Physiol. Res. 55 (Suppl. 2): S27-S41, 2006

Biogenesis of Eukaryotic Cytochrome c Oxidase

L. STIBUREK, H. HANSIKOVA, M. TESAROVA, L. CERNA, J. ZEMAN

Department of Pediatrics and Center of Applied Genomics, First Faculty of Medicine, Charles University, Prague, Czech Republic

Received July 24, 2006
Accepted September 8, 2006

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 Cu_B copper ion (Michel *et al.* 1998). The mitochondrial enzyme, an aa_3-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 CcO 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, Böhm *et al.* 2005). Pedigree studies suggest that the majority of genetic defects associated with fatal infantile CcO 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 CcO 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 CcO biogenesis, with a special focus on mammalian enzyme whenever possible.

1. CcO structure and function

Mammalian CcO 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 Cu_A 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* aa_3-type CcO 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 Cu_B site (Bratton *et al.* 1999, Hosler 2004). The remaining 10 evolutionary 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 CcO 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 CcO activity and the

absorption bands representing heme aa_3 (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_3-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_3-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, is 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_1F_O-ATP synthase (complex V) to drive ATP synthesis.

Although no functional role for CcO 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 CcO (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_1III_2 and $I_1III_2IV_1$) of respiratory complexes was characterized, suggesting that the $I_1III_2IV_1$ 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 CcO enzymes. In contrast, many bacterial terminal oxidases utilize heme *b* or heme *o*, 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 monooxygenase 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_3, that forms a heterobimetallic site with Cu_B 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_3 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 metallation 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 Cx_9C 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 Cu_A 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 Cu_A 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 Cu_A 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 CcO 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 Cu_A 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 Cu_A center, Sco proteins can bind either Cu(I) or Cu(II) ions (Horng *et al.* 2005). Since the Cu_A 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 Cu_B ion into Cox1 (Hiser *et al.* 2000). Yeast Cox11 null mutant has diminished CcO activity and reduced levels of Cox1 (Tzagoloff *et al.* 1990). The role for Cox11 in Cu_B site formation was implicated by the observation that CcO isolated from *R. sphaeroides* Cox11 null mutant lacked Cu_B 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 CcO 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 bc_1 complex and F_1F_O-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.

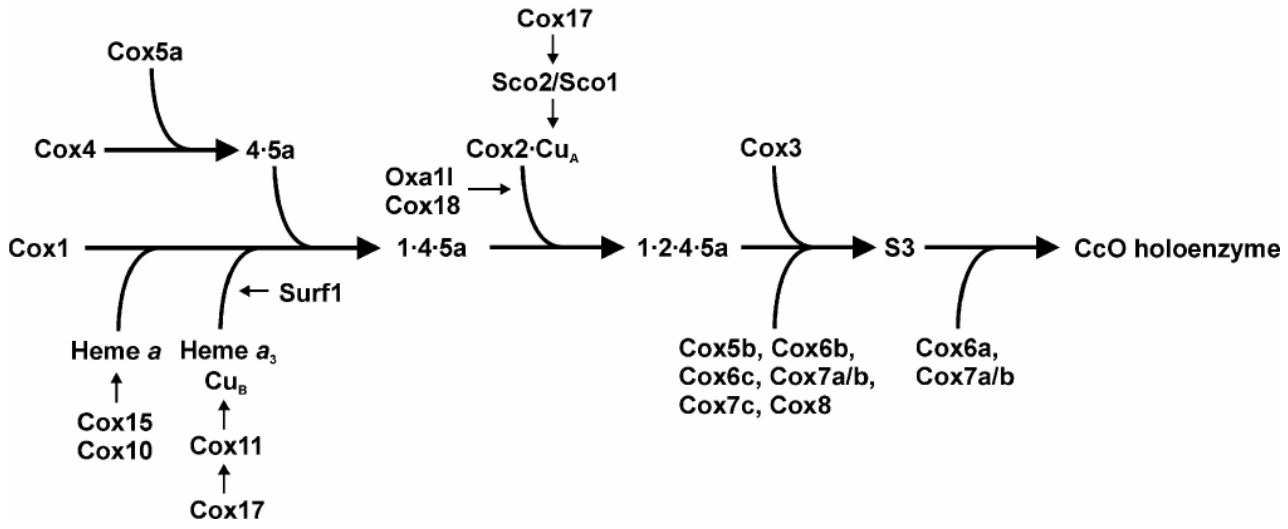

Fig. 1. Proposed model of the assembly pathway of human cytochrome *c* oxidase**.** The Arabic numerals denote subunits of the enzyme. Prosthetic groups and assembly factors are also indicated. S3 indicate previously identified assembly intermediate.

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*

al. 2005). 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* CcO (Hiser and Hosler 2001). The insertion of active site heme might require the inner-membrane protein Surf1, since a significant fraction of CcO isolated from *R. sphaeroides* Surf1 null mutant is devoid of heme a_3 (Smith *et al.* 2005). Owing to the location of Cu_B site, its formation is likely to occur more or less concurrently with the insertion of heme groups. But the presence of Cu_B ion within Cox1 does not seem to be essential for stable incorporation of heme a_3 (Hiser *et al.* 2000). The intrinsic inner-membrane protein Cox11 might be responsible for the formation of Cu_B site (Hiser *et al.* 2000). Upon assembly of heme moieties and formation of Cu_B center, the Cu_A-containing Cox2 is believed to join the Cox1·Cox4·Cox5a subcomplex. The formation of Cu_A site in Cox2 appears to constitute a prerequisite for efficient association of this subunit with Cox1·Cox4·Cox5a subcomplex. Since the diminished formation of Cu_A 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, transmitochonrial 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 a_3, or whole active site, via capping 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 CcO 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 His^{240}, one of the three histidine ligands of Cu_B, with conserved Tyr^{244} located at the end of the proton translocation K-channel (Yoshikawa *et al.* 1998). This posttranslational modification is thought to secure the Cu_B ion in a certain configuration and distance from heme a_3, thus preventing the coordination of Cu_B 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 CcO, as well as the function of cytochrome *c* during CcO assembly, remains elusive.

Acknowledgements

This work was supported by grants from the Grant Agency of Czech Republic (GACR 303/03/D132 and GACR 303/03/H065) and Grant Agency of Charles University (GAUK 17/2004/C).

References

ALTAMURA N, CAPITANIO N, BONNEFOY N, PAPA S, DUJARDIN G. The Saccharomyces cerevisiae OXA1 gene is required for the correct assembly of cytochrome c oxidase and oligomycin sensitive ATP synthase. *FEBS Lett* **382**: 111-115, 1996.

AMARAVADI R, GLERUM DM, TZAGOLOFF A: Isolation of a cDNA encoding the human homolog of COX17, a yeast gene essential for mitochondrial copper recruitment. *Hum Genet* **99**: 329-333, 1997.

ANDERSSON SG, KURLAND CG: Origins of mitochondria and hydrogenosomes. *Curr Opin Microbiol* **2**: 535-544, 1999.

ANTONICKA H, LEARY SC, GUERCIN GH, AGAR JN, HORVATH R, KENNAWAY NG, HARDING CO, JAKSCH M, SHOUBRIDGE EA: Mutations in COX10 result in a defect in mitochondrial heme A biosynthesis and account for multiple, early-onset clinical phenotypes associated with isolated COX deficiency. *Hum Mol Genet* **12**: 2693-2702, 2003a.

ANTONICKA H, MATTMAN A, CARLSON CG, GLERUM DM, HOFFBUHR KC, LEARY SC, KENNAWAY NG, SHOUBRIDGE EA: Mutations in COX15 produce a defect in the mitochondrial heme biosynthetic pathway, causing early-onset fatal hypertrophic cardiomyopathy. *Am J Hum Genet* **72**: 101-114, 2003b.

BALATRI E, BANCI L, BERTINI I, CANTINI F, CIOFFI-BAFFONI S: Solution structure of Sco1: a thioredoxin-like protein involved in cytochrome c oxidase assembly. *Structure* **11**: 1431-1443, 2003.

BARRIENTOS A, BARROS MH, VALNOT I, ROTIG A, RUSTIN P, TZAGOLOFF A: Cytochrome oxidase in health and disease. *Gene* **286**: 53-63, 2002.

BARROS MH, JOHNSON A, TZAGOLOFF A: Cox23, a homologue of Cox17, is required for cytochrome oxidase assembly. *J Biol Chem* **279**: 31943-31947, 2004.

BARROS MH, NOBREGA FG, TZAGOLOFF A: Mitochondrial ferredoxin is required for heme a synthesis in Saccharomyces cerevisiae. *J Biol Chem* **277**: 9997-10002, 2002.

BEERS J, GLERUM DM, TZAGOLOFF A: Purification and characterization of yeast Sco1, a mitochondrial copper protein. *J Biol Chem* **277**: 22185-22190, 2002.

BEERS J, GLERUM DM, TZAGOLOFF A: Purification, characterization, and localization of yeast Cox17, a mitochondrial copper shuttle. *J Biol Chem* **272**: 33191-33196, 1997.

BELEVICH I, VERKHOVSKY MI, WIKSTRÖM M: Proton-coupled electron transfer drives the proton pump of cytochrome c oxidase. *Nature* **440**: 829-832, 2006.

BOHM M, PRONICKA E, KARCZMAREWICZ E, PRONICKI M, PIEKUTOWSKA-ABRAMCZUK D, SYKUT-CEGIELSKA J, MIERZEWSKA H, HANSIKOVA H, VESELA K, TESAROVA M, HOUSTKOVA H, HOUSTEK J, ZEMAN J: Retrospective, multicentric study of 180 children with cytochrome C oxidase deficiency. *Pediatr Res* **59**: 21-6, 2006.

BONNEFOY N, CHALVET F, HAMEL P, SLOMINSKI PP, DUJARDIN G: OXA1, a Saccharomyces cerevisiae nuclear gene whose sequence is conserved from prokaryotes to eukaryotes controls cytochrome oxidase biogenesis. *J Mol Biol* **239**: 201-212, 1994a.

BONNEFOY N, KERMORGANT M, GROUDINSKY O, MINET M, SLONIMSKI PP, DUJARDIN G: Cloning of a human gene involved in cytochrome oxidase assembly by functional complementation of an oxa1- mutation in Saccharomyces cerevisiae. *Proc Natl Acad Sci U S A* **91**: 11978-11982, 1994b.

BONNEFOY N, KERMORGANT M, GROUDINSKY O, DUJARDIN G: The respiratory gene *OXA1* has two fission yeast orthologues which together encode a function essential for cellular viability. *Mol Microbiol* **35**: 1135-1145, 2000.

BRATTON MR, HISER L, ANTHOLINE WE, HOGANSON C, HOSLER JP: Identification of the structural subunits required for formation of the metal centers in subunit I of cytochrome c oxidase of Rhodobacter sphaeroides. *Biochemistry* **39**: 12989-12995, 2000.

BRATTON MR, PRESSLER MA, HOSLER JP: Suicide inactivation of cytochrome c oxidase: catalytic turnover in the absence of subunit III alters the active site. *Biochemistry* **38**: 16236-16245, 1999.

BROWN KR, ALLAN BM., DO P, HEGG EL: Identification of Novel Hemes Generated by Heme A Synthase: Evidence for Two Successive Monooxygenase Reactions. *Biochemistry* **41**: 10906-10913, 2002.

BRUNORI M, GIUFFRE A, SARTI P: Cytochrome c oxidase, ligands and electrons. *J Inorgan Biochem* **99**: 324-336, 2005.

BUCHWALD P, KRUMMECK G, RODEL G: Immunological identification of yeast SCO1 protein as a component of the inner mitochondrial membrane. *Mol Gen Genet* **229**: 413-420, 1991.

CAPALDI RA: Structure and assembly of cytochrome c oxidase. *Arch Biochem Biophys* **280**: 252-262, 1990.

CARR HS, GEORGE GN, WINGE DR: Yeast Cox11, a protein essential for cytochrome c oxidase assembly, is a Cu(I) binding protein. *J Biol Chem* **277**: 31237-31242, 2002.

CARR HS, WINGE DR: Assembly of cytochrome c oxidase within the mitochondrion. *Acc Chem Res* **36**: 309-316, 2003.

CAUGHEY WS, SMYTHE GA, O'KEEFFE DH, MASKASKY JE, SMITH MI: Heme A of cytochrome c oxidase. Structure and properties: comparisons with hemes B, C, and S and derivatives. *J Biol Chem* **250**: 7602-7622, 1975.

CAVALIER-SMITH T: The phagotrophic origin of eukaryotes and phylogenetic classification of Protozoa. *Int J Syst Evol Microbiol* **52**: 297-354, 2002.

CHINENOV YV: Cytochrome c oxidase assembly factors with a thioredoxin fold are conserved among prokaryotes and eukaryotes. *J Mol Med* **78**: 239-242, 2000.

CLAROS MG, PEREA J, SHU YM, SAMATEY FA, POPOT JL, JACQ C: Limitations to in vivo import of hydrophobic proteins into yeast mitochondria - the case of a cytoplasmically synthesized apocytochrome b. *Eur J Biochem* **228**: 762-771, 1995.

COBINE PA, OJEDA LD, RIGBY KM, WINGE DR: Yeast contain a nonproteinaceous pool of copper in the mitochondrial matrix. *J Biol Chem* **279**: 14447-14455, 2004.

COBINE PA, PIERREL F, WINGE DR: Copper trafficking to the mitochondrion and assembly of copper metalloenzymes. accepted by *Biochim Biophys Acta*, 2006a.

COBINE PA, PIERREL F, BESTWICK ML, WINGE DR: Mitochondrial matrix copper complex used in metallation of cytochrome c oxidase and superoxide dismutase. accepted by *J Biol Chem*, 2006b.

FUNES S, NARGANG FE, NEUPERT W, HERRMANN JM: The Oxa2 protein of Neurospora crassa plays a critical role in the biogenesis of cytochrome oxidase and defines a ubiquitous subbranch of the Oxa1/YidC/Alb3 protein family. *Mol Biol Cell* **15**: 1853-1861, 2004.

GILDERSON G, SALOMONSSON L, AAGAARD A, GRAY J, BRZEZINSKI P, HOSLER J: Subunit III of cytochrome c oxidase of Rhodobacter sphaeroides is required to maintain rapid proton uptake through the D pathway at physiologic pH. *Biochemistry* **42**: 7400-7409, 2003.

GILMORE R, BLOBEL G: Transient involvement of signal recognition particle and its receptor in the microsomal membrane prior to protein translocation. *Cell* **35**: 677-685, 1983.

GLERUM DM, SHTANKO A, TZAGOLOFF A: SCO1 and SCO2 act as high copy suppressors of a mitochondrial copper recruitment defect in Saccharomyces cerevisiae. *J Biol Chem* **271**: 20531-20535, 1996.

GLERUM DM, TZAGOLOFF A: Isolation of a human cDNA for heme A:farnesyltransferase by functional complementation of a yeast cox10 mutant. *Proc Natl Acad Sci U S A* **91**: 8452-8456, 1994.

GLICK BS, VON HEIJNE G: Saccharomyces cerevisiae mitochondria lack a bacterial-type Sec machinery. *Protein Sci* **5**: 1-2, 1996.

GREEN-WILLMS NS, BUTLER CA, DUNSTAN HM, FOX TD: Pet111, an inner membrane-bound translational activator that limits expression of the Saccharomyces cerevisiae mitochondrial gene COX2. *J Biol Chem* **276**: 6392-6397, 2001.

HAMEL P, SAKAMOTO W, WINTZ H, DUJARDIN G: Functional complementation of an oxa1- yeast mutation identifies an Arabidopsis thaliana cDNA involved in the assembly of respiratory complexes. *Plant J* **12**: 1319-1327, 1997.

HARTL FU, HAYER-HARTL M: Molecular chaperones in the cytosol: from nascent chain to folded protein. *Science* **295**: 1852-1858, 2002.

HELL K, NEUPERT W, STUART RA: Oxa1p acts as a general membrane insertion machinery for proteins encoded by mitochondrial DNA. *EMBO J* **20**: 1281-1288, 2001.

HELL K, TZAGOLOFF A, NEUPERT W, STUART RA: Identification of Cox20p, a novel protein involved in the maturation and assembly of cytochrome oxidase subunit 2. *J Biol Chem* **275**: 4571-4578, 2000.

HERRMANN JM, FUNES S: Biogenesis of cytochrome oxidase-sophisticated assembly lines in the mitochondrial inner membrane. *Gene* **354**: 43-52, 2005.

HERRMANN JM, NEUPERT W, STUART RA: Insertion into the mitochondrial inner membrane of a polytopic protein, the nuclear encoded Oxa1p. *EMBO J* **16**: 2217-2226, 1997.

HERRMANN JM, NEUPERT W: Protein insertion into the inner membrane of mitochondria. *IUBMB Life* **55**: 219–225, 2003.

HISER L, DI VALENTIN M, HAMER AG, HOSLER JP: Cox11 is required for stable formation of the CuB and magnesium centers of cytochrome c oxidase. *J Biol Chem* **275**: 619-623, 2000.

HISER L, HOSLER JP: Heme A is not essential for assembly of the subunits of cytochrome c oxidase of Rhodobacter sphaeroides. *J Biol Chem* **276**: 45403-45407, 2001.

HORAN S, BOURGES I, TAANMAN JW, MEUNIER B: Analysis of COX2 mutants reveals cytochrome oxidase subassemblies in yeast. *Biochem J* **390**: 703-708, 2005.

HORNG YC, COBINE PA, MAXFIELD AB, CARR HS, WINGE DR: Specific copper transfer from the Cox17 metallochaperone to both Sco1 and Cox11 in the assembly of yeast cytochrome c oxidase. *J Biol Chem* **279**: 35334-35340, 2004.

HORNG YC, LEARY SC, COBINE PA, YOUNG FBJ, GEORGE GN, SHOUBRIDGE EA, WINGE DR: Human Sco1 and Sco2 function as copper binding proteins. *J Biol Chem* **280**: 34113-34122, 2005.

HOSLER J: The influence of subunit III of cytochrome c oxidase on the D pathway, the proton exit pathway and mechanism-based inactivation in subunit I. *Biochim Biophys Acta* **1655**: 332-339, 2004.

HUFFMAN DL, O'HALLORAN TV: Function, structure, and mechanism of intracellular copper trafficking proteins. *Annu Rev Biochem* **70**: 677-701, 2001.

JAKSCH M, OGILVIE I, YAO J, KORTENHAUS G, BRESSER H-G, GERBITZ K-D, SHOUBRIDGE EA: Mutations in SCO2 are associated with a distinct form of hypertrophic cardiomyopathy and cytochrome c oxidase deficiency. *Hum Mol Genet* **9**: 795-801, 2000.

JAKSCH M, PARET C, STUCKA R, HORN N, MULLER-HOCKER J, HORVATH R, TREPESCH N, STECKER G, FREISINGER P, THIRION C, MÜLLER J, LUNKWITZ R, RÖDEL G, SHOUBRIDGE EA, LOCHMÜLLER H: Cytochrome c oxidase deficiency due to mutations in SCO2, encoding a mitochondrial copper-binding protein, is rescued by copper in human myoblasts. *Hum Mol Genet* **10**: 3025-3035, 2001.

JIA L, DIENHART M, SCHRAMP M, MCCAULEY M, HELL K, STUART RA: Yeast Oxa1 interacts with mitochondrial ribosomes: The importance of the C-terminal hydrophilic region of Oxa1. *EMBO J* **22**: 6438-6447, 2003.

KADENBACH B, HUTTEMANN M, ARNOLD S, LEE I, BENDER E: Mitochondrial energy metabolism is regulated via nuclear-coded subunits of cytochrome c oxidase. *Free Radic Biol Med* **29**: 211-221, 2000.

KADENBACH B, STROH A, BECKER A, ECKERSKORN C, LOTTSPEICH F: Tissue- and species-specific expression of cytochrome c oxidase isoenzymes in vertebrates. *Biochim Biophys Acta* **1015**: 368-372, 1990.

KHALIMONCHUK O, RODEL G: Biogenesis of cytochrome c oxidase. *Mitochondrion* **5**: 363-388, 2005.

KOEHLER CM: New Developments In mitochondrial Assembly. *Annu Rev Cell Dev Biol* **20**: 309-35, 2004.

KUHN A, STUART R, HENRY R, DALBEY RE: The Alb3/Oxa1/YidC protein family: membrane-localized chaperones facilitating membrane protein insertion? *Trends Cell Biol* **13**: 510-516, 2003.

LEARY SC, HILL BC, LYONS CN, CARLSON CG, MICHAUD D, KRAFT CS, KO K, GLERUM DM, MOYES CD: Chronic treatment with azide in situ leads to an irreversible loss of cytochrome c oxidase activity via holoenzyme dissociation. *J Biol Chem* **277**: 11321-11328, 2002.

LEARY SC, KAUFMAN BA, PELLECHIA G, GUERCIN G-H, MATTMAN A, JAKSCH M, SHOUBRIDGE EA: Human SCO1 and SCO2 have independent, cooperative functions in copper delivery to cytochrome c oxidase. *Hum Mol Genet* **13**: 1839-1848, 2004.

LINDER D, FREUND R, KADENBACH B: Species-specific expression of cytochrome c oxidase isozymes. *Comp Biochem Physiol B* **112**: 461-469, 1995.

LIU M, SPREMULLI L: Interaction of mammalian mitochondrial ribosomes with the inner membrane. *J Biol Chem* **275**: 29400–29406, 2000.

LODE A, KUSCHEL M, PARET C, RODEL G: Mitochondrial copper metabolism in yeast: interaction between Sco1 and Cox2. *FEBS Lett* **448**: 1-6, 2000.

LUDWIG B, BENDER E, ARNOLD S, HUTTEMANN M, LEE I, KADENBACH B: Cytochrome C oxidase and the regulation of oxidative phosphorylation. *Chembiochem Eur J Chem Biol* **2**: 392-403, 2001.

MANTHEY GM, PRZYBYLA-ZAWISLAK BD, MCEWEN JE: The Saccharomyces cerevisiae Pet309 protein is embedded in the mitochondrial inner membrane. *Eur J Biochem* **255**: 156-161, 1998.

MARGEOT A, GARCIA M, WANG W, TETAUD E, DI RAGO JP, JACQ C: Why are many mRNAs translated to the vicinity of mitochondria: a role in protein complex assembly? *Gene* **354**: 64-71, 2005.

MATOBA S, KANG JG, PATINO WD, WRAGG A, BOEHM M, GAVRILOVA O, HURLEY PJ, BUNZ F, HWANG PM: p53 regulates mitochondrial respiration. *Science* **312:** 1650-1653, 2006.

MAXFIELD AB, HEATON DN, WINGE DR: Cox17 is functional when tethered to the mitochondrial inner membrane. *J Biol Chem* **279**: 5072-5080, 2004.

MCMULLIN TW, FOX TD: COX3 mRNA-specific translational activator proteins are associated with the inner mitochondrial membrane in Saccharomyces cerevisiae. *J Biol Chem* **268**: 11737-11741, 1993.

MICHEL H, BEHR J, HARRENGA A, KANNT A: Cytochrome c oxidase: structure and spectroscopy. *Annu Rev Biophys Biomol Struct* **27**: 329-359, 1998.

MORRISON MS, CRICCO JA, HEGG EL: The biosynthesis of heme O and heme A is not regulated by copper. *Biochemistry* **44**: 12554-12563, 2005.

MUSATOV A, ROBINSON NC: Cholate-Induced dimerization of detergent- or phospholipid-solubilized bovine cytochrome c oxidase. *Biochemistry* **41**: 4371-4376, 2002.

NAITHANI S, SARACCO SA, BUTLER CA, FOX TD: Interactions among COX1, COX2, and COX3 mRNA-specific translational activator proteins on the inner surface of the mitochondrial inner membrane of Saccharomyces cerevisiae. *Mol Biol Cell* **14**: 324-333, 2003.

NARGANG FE, PREUSS M, NEUPERT W, HERRMANN JM: The Oxa1 protein forms a homooligomeric complex and is an essential part of the mitochondrial export translocase in Neurospora crassa. *J Biol Chem* **277**: 12846-12853, 2002.

NIJTMANS LG, TAANMAN JW, MUIJSERS AO, SPEIJER D, VAN DEN BOGERT C: Assembly of cytochrome-c oxidase in cultured human cells. *Eur J Biochem* **254**: 389-394, 1998.

NOBREGA MP, BANDEIRA SCB, BEERS J, TZAGOLOFF A: Characterization of COX19, a widely distributed gene required for expression of mitochondrial cytochrome c oxidase. *J Biol Chem* **277**: 40206-40211, 2002.

NUNNARI J, FOX D, WALTER P: A mitochondrial protease with two catalytic subunits of nonoverlapping specificities. *Science* **262**: 1997-2004, 1993.

OTT M, PRESTELE M, BAUERSCHMITT H, FUNES S, BONNEFOY N, HERRMANN JM: Mba1, a membrane-associated ribosome receptor in mitochondria. *EMBO J* **25**: 1603-1610, 2006.

PAPADOPOULOU LC, SUE CM, DAVIDSON MM, TANJI K, NISHINO I, SADLOCK JE, KRISHNA S, WALKER W, SELBY J, GLERUM DM, COSTER RV, LYON G, SCALAIS E, LEBEL R, KAPLAN P, SHANSKE S, DE VIVO DC, BONILLA E, HIRANO M, DIMAURO S, SCHON EA: Fatal infantile cardioencephalomyopathy with COX deficiency and mutations in SCO2, a COX assembly gene. *Nat Genet* **23**: 333-337, 1999.

PECINA P, HOUSTKOVA H, HANSIKOVA H, ZEMAN J, HOUSTEK J: Genetic defects of cytochrome *c* oxidase assembly. *Physiol Res* **53**: S213-S223, 2004.

PETRUZZELLA V, TIRANTI V, FERNANDEZ P, IANNA P, CARROZZO R, ZEVIANI M: Identification and characterization of human cDNAs specific to BCS1, PET112, SCO1, COX15, and COX11, five genes involved in the formation and function of the mitochondrial respiratory chain. *Genomics* **54**: 494-504, 1998.

PFANNER N, WIEDEMANN N: Mitochondrial protein import: two membranes, three translocases. *Curr Opin Cell Biol* **14**: 400-411, 2002.

PINAKOULAKI E, PFITZNER U, LUDWIG B, CONSTANTINOS VAROTSIS C: The role of the cross-link His-Tyr in the functional properties of the binuclear center in cytochrome c oxidase. *J Biol Chem* **277**: 13563-13568, 2002.

PREUSS M, OTT M, FUNES S, LUIRINK J, HERRMANN JM: Evolution of mitochondrial Oxa proteins from bacterial YidC: inherited and acquired functions of a conserved insertion machinery. *J Biol Chem* **280**: 13004-13011, 2005.

RAE TD, SCHMIDT PJ, PUFAHL RA, CULOTTA VC, O'HALLORAN TV: Undetectable intracellular free copper: The requirement of a copper chaperone for superoxide dismutase. *Science* **284**: 805-808, 1999.

RÖTIG A, PARFAIT B, HEIDET L, DUJARDIN G, RUSTIN P, MUNNICH A: Sequence and structure of the human OXA1L gene and its upstream elements. *Biochim Biophys Acta* **1361**: 6-10, 1997.

RYTER SW, TYRRELL RM: The heme synthesis and degradation pathways: role in oxidant sensitivity. *Radic Biol Med* **28**: 289-309, 2000.

SACCONI S, TREVISSON E, PISTOLLATO F, BALDOIN MC, REZZONICO R, BOURGET I, DESNUELLE C, TENCONI R, BASSO G, DIMAURO S, SALVIATI L: hCOX18 and hCOX19: Two human genes involved in cytochrome c oxidase assembly. *Biochem Biophys Res Commun* **337**: 832-839, 2005.

SANCHIRICO ME, FOX TD, MASON TL: Accumulation of mitochondrially synthesized Saccharomyces cerevisiae Cox2 and Cox3 depends on targeting information in untranslated portions of their mRNAs. *EMBO J* **17**: 5796-5804, 1998.

SARACCO SA, FOX TD: Cox18p is required for export of the mitochondrially encoded Saccharomyces cerevisiae Cox2p C-tail and interacts with Pnt1p and Mss2p in the inner membrane. *Mol Biol Cell* **13**: 1122-1131, 2002.

SCHAFER E, SEELERT H, REIFSCHNEIDER NH, KRAUSE F, DENCHER NA, VONCK J: Architecture f active mammalian respiratory chain supercomplexes. accepted by *J Biol. Chem.*, 2006.

SCHAGGER H, PFEIFFER K: Supercomplexes in the respiratory chains of yeast and mammalian mitochondria. *EMBO J* **19**: 1777-1783, 2000.

SHOUBRIDGE EA: Cytochrome c oxidase deficiency. *Am J Med Genet* **106**: 46-52, 2001a.

SHOUBRIDGE EA: Nuclear genetic defects of oxidative phosphorylation. *Hum Mol Genet* **10**: 2277-2284, 2001b.

SMITH D, GRAY J, MITCHELL L, ANTHOLINE WE, HOSLER JP: Assembly of cytochrome c oxidase in the absence of the assembly protein Surf1 leads to loss of the active site heme. *J Biol Chem* **280**: 17652-17656, 2005.

SOUZA RL, GREEN-WILLMS NS, FOX TD, TZAGOLOFF A, NOBREGA FG: Cloning and characterization of *COX18*, a *Saccharomyces cerevisiae pet* gene required for the assembly of cytochrome oxidase. *J Biol Chem* **275**: 14898-14902, 2000.

STIBUREK L, VESELA K, HANSIKOVA H, PECINA P, TESAROVA M, CERNA L, HOUSTEK J, ZEMAN J: Tissue-specific cytochrome c oxidase assembly defects due to mutations in SCO2 and SURF1. *Biochem J* **392**: 625-632, 2005.

STUART RA: Insertion of proteins into the inner membrane of mitochondria: the role of the Oxa1 complex. *Biochim Biophys Acta* **1592**: 79-87, 2002.

SVENSSON B, ANDERSSON KK, HEDERSTEDT L: Low spin heme A in the heme A biosynthetic protein CtaA from *Bacillus subtilis*. *Eur J Biochem* **238**: 287-295, 1996.

SZYRACH G, OTT M, BONNEFOY N, NEUPERT W, HERRMANN JM: Ribosome binding to the Oxa1 complex facilitates cotranslational protein insertion in mitochondria. *EMBO J* **22**: 6448-6457, 2003.

TAANMAN J-W, WILLIAMS SL: Assembly of cytochrome c oxidase: what can we learn from patients with cytochrome c oxidase deficiency? *Biochem Soc Trans* **29**: 446-451, 2001.

TAANMAN JW: Human cytochrome c oxidase: structure, function, and deficiency. *J Bioenerg Biomembr* **29**: 151-163, 1997.

TIRANTI V, CORONA P, GRECO M, TAANMAN JW, CARRARA F, LAMANTEA E, NIJTMANS L, UZIEL G, ZEVIANI M: A novel frameshift mutation of the mtDNA COIII gene leads to impaired assembly of cytochrome c oxidase in a patient affected by Leigh-like syndrome. *Hum Mol Genet* **9**: 2733-2742, 2000.

TRUSCOTT KN, BRANDNER K, PFANNER N: Mechanisms of Protein Import into Mitochondria. *Current Biology* **13**: 326-337, 2003.

TSUKIHARA T, AOYAMA H, YAMASHITA E, TOMIZAKI T, YAMAGUCHI H, SHINZAWA-ITOH K, HAKASHIMA R, YAONO R, YOSHIKAWA S: Structures of metal sites of oxidized bovine heart cytochrome c oxidase at 2.8 Å. *Science* **269**: 1069-1074, 1995.

TSUKIHARA T, AOYAMA H, YAMASHITA E, TOMIZAKI T, YAMAGUCHI H, SHINZAWA-ITOH K, NAKASHIMA R, YAONO R, YOSHIKAWA S: The whole structure of the 13-subunit oxidized cytochrome c oxidase at 2.8 Å. *Science* **272**, 1136-1144, 1996.

TZAGOLOFF A, CAPITANIO N, NOBREGA MP, GATTI D: Cytochrome oxidase assembly in yeast requires the product of COX11, a homolog of the P. denitrificans protein encoded by ORF3. *EMBO J* **9**: 2759-2764, 1990.

TZAGOLOFF A., NOBREGA M., GORMAN N, SINCLAIR P: On the functions of the yeast COX10 and COX11 gene products. *Biochem Mol Biol Int* **31**: 593-598, 1993.

VALNOT I, OSMOND S, GIGAREL N, MEHAYE B, AMIEL J, CORMIER-DAIRE V, MUNNICH A, BONNEFONT JP, RUSTIN P, ROTIG A: Mutations of the SCO1 gene in mitochondrial cytochrome c oxidase deficiency with neonatal-onset hepatic failure and encephalopathy. *Am J Hum Genet* **67**: 1104-1109, 2000.

WIELBURSKI A, NELSON BD: Evidence for the sequential assembly of cytochrome oxidase subunits in rat liver mitochondria. *Biochem J* **212**: 829-834, 1983.

WIKSTRÖM M: Proton translocation by cytochrome c oxidase: a rejoinder to recent criticism. *Biochemistry* **39**: 3515-3519, 2000.

WILLIAMS JC, SUE C, BANTING GS, YANG H, GLERUM DM, HENDRICKSON WA, SCHON EA: Crystal structure of human SCO1: implications for redox signaling by a mitochondrial cytochrome c oxidase "assembly" protein. *J Biol Chem* **280**: 15202-15211, 2005.

WILLIAMS SL, VALNOT I, RUSTIN P, TAANMAN J-W: Cytochrome c oxidase subassemblies in fibroblast cultures from patients carrying mutations in COX10, SCO1 or SURF1. *J Biol Chem* **279**: 7462-7469, 2004.

YAO J, SHOUBRIDGE EA: Expression and functional analysis of SURF1 in Leigh syndrome patients with cytochrome c oxidase deficiency. *Hum Mol Genet* **8**: 2541-2549, 1999.

YOSHIKAWA S, SHINZAWA-ITOH K, TSUKIHARA T: Crystal structure of bovine heart cytochrome c oxidase at 2.8 Å resolution. *J Bioenerg Biomembr* **30**: 7-14, 1998.

Reprint requests

J. Zeman, Department of Pediatrics, First Faculty of Medicine, Charles University, Ke Karlovu 2, 128 08 Prague 2, Czech Republic. Tel. 224967792. Fax. 224967099. E-mail: jzem@lf1.cuni.cz