 Å into the mitochondrial matrix (Tsukihara et al. 1996). The three mitochondrially encoded subunits, Cox1, Cox2 and Cox3 constitute the catalytic and structural core of the enzyme that incorporates all redox-active cofactors (Taanman 1997). Cox1, the largest and the most conserved subunit of the enzyme, is a highly hydrophobic protein composed of twelve transmembrane helices connected by short extramembrane loops. This subunit coordinates the catalytic site of the enzyme, and constitutes the two proton translocation pathways (D- and K-pathway) (Wikström et al. 2000). Cox2 is the smallest and the least hydrophobic subunit of the enzyme core. It consists of a large polar C-terminal domain that protrudes into IMS, and a transmembrane α-helical hairpin that anchors the subunit within the inner membrane. The C-terminal domain of Cox2, composed of ten stranded β barrel, coordinates the Cuα center and constitutes the docking site for cytochrome c. Similarly to Cox1, subunit Cox3 is a highly hydrophobic protein spanning the inner membrane with seven transmembrane helices. It does not bear any prosthetic groups (Tsukihara et al. 1996) and is not directly involved in proton translocation. However, studies of the *Rhodobacter sphaeroides* aα1-type CeO indicate that the presence of Cox3 maintains the rapid proton uptake into the D-channel at physiological pH, which presumably reduces the half-life of reactive dioxygen reduction intermediates (Gilderson et al. 2003, Hosler 2004). This is thought to prevent the turnover-induced inactivation of the enzyme (suicide inactivation), and the subsequent loss of Cuα site (Bratton et al. 1999, Hosler 2004). The remaining 10 evolutionarily younger subunits that associate with the surface of the complex core are encoded by the nuclear genome, and imported into mitochondria upon synthesis on cytoplasmic polysomes (Taanman 1997, Margeot et al. 2005). They include small polypeptides required for the stability/assembly of the holoenzyme, with several of them believed to be involved in regulation of catalytic activity (Ludwig et al. 2001). It was shown that the exchange of bound ADP by ATP at the matrix domain of subunit Cox4 leads to allosteric inhibition of the bovine enzyme at high intramitochondrial ATP/ADP ratios (Kadenbach et al. 2000). In addition, some of the nuclear encoded subunits were shown to be expressed in tissue- and developmentally-specific isoforms (Kadenbach et al. 1990, Linder et al. 1995). Subunits Cox5a, Cox5b and Cox6b are hydrophilic extramembrane proteins, while the rest of the nuclear encoded subunits are hydrophobic polypeptides, spanning the membrane once. Subunit Cox5a is unique in that it does not interact with any of the core subunits being held by the matrix domain of Cox4 and an extramembrane segment of Cox6c (Tsukihara et al. 1996). Although mutations in the three mitochondrially encoded subunits have been reported in several cases, mutations in the nuclear encoded subunits have not been found yet (Shoubridge 2001a, Barrientos et al. 2002). The CeO complex from *S. cerevisiae*, composed of three mitochondrially encoded and eight nuclear encoded subunits closely resembles the mammalian counterpart (Taanman 1997). Yeast null mutants for homologues of mammalian nuclear encoded subunits Cox4, Cox5a, Cox5b, Cox6c or Cox7a are respiratory deficient, lacking CeO activity and the
absorption bands representing heme $a_{a3}$ (Taanman and Williams 2001). This suggests that loss-of-function mutations in at least some of the human nuclear encoded CcO subunits might confer lethality during the early stages of intrauterine development.

In addition to the constituent protein subunits CcO contains several metal centers involved in electron transfer and dioxygen reduction (Taanman 1997). Besides their function in catalysis, most of these prosthetic groups confer an important structural/assembly function within the complex, since the defects in the synthesis and/or insertion of these cofactors often result in markedly reduced levels of fully assembled complex. In addition to catalytic cofactors, the matrix portion-associated peripheral subunit Cox5b contains a bound Zn(II) ion, while a Mg(II) ion is found at the interface of Cox1 and Cox2 subunits. Moreover, Cox1 contains single Na(I) ion in a site that can also bind Ca(II) (Tsukihara et al. 1996). However, the functional relevance of these three cofactors, as well as their import/insertion pathways, remains to be clarified. The low-spin heme $a$ and the heterobimetallic heme $a_{2}$-Cu$_{b}$ center are located relatively deep within the hydrophobic interior of Cox1. In contrast, the binuclear, mixed-valent Cu$_{A}$ center extends 8 Å above the surface of the inner membrane, being held by the IMS-located, C-terminal domain of Cox2. The two copper ions (Cu(I) and Cu(II)) of the Cu$_{A}$ center are coordinated by two bridging cysteines of the CxxxC motif, two histidines, one methionine and a carbonyl oxygen of the peptide backbone of Cox2 (Tsukihara et al. 1995).

The Cu$_{A}$ center serves as the primary acceptor of electrons channeled through the respiratory chain. The electrons donated by cytochrome $c$ are rapidly distributed between the Cu$_{A}$ center and heme $a$, further continuing to the catalytic site composed of high-spin heme $a_{3}$ and an electronically coupled Cu$_{b}$ ion. This part of the catalytic cycle referred to as the reductive phase, ensures the reduction of heme $a_{2}$-Cu$_{b}$ center, a prerequisite for binding of dioxygen (and CO, but not NO) to this site, and subsequent water formation (oxidative phase) (Michel et al. 1998, Brunori et al. 2005). Protons required for the reduction of dioxygen, together with those translocated through the complex to the IMS, are taken up from the matrix and transferred via two distinct pathways to the vicinity of the heme $a_{3}$-Cu$_{b}$ catalytic site. The K-pathway, named after a conserved lysine residue, responsible for one or two-proton supply during the reductive phase. The D-pathway, named after a conserved aspartic residue, transfers the remaining two or three "substrate" protons required for water formation, as well as four protons pumped to the IMS (Wikström et al. 2000). Recently, the fundamental mechanism of coupling of electron transfer with proton translocation was revealed (Belevich et al. 2006). It was shown that the electron transfer from heme $a$ to the catalytic site is kinetically linked to an internal vectorial proton transfer, initiating the proton pump mechanism of the enzyme (Belevich et al. 2006). The free energy released during the electron-transfer reactions is thus transformed into the electrochemical transmembrane gradient of protons, that is utilized by F$_{1}$F$_{0}$-ATP synthase (complex V) to drive ATP synthesis.

Although no functional role for CeO dimerization has been suggested, catalytically active enzyme is believed to exist within the inner membrane as a dimer of two thirteen-subunit assemblies, with contact between monomers mediated merely by subunits Cox6a and Cox6b (Tsukihara et al. 1996, Musatov and Robinson 2002). In mammalian mitochondria CeO (one to four copies) is found associated with NADH:ubiquinone oxidoreductase (complex I) and dimeric cytochrome bc$_{1}$ complex (complex III), within a macromolecular assembly referred to as "supercomplex" (Schägger and Pfeiffer 2000). The functional relevance of such association of respiratory complexes is thought to reside in facilitating the electron flux between the complexes, by reducing the distance of diffusion of cytochrome $c$, and by substrate channeling (Schägger and Pfeiffer 2000).

Recently, using electron microscopy and single particle image analysis, the molecular architecture of both predominant mammalian stoichiometric assemblies (I$_{1}$II$_{3}$I$_{2}$IV$_{1}$ and I$_{1}$II$_{3}$I$_{2}$IV$_{1}$) of respiratory complexes was characterized, suggesting that the I$_{1}$II$_{3}$I$_{2}$IV$_{1}$ supercomplex of approx. 1.7 MDa represents a major physiological module of the mammalian respiratory chain (Schäfer et al. 2006).

2. Synthesis and insertion of heme moieties

Heme $a$ is a unique heme derivative found exclusively in all eukaryotic and certain prokaryotic CeO enzymes. In contrast, many bacterial terminal oxidases utilize heme $b$ or heme $a$, instead of heme $a$ (Michel et al. 1998). Heme $a$ differs from protoheme (heme $b$ or ferroprotoporphyrin IX) in that the C2 vinyl side chain is replaced by an isoprenoid substituent and a methyl group is oxidized into a formyl group (Caughey et al. 1975). Heme represents a potentially toxic, hydrophobic iron...
chelate, which may facilitate harmful cellular process through ROS formation, e.g. oxidative membrane damage (Ryter and Tyrrell 1999). Hence, the synthesis, delivery, and final incorporation of heme $a$ into CcO must be carefully regulated. Despite this fact, almost nothing is known concerning the regulation of heme $a$ homeostasis (Morrison et al. 2005). Recently, the possible role for copper as a regulator of heme $a$ biosynthesis was investigated. However, no functional correlations could have been found (Morrison et al. 2005). Most of the yeast CcO accessory factors mutants characterized by blocked holoenzyme assembly and rapid turnover of intermediates is unable to accumulate heme $a$. Initially, this phenomenon was ascribed to increased turnover of free heme $a$, as a consequence of reduced Cox1 levels. However, this notion is inconsistent with the fact that some of the yeast mutants retain high heme $a$ levels, even when Cox1 is almost undetectable (Barros and Tzagoloff 2002). Instead, preliminary studies have suggested that the synthesis of heme $a$ is subject to either positive or negative regulation by intermediate/subunit of CcO at the level of heme $a$ synthase (Cox15) (Barros and Tzagoloff 2002).

The biosynthesis of heme $a$ involves a sequential conversion of heme $b$. The first step in this reaction is catalyzed by an inner membrane-associated farnesyl transferase (Cox10). It involves the conversion of C2 vinyl group on pyrrole ring A into a 17-hydroxyethylfarnesyl moiety (Tzagoloff et al. 1993). This reaction yields heme $o$, found as a final cofactor in some prokaryotic terminal oxidases. In the next reaction, the C8 methyl substituent on pyrrole ring D of heme $o$ is oxidized into an aldehyde, thus generating heme $a$. This oxidation proceeds via two successive monoxygenase steps catalyzed by Cox15, an inner membrane-anchored heme $a$ synthase (Brown et al. 2002). The matrix localized ferredoxin (Yah1) and ferredoxin reductase (Arh1) are thought to provide reducing equivalents during this reaction (Barros et al. 2002). Interestingly, Cox15 is itself presumably a heme-containing enzyme, employing a heme $b$ cofactor at the active site (Svensson et al. 1996). Human homologues of both yeast Cox10 and Cox15 were identified sharing 33 and 42 % sequence identity, respectively with yeast proteins (Glerum and Tzagoloff 1994, Petruzzella et al. 1998). Mutations in both human genes were reported to result in isolated CcO deficiency associated with severely reduced heme $a$ levels (Antonicka et al. 2003a, 2003b). In COX10-deficient fibroblasts and COX15-deficient heart mitochondria, the CcO-specific assembly defect is not accompanied by any accumulation of subassemblies (Antonicka et al. 2003b, Williams et al. 2004).

Two heme $a$ moieties are found within the eukaryotic CcO. The bis-histidine low-spin heme $a$ is a six-coordinate heme responsible for electron transfer. The second heme $a$, present in the complex, is a five-coordinate, high-spin heme $a$s, that forms a heterobimetallic site with Cu$_{II}$ ion, a place where dioxygen, CO or NO binds (Michel et al. 1998, Brunori et al. 2005). Both heme planes are oriented perpendicular to the membrane with their iron centers being 14 Å apart (Yoshikava et al. 1998). The insertion of heme $a$ moieties into the Cox1 subunit has not been characterized so far. As both metal centers are enfolded within the hydrophobic interior of Cox1, buried 13 Å below the membrane surface, their incorporation is likely to occur either on nascent Cox1 or an early subassembly (Carr and Winge 2003). The Cox1-Cox4-Cox5a subcomplex, as structurally present in the mature CcO, constitutes an open channel through which it would be possible for both heme moieties to be incorporated into Cox1 from the IMS side (Cobine et al. 2006a). Recent evidence from R. sphaeroides suggests that Surf1 protein might facilitate the insertion of heme $a$s into CcO (Smith et al. 2005). Human Surf1 is a 30 kDa integral protein of the inner mitochondrial membrane, composed of two transmembrane domains with a central loop region facing the IMS (Yao and Shoubridge 1999). In SCO2-deficient heart mitochondria solubilized with 1.3 % lauryl maltoside, virtually no Surf1 exists as a monomer, but rather as a trimer, and as part of higher molecular weight complex that might involve some of the accumulated CcO subassembly species (L. Stiburek, unpublished observation). Mutations in human SURF1 represent a common cause of CcO-deficient Leigh syndrome, a subacute necrotizing encephalomyopathy (Shoubridge 2001a, Pecina et al. 2004). Recently, we have demonstrated that this fatal neurological phenotype is associated with remarkable tissue pattern of CcO assembly impairment, pointing to rather tissue-specific character of regulation of CcO biogenesis (Stiburek et al. 2005).

3. Delivery and insertion of copper ions

Copper ions are required in mitochondria for formation of Cu$_{A}$ and Cu$_{B}$ sites in CcO and for incorporation into IMS-located fraction of Cu/Zn-
superoxide dismutase (Cobine et al. 2006a). Due to its chemical reactivity that may lead to deleterious side effects, the amount of free cellular copper is maintained at extraordinary low levels under physiological conditions (Rae et al. 1999). As a result, the delivery and compartmentalization of copper is mediated by a specific subset of proteins termed copper metallochaperones, that are thought to transfer copper ions to their target proteins via transient protein-protein interactions (Huffman and O’Halloran 2001). Despite the recent progress in detailed structure-function characterization of several members of the mitochondrial CcO-specific copper delivery pathway, the fundamental mechanism which ensures copper uptake into mitochondria still remains unknown (Cobine et al. 2006a). Recently it was shown that yeast mitochondria contain a significant pool of copper bound neither to proteins nor mitochondrial DNA (Cobine et al. 2004). This pool was shown to be found in matrix as a soluble, anionic, low molecular weight complex, responding to changes in cytoplasmic copper content. Although the identity of this yeast matrix copper ligand was not revealed yet (Cobine et al. 2004), a compound with the same fluorescent and chromatographic properties was found to be conserved in mouse liver mitochondria (Cobine et al. 2006b). This copper pool likely serves as a reserve for metatllation of mitochondrial copper metalloenzymes (Cobine et al. 2004), since the overexpression of heterologous copper-binding proteins in yeast matrix results in respiratory growth defect, suppressible by exogenous copper supplementation (Cobine et al. 2006b). A number of proteins engaged in mitochondrial, CcO-specific copper trafficking have been identified in eukaryotes, while mutations in two of them (Sco1 and Sco2) were shown to lead to fatal neonatal CcO deficiency in human (Shoubridge 2001b, Carr and Winge 2003).

The small hydrophilic protein Cox17 that localizes both to the cytoplasm and the mitochondrial IMS was the first to be implicated in copper ion delivery to CcO. Based on its dual localization, Cox17 was initially proposed to act as a copper shuttle between the cytoplasm and IMS (Beers et al. 1997). The tethering of Cox17 to the inner membrane by a heterologous transmembrane domain renders the protein fully functional, suggesting that movement between the cytoplasm and IMS is not essential for its function (Maxfield et al. 2004). Deletion of COX17 does not affect mitochondrial copper level (Cobine et al. 2004). However, in vitro studies with purified proteins and yeast cytoplasm assay have demonstrated that Cox17 is able to deliver Cu(I) to both Sco1 and Cox11 (Horng et al. 2004). Yeast Cox17 thus represents CcO-specific copper metallochaperone that functions in a certain step downstream of putative mitochondrial copper shuttle/transporter, which acquires copper ions either in matrix or cytoplasm. Human Cox17 orthologue has been identified that shares 48% sequence identity with yeast counterpart (Amaravadi et al. 1997). Overexpression of the human Cox17 rescues the CcO activity defect of human SCO2 but not SCO1-deficient cells (Leary et al. 2004).

Cox19 is another small soluble copper-binding protein implicated in copper transfer to CcO. It exhibits dual localization in IMS and cytoplasm, albeit only upon overexpression (Nobrega et al. 2002). The CcO-specific respiratory defect of Cox19 null strain is not associated with decreased mitochondrial copper level. Moreover, the mutant phenotype can not be rescued by addition of exogenous copper salts. Cox19 exists as a stable dimer, and recombinant protein was reported to bind Cu(I). The tethering of Cox19 to the inner membrane via transmembrane domain of Sco2 does not abrogate its function concerning CcO assembly (Cobine et al. 2006a). Human Cox19 orthologue was identified that shares 40% sequence identity with yeast protein. Subcellular localization studies with full-length, GFP-fused, human Cox19 showed predominant cytoplasmic localization in HEK 293 cells (Sacconi et al. 2005).

Like Cox17 and Cox19, yeast Cox23 is a small soluble protein containing four cystein residues within a specific helical hairpin conformation referred to as twin Cx6C motif. Cox23 is localized both to IMS and cytoplasm (Barros et al. 2004). The CcO-specific, respiratory defect of Cox23 null mutant is rescued by increased concentrations of copper, but only when COX17 is overexpressed simultaneously (Barros et al. 2004). The deletion of COX23 does not affect mitochondrial copper level (Cobine et al. 2006a).

The copper-binding protein Sco1, member of the conserved Sco protein family appears to act downstream of Cox17 in copper delivery pathway to Cu₆ site in Cox2. Sco1 is an integral inner-membrane protein containing a globular copper-binding domain that protrudes into the IMS (Buchwald et al. 1991). This domain consists of a thioredoxin fold composed of a central four stranded β sheet surrounded by four α helices (Williams et al. 2005). The protein is tethered to the membrane by a single N-terminal transmembrane helix that was shown to be
functionally important (Beers et al. 2002). The observation that yeast Sco1 physically interacts with Cox2 substantiates its postulated role in CuA site formation (Lode et al. 2000). Alternatively, based on its similarity with peroxiredoxin protein family, Sco1 was proposed to be involved in the maintenance of CuA site cysteines in the reduced state (Chinenov 2000, Balatri et al. 2003). More recently, based on the structural data, human Sco1 orthologue has been implicated as a redox switch in IMS (Williams et al. 2005). Despite the fact that the CcO defect of human SCO1-deficient cells is not reversed upon overexpression of human Cox17 (Leary et al. 2004), its expression is required for copper metallation of human Sco1 in yeast cytoplasm assay (Horng et al. 2005). Mutations in human SCO1 result in neonatal hepatic failure associated with isolated, tissue-specific CcO deficiency (Valnot et al. 2000). In addition to severely reduced holoenzyme levels, human SCO1-deficient fibroblasts accumulate several CeO subassemblies, particularly the Cox1-Cox4-Cox5a subcomplex (Williams et al. 2004).

Human Sco2, the second member of the Sco protein family, is an inner-membrane, copper-binding protein implicated in the formation of CuA site in Cox2. Although yeast also encode a Sco2 protein, capable of binding copper ions (Cobine et al. 2006a), this has no obvious function in CcO assembly (Glerum et al. 1996). Sco proteins are characterized by the presence of copper-binding motif composed of two conserved cysteines within a CxxxC motif and a conserved histidine residue. Consistent with the composition of CuA center, Sco proteins can bind either Cu(I) or Cu(II) ions (Horng et al. 2005). Since the CuA site is binuclear, human Sco proteins might physically interact in order to deliver two copper ions to Cox2 simultaneously (Leary et al. 2004). Two obvious reasons support the presumed involvement of human Sco proteins in copper delivery to CcO. First, the missense mutations in human SCO1 (P174L) and SCO2 (E140K and S240F) are located in the vicinity of the copper-binding motif (Jaksch et al. 2000, Valnot et al. 2000). Second, the CcO defect of SCO1 and SCO2-deficient cells is at least partially rescued by exogenous copper supplementation (Jaksch et al. 2001, Leary et al. 2004). Moreover, the overexpression of the mutant human Sco proteins with conserved cysteines and histidine residues substituted by alanines, fail to rescue the CcO deficiency of either SCO1 or SCO2-deficient fibroblasts (Horng et al. 2005). In contrast to SCO1, mutations in SCO2 are associated with encephalopathy and hypertrophic cardiomyopathy (Papadopoulou et al. 1999). The molecular basis for such distinct clinical presentation remains unresolved, since both transcripts are ubiquitous, displaying similar steady-state levels among various human tissues. However, it seems conceivable that one or both Sco proteins might exhibit tissue-specific functional differences, in order to sustain different tissue-specific requirements for the regulation of CcO biogenesis (Leary et al. 2004, Stiburek et al. 2005). We have demonstrated that human Sco2 acts in a highly tissue-specific manner at an early stage of CcO assembly, very likely during the biogenesis of Cox2 subunit (Stiburek et al. 2005). Recently, the tumor suppressor p53 was shown to directly regulate mitochondrial respiration through transactivation of human SCO2 transcription (Matoba et al. 2006).

The inner-membrane copper-binding protein Cox11 represents another mitochondrial CcO-specific copper metallochaperone. Similarly to Sco proteins, Cox11 is thought to function downstream of Cox17 in copper delivery to CcO, presumably inserting CuB ion into Cox1 (Hiser et al. 2000). Yeast Cox11 null mutant has diminished CeO activity and reduced levels of Cox1 (Tzagoloff et al. 1990). The role for Cox11 in CuB site formation was implicated by the observation that CeO isolated from R. sphaeroides Cox11 null mutant lacked CuB site, along with diminished magnesium content, but contained both heme moieties (Hiser et al. 2000). Like Sco1, yeast Cox11 is tethered to the inner membrane by a single N-terminal transmembrane helix, while the soluble C-terminal domain harboring three copper-binding cystein residues protrudes into IMS (Carr et al. 2002). Cox11 functions in a dimeric state, binding one Cu(I) ion per each monomer (Carr et al. 2002). As mentioned above, Cox11 is capable of accepting copper ions from Cox17 (Horng et al. 2004). Two COX11 homologues have been identified in human genome, however only one represents active gene predicted to encode protein with 55 % sequence identity with yeast polypeptide (Petruzzella et al. 1998).

4. Import and membrane-insertion of constituent subunits

The vast majority of mitochondrial proteins, including ten of the thirteen CeO subunits, are encoded by nuclear genes and synthesized in cytoplasm as precursor proteins. The targeting of most of these proteins to mitochondria is mediated by a specific cleavable N-
terminal presequence, rich in basic, hydrophobic and hydroxylated amino acids (Truscott et al. 2003). Such extensions, often in the form of amphipathic α helix are recognized by receptor subunits of a multimeric outer membrane TOM (translocase of the outer membrane) complex, that consists of a stable core, so-called general import pore complex (GIP complex) and loosely associated receptor proteins. The Tom40 subunit of the complex constitutes a 22 Å, aqueous translocation pore that represents the entry point into mitochondria for most nuclear encoded proteins (Pfanner and Wiedemann 2002). After crossing the outer membrane, preproteins destined to the inner membrane and matrix interact with one of the TIM (translocase of the inner membrane) complexes. The inner membrane proteins that contain internal targeting signals (TIM subunits or metabolite carriers) are inserted from IMS via a TIM22 complex (carrier translocase), upon release from a soluble Tim9-Tim10 hexameric complex. The inner membrane-destined preproteins imported via a TIM23 complex (presequence translocase), in a membrane potential and ATP-dependent manner, are either arrested at the level of translocase and then laterally inserted into the inner membrane ("stop-transfer mechanism"), or translocated into the matrix and subsequently exported into the inner membrane by a specific export machinery ("conservative sorting" pathway) (Herrmann and Neupert 2003, Koehler 2004).

Only a very limited number of gene products is encoded on mitochondrial genome (thirteen in human, eight in the yeast). During the evolution, most genes of α-proteobacterial descent were transferred to the nucleus (Andersson and Kurland 1999, Cavalier-Smith 2002). The hydrophobic nature of most mitochondrially encoded proteins have hindered the transfer of their respective genes to the nucleus (Claros et al. 1995). Since, due to the high tendency to form nonspecific aggregates, the synthesis of hydrophobic membrane proteins represents a considerable problem. Consequently, an evolutionary conserved membrane-insertion machinery, represented by the Alb3/Oxa1/YidC protein family, have evolved to ensure the cotranslational insertion of hydrophobic proteins in mitochondria, chloroplasts and bacteria (Stuart 2002, Herrmann and Neupert 2003). Hence, mitochondrial translation is thought to occur exclusively at the matrix face of the inner membrane bilayer (Liu and Spremulli 2000). In the cytoplasm of eukaryotic and prokaryotic cells the recognition and membrane-recruitment of translating ribosomes is mediated by signal recognition particles (Gilmore and Blobel 1983), that appear to be absent from mitochondria (Glick and von Hejne 1996).

The insertion of mitochondrial translation products, as well as a subset of conservatively sorted nuclear gene products, into the inner membrane ensures a conserved integral inner-membrane protein Oxa1, the founding member of the Alb3/Oxa1/YidC protein family (Herrmann and Neupert 2003). Members of this family possess a hydrophobic core domain containing five transmembrane helices that facilitate the membrane export of protein substrates (Herrmann et al. 1997, Kuhn et al. 2003). Unlike bacterial homologues, mitochondrial Oxa1 contain a C-terminal α-helical domain of approx. 100 residues that protrudes into the matrix (Jia et al. 2003, Preuss et al. 2005). This domain binds to 60S ribosomal subunit protein L41, located near the polypeptide exit tunnel, and physically recruits the mitochondrial translation apparatus to the translocation complex (Jia et al. 2003), represented by approx. 200 kDa homooligomeric assembly of four Oxa1 subunits (Szyrach et al. 2003). The yeast Oxa1 null mutant is respiratory deficient, with no detectable CcO activity and markedly reduced levels of the cytochrome bc1 complex and FoF1-ATP synthase (Bonnefoy et al. 1994a, Altamura et al. 1996). The obligate aerobic fungi N. crassa and S. pombe are not viable in the absence of Oxa1 (Bonnefoy et al. 2000, Nargang et al. 2002). Cox1, Cox2 and Cox3 were shown to transiently interact with Oxa1 as nascent chains (Hell et al. 2001). The membrane translocation of both N- and C-termini of yeast Cox2 relies on Oxa1 function (Hell et al. 2001). The other mitochondrial translation products do not show such a strong dependency on Oxa1, suggesting the existence of an alternative insertion pathway (Stuart 2002). Mitochondrial Oxa1 proteins are functionally conserved since the homologues of humans, plants, N. crassa and S. pombe are able to rescue the respiratory deficiency of yeast Oxa1 null mutant (Bonnefoy et al. 1994b, 2000; Hamel et al. 1997; Nargang et al. 2002). The human Oxa1 orthologue, referred to as OXA1L, shares 33 % sequence identity with yeast polypeptide (Bonnefoy et al. 1994b, Rötig et al. 1997). The full-length, FLAG-tagged versions of both identified coding sequences of human OXA1L localize to mitochondria when expressed in HEK 293 cells (L. Stiburek, unpublished observation). Yeast Mba1, a protein associated with the matrix face of the inner membrane presumably functions as a ribosome receptor that cooperates with Oxa1 in the cotranslational
insertion process (Ott et al. 2006). The lack of Mba1 together with the C-terminus of Oxa1 results in association of mitochondrial translation products with mtHSP70 (Ott et al. 2006), a matrix chaperone known to specifically interact with unfolded polypeptides (Hartl and Hayer-Hartl 2002). Yeast Mba1 exhibits sequence similarity with mitochondrial ribosomal L45 proteins from higher eukaryotes (Ott et al. 2006).

A distant homologue of Oxa1, referred to as Cox18 or Oxa2, is an integral inner-membrane protein containing five predicted transmembrane helices within a conserved core domain of approx. 200 amino acid residues (Souza et al. 2000, Funes et al. 2004). Cox18 is believed to be involved in topogenesis of the C-terminal domain of Cox2. Since the HA-tagged C-terminus of Cox2, normally found exposed into IMS, becomes protease-protected in mitoplasts from Cox18 null strain (Saracco and Fox 2002). Unlike Oxa1, Cox18 lacks the C-terminal ribosome-binding domain, and yeast Cox18 null mutants exhibit isolated CcO deficiency (Souza et al. 2002, Funes et al. 2004). Overexpression of Oxa1 does not suppress Cox18 null phenotype, suggesting functional differences between both proteins (Saracco and Fox 2002). In contrast to mammalian protein, yeast Cox2 is synthesized as a precursor with N-terminal extension of 15 amino acid residues. This presequence is removed, upon translocation into IMS, by Imp1/Imp2 protease in conjunction with Cox20 (Nunnari et al. 1993, Hell et al. 2000). Only processed Cox2 is allowed to assemble into the yeast CcO complex. Human Cox18 orthologue was identified that exhibits 25 % sequence identity with yeast protein. A GFP-fused, N-terminal fragment of 210 bp of human Cox18 accumulates exclusively in mitochondria in HEK293 cells (Sacconi et al. 2005).

In yeast, another group of inner membrane proteins, so-called translational activators exists, that mediate the membrane-recruitment of translating mitochondrial ribosomes (Sanchirico et al. 1998, Naithani et al. 2003). These proteins bind to sequences in 5’ untranslated regions (UTR) of particular mitochondrial CcO transcripts (McMullin et al. 1993, Manthey et al. 1998, Green-Willms et al. 2001). This mechanism does not seem to be conserved in mammals, since the mammalian mitochondrial mRNAs do not possess similar 5’ UTR sequences.

**Fig. 1.** Proposed model of the assembly pathway of human cytochrome c oxidase. The Arabic numerals denote subunits of the enzyme. Prosthetic groups and assembly factors are also indicated. S3 indicate previously identified assembly intermediate.

### 5. Assembly of mammalian CcO in the inner membrane

The spatiotemporal assembly of mammalian CcO within the inner mitochondrial membrane is a sequential and relatively slow process (Wielburski and Nelson 1983, Nijtmans et al. 1998). The half-life of the holoenzyme is thought to be about three days (Leary et al. 2002). Little is known about the sequential order in which prosthetic groups are delivered/synthesized and inserted, and constituent subunits are assembled to form the mature membrane-embedded complex. The fact that CcO subassemblies are allowed to accumulate in human mitochondria, have permitted the *bona fide* definition of several key stages of this intricate process (Fig. 1) (Nijtmans et al. 1998, Williams et al. 2004, Stiburek et
In contrast, yeast CcO subassemblies are difficult to detected as they undergo rapid proteolytic degradation (Horan et al. 2005). The nuclear encoded CcO subunits are imported into mitochondria upon synthesis on free cytoplasmic polysomes (Margeot et al. 2005). It is not known whether all of these subunits undergo conservative sorting or whether a subset of them is inserted from the IMS side (Cobine et al. 2006a). In contrast, most of the CcO accessory proteins are translated on outer membrane-attached polysomes, and might be imported through the TOM machinery in a cotranslational manner (Margeot et al. 2005). The intramitochondrial steady-state levels of various unassembled CcO subunits seem to differ considerably. Significant pools of free Cox1 and Cox5a appear to exist in mitochondria of various human tissues, whereas the levels of unassembled Cox4, and mainly of Cox2 are substantially lower (Stiburek et al. 2005). Cox1 appears to stably interact with several nonsubunit proteins before it associates with Cox4 and Cox5a, since it is readily detected as part of three 60-100 kDa complexes that apparently lack other CcO subunits. Subsequently, upon membrane insertion, Cox1 associates with the Cox4-Cox5a heterodimer, forming Cox1·Cox4-Cox5a subassembly (Stiburek et al. 2005). This subcomplex readily accumulates under conditions of blocked/retarded assembly, pointing to its high intrinsic stability. Two lines of evidence suggest that the insertion of heme a occurs either on unassembled Cox1 or during the formation of Cox1·Cox4-Cox5a subassembly. First, both heme moieties are buried deep within the transmembrane interior of Cox1, making the incorporation at the later stages unlikely (Tsukihara et al. 1996). Second, human cells deficient in heme a synthesis do not accumulate Cox1·Cox4-Cox5a subassembly (Antonicka et al. 2003b, Williams et al. 2004). The later finding also suggests that the presence of heme a within Cox1 might stabilize the binding of Cox4-Cox5a heterodimer to this subunit. In contrast, heme a is not required for assembly of the core subunits in *R. sphaeroides* CeO (Hiser and Hosler 2001). The insertion of active site heme might require the inner-membrane protein Surf1, since a significant fraction of CeO isolated from *R. sphaeroides* Surf1 null mutant is devoid of heme a3 (Smith et al. 2005). Owing to the location of CuA site, its formation is likely to occur more or less concurrently with the insertion of heme groups. But the presence of CuA ion within Cox1 does not seem to be essential for stable incorporation of heme a3 (Hiser et al. 2000). The intrinsic inner-membrane protein Cox11 might be responsible for the formation of CuA site (Hiser et al. 2000). Upon assembly of heme moieties and formation of CuA center, the CuA-containing Cox2 is believed to join the Cox1·Cox4·Cox5a subcomplex. The formation of CuA site in Cox2 appears to constitute a prerequisite for efficient association of this subunit with Cox1·Cox4·Cox5a subcomplex. Since the diminished formation of CuA site apparently leads to an accelerated turnover of Cox2 (Williams et al. 2004, Stiburek et al. 2005). The increased proteolytic degradation of such Cox2 might result either from the lowered intrinsic stability of the protein or its reduced binding to Cox1. Conversely, the proper assembly of Cox2 appears indispensable for subsequent association of Cox3, and hence for the stable binding of most of the remaining subunits. Indeed, transmitchondrial cell line (cybrid) with 100 % mutant load of a large C-terminal truncation in Cox3 was shown to lack the holoenzyme complex and accumulate subcomplex composed of Cox1, Cox2, Cox4 and Cox5a (Tiranti et al. 2000, Taanman and Williams 2001). Cox2 might be required to secure the incorporation of heme a3, or whole active site, via cupping the proposed heme-insertion channel formed in Cox1·Cox4-Cox5a subassembly (Bratton et al. 2000, Cobine et al. 2006a). Upon assembly of Cox2 and Cox3 the remaining nuclear encoded subunits, with the exception of Cox6a and Cox7a or Cox7b, are thought to join the complex (Nijtmans et al. 1998). The resulting assembly intermediate S3 represents a ubiquitous, although minor form of CeO in lauryl maltoside preparations. Subsequent association of the rest of the subunits completes the assembly of the holoenzyme complex (Nijtmans et al. 1998). In the next, maturation step a covalent bond is formed on assembled Cox1 bridging His240, one of the three histidine ligands of CuA, with conserved Tyr244 located at the end of the proton translocation K-channel (Yoshikawa et al. 1998). This posttranslational modification is thought to secure the CuA ion in a certain configuration and distance from heme a3, thus preventing the coordination of CuA via histidine ligands of the active site heme (Pinakoulaki et al. 2002). Finally, the mature holoenzyme complex associates with complex I and dimeric complex III, to form the 1.7 MDa respiratory supercomplex (Schägger and Pfeiffer 2001, Schäfer et al. 2006). The role of dimerization and cardiolipin in final maturation of CeO, as well as the function of cytochrome c during CeO assembly, remains elusive.
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