

# Copper Chaperones for Cytochrome *c* Oxidase and Human Disease

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Biological processes in living cells are compartmentalized between lipid membranes. Integral membrane proteins often confer specific functions to these compartments and as such have a critical role in cellular metabolism and function. Cytochrome *c* oxidase is a macromolecular metalloprotein complex essential for the respiratory function of the cell. Elucidating the mechanisms of assembly of cytochrome *c* oxidase within the inner mitochondrial membrane represents a unique challenge for understanding metalloprotein biosynthesis. Elegant genetic experiments in yeast have defined several proteins required for copper delivery to cytochrome *c* oxidase. While the precise role of each of these proteins in copper incorporation remains unclear, recent studies have revealed that inherited mutations in two of these proteins can result in severe pathology in human infants in association with cytochrome *c* oxidase deficiency. Characterization of the molecular pathogenesis of these disorders offers new insights into the mechanisms of cellular copper metabolism and the role of these cytochrome *c* oxidase copper chaperones in human disease.

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**KEY WORDS:** copper; metallo chaperone; mitochondria; genetic disease.

## INTRODUCTION

Copper is an essential trace element that plays a critical role in the biochemistry of aerobic organisms. In man, this metal is utilized by a diverse but limited number of enzymes to permit facile electron transfer reactions in key metabolic pathways (Culotta and Gitlin, 2001). These well-characterized cuproenzymes include cytochrome *c* oxidase, ceruloplasmin, tyrosinase, dopamine  $\beta$ -hydroxylase, superoxide dismutase, peptidyl  $\alpha$ -amidating monooxygenase, and lysyl oxidase. Copper deficiency causes loss of activity of these enzymes, resulting in impairment in cellular respiration, iron oxidation, pigment formation, neurotransmitter biosynthesis, antioxidant defense, peptide amidation, and connective tissue formation. The chemical reactivity that makes copper a

useful cofactor in these metabolic pathways also accounts for the toxicity of this metal in circumstances where copper homeostasis is impaired (Harris and Gitlin, 1996). For this reason, specific cellular mechanisms have evolved that regulate the intracellular trafficking and compartmentalization of copper, ensuring an adequate tissue supply of this metal while avoiding cellular toxicity.

Under physiological conditions the amount of free or available copper within the cell is extraordinarily restricted (Rae *et al.*, 1999). As a result, the trafficking of copper to specific enzymes within the cell is mediated by a group of proteins termed copper chaperones (O'Halloran and Culotta, 2000). These metallochaperones function to provide copper directly to specific cellular pathways while protecting this metal from intracellular scavenging (Rosenzweig, 2001). The copper chaperone for superoxide dismutase is essential for the delivery of copper to cytosolic copper/zinc superoxide dismutase (Culotta *et al.*, 1997; Wong *et al.*, 2000) and the small cytoplasmic copper chaperone atox1 is required for copper delivery to the Wilson ATPase (ATP7b) in the secretory pathway (Hamza *et al.*, 1999, 2001). Through a series of biochemical and

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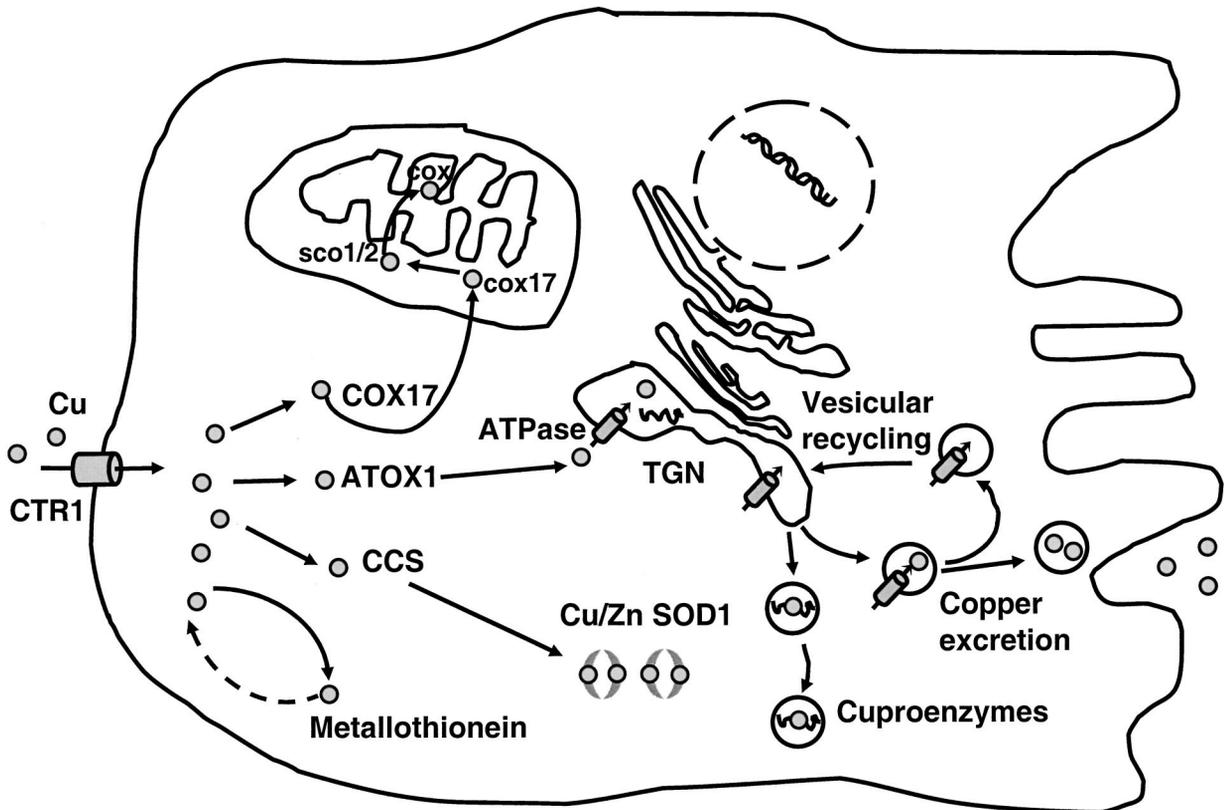
structural studies these chaperones have been demonstrated to deliver copper to their respective targets through direct protein–protein interaction, inserting this cofactor directly into the active site of the respective enzyme or transport protein (Huffman and O’Halloran, 2001).

Taken together, data from a number of laboratories now permits a comprehensive outline of mammalian cellular copper metabolism (Fig. 1). Copper is taken up at the plasma membrane by *ctrl* a multimeric protein that transports copper with high-affinity in a metal-specific and saturable fashion (Lee *et al.*, 2002). Genetic ablation of *ctrl* in mice results in fetal demise, indicating a critical role for this protein in embryonic development and copper homeostasis (Kuo *et al.*, 2001; Lee *et al.*, 2001). Copper homeostasis is largely determined by the copper transporting P-type ATPases, ATP7a and ATP7b, homologous proteins expressed in all cell types (Schaefer and Gitlin, 1999). These ATPases are localized to the *trans*-Golgi network of all cells and function to transport copper into the secretory pathway for incorporation into cuproenzymes and cellular excretion. In response to increases in intracellular copper, these ATPases localize to a vesicular compartment

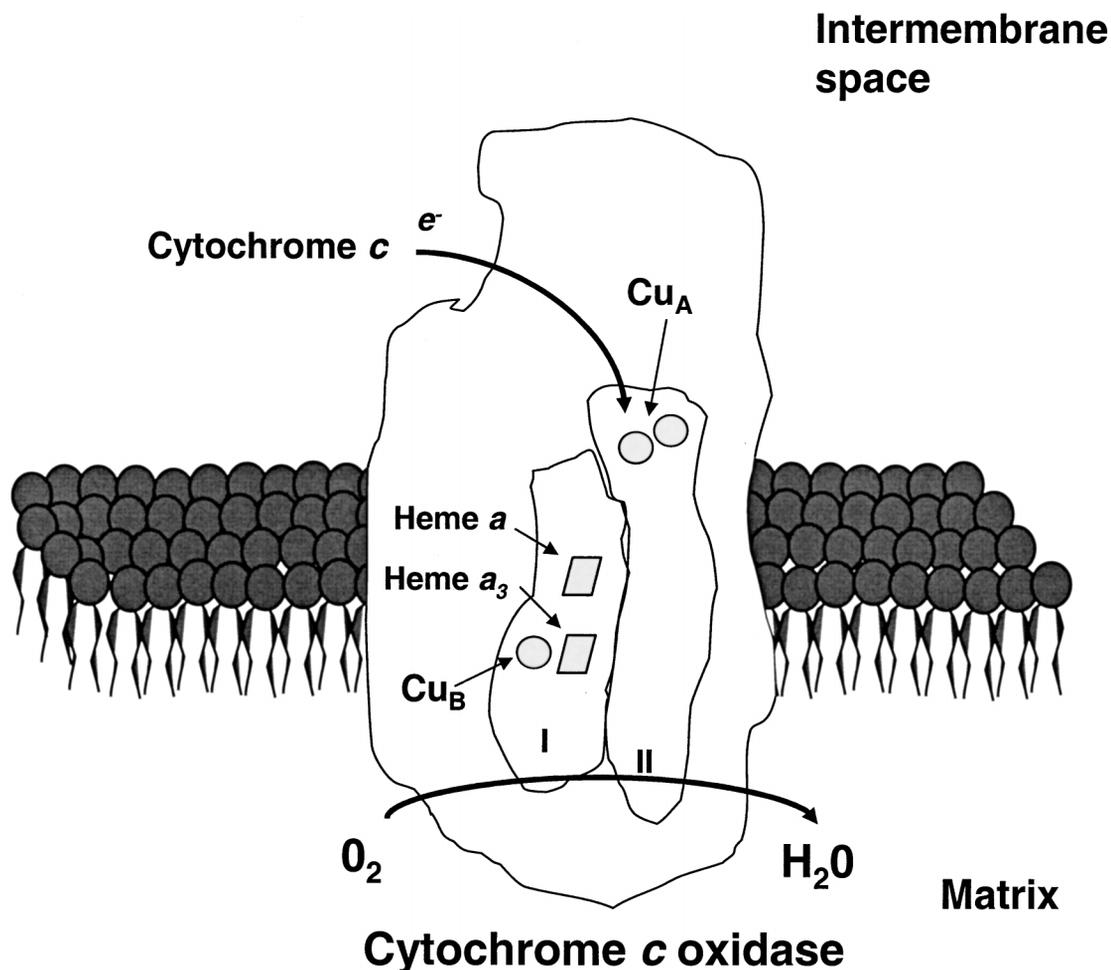
where copper is accumulated for subsequent excretion, thereby providing a sensitive posttranslational mechanism for maintaining copper homeostasis. The mechanisms of copper distribution to individual chaperones within the cell as well as the factors determining the hierarchy of this distribution are currently unknown. Nevertheless, the critical role of cuproenzymes such as cytochrome *c* oxidase in cellular survival suggests that elucidation of these mechanisms will permit a more integrated view of copper homeostasis and cellular metabolism.

### CYTOCHROME C OXIDASE

Cytochrome *c* oxidase (complex IV) is a multi-subunit enzyme that catalyzes electron transfer from cytochrome *c* to dioxygen in the final step of mitochondrial oxidative phosphorylation (Michel *et al.*, 1998) (Fig. 2). This process is crucial in creating the mitochondrial membrane electrochemical gradient that provides the driving force for  $F_1F_0$ -ATPase dependent ATP synthesis. Cytochrome *c* oxidase is a member of the family of heme



**Fig. 1.** Pathways of copper transport in a mammalian cell. Known copper chaperones and transporters are illustrated along with the predicted trafficking of copper along intracellular routes. (Reprinted from Harris and Gitlin, 1996, with permission).



**Fig. 2.** Copper sites in mammalian cytochrome *c* oxidase. The mitochondrial subunits *cox1* and *cox2* are illustrated within complex IV in the inner mitochondrial membrane. The arrows delineate the presumed pathway of electron transfer from cytochrome *c* to dioxygen.

and copper-containing terminal oxidases found in all aerobic organisms (Ferguson-Miller and Babcock, 1996). In mammals, this protein consists of 13 individual subunits, 3 of which, subunits I–III (*cox1*, *cox2*, *cox3*), are encoded in the mitochondrial genome and serve as the catalytic centers binding copper and heme. Functional cytochrome *c* oxidase is a macromolecular complex requiring the assembly of proteins encoded on mitochondrial and nuclear genes as well as the insertion of cofactors including copper, heme, zinc, and magnesium. The process of cofactor insertion and assembly is mediated by a large number of proteins encoded within the nucleus. Copper is present in the mitochondrial encoded subunits, indicating that this metal must be derived from the cytoplasm and inserted into these proteins within this organelle. Resolution of the structure of bovine heart cytochrome *c* oxidase has

permitted a detailed understanding of the metal binding sites as well as the association of each subunit and the mechanisms of electron transfer (Tsukihara *et al.*, 1995, 1996) (Fig. 2). Subunit II is a transmembrane protein with a carboxyl-terminal domain extending into the intermembrane space and containing the binuclear  $\text{Cu}_A$  center that serves as the primary electron acceptor from cytochrome *c*. Subunit I is an integral membrane protein that contains two heme moieties and a copper ion. Electrons are transferred from the  $\text{Cu}_A$  center to heme *a*, a low-spin heme moiety in subunit I, and then to heme  $a_3$  that forms a binuclear center with the associated  $\text{Cu}_B$  ion. This binuclear heme–copper center serves as the site of dioxygen binding and subsequent reduction to  $\text{H}_2\text{O}$ . Subunit III does not contain prosthetic sites and biochemical and genetic evidence suggests that this subunit provides long-range

interactions essential for the maintenance of the copper-heme binuclear center during the catalytic cycle (Michel *et al.*, 1998; Tsukihara *et al.*, 1996).

Cytochrome oxidase deficiency is the most frequent cause of respiratory chain defects in humans. Human cytochrome *c* oxidase deficiency is a phenotypically variable disorder resulting from a number of unique genetic abnormalities (Robinson, 2000). In general these disorders are associated with lactic acidosis, weakness, cardiomyopathy, and severe progressive neurodegeneration early in infancy. As expression of the functional oxidase complex requires many proteins, this considerable phenotypic variability is dependent upon the specific molecular defect, tissue-specific expression of assembly proteins, and the absolute quantity of mutated mitochondrial DNA present in each cell. As a result, partial loss of function mutations are often most severe in those tissues where the affected protein may be rate-limiting. Although mutations in mitochondrial DNA do account for a small number of cases of cytochrome oxidase deficiency, most arise as autosomal recessive disease secondary to loss-of-function mutations not in the nuclear-encoded structural subunits but rather in genes encoding proteins required for cytochrome *c* oxidase subunit assembly (Shoubridge, 2001). Elucidation of the molecular pathogenesis of these disorders has been greatly facilitated by elegant genetic studies of cytochrome *c* oxidase assembly in *Saccharomyces cerevisiae* (Barrientos *et al.*, 2002). These studies have identified four genes, *COX17*, *SCO1*, *SCO2*, and *COX11*, that are critical for the metallation of cytochrome *c* oxidase with copper. Consistent with these observations, patients have now been identified with cytochrome *c* oxidase deficiency resulting in cardiomyopathy, hepatic failure, and ketoacidosis secondary to inherited loss-of-function mutations in the human homologues of two of these genes. Taken together, these results have identified an essential pathway of copper delivery to mitochondrial cytochrome *c* oxidase.

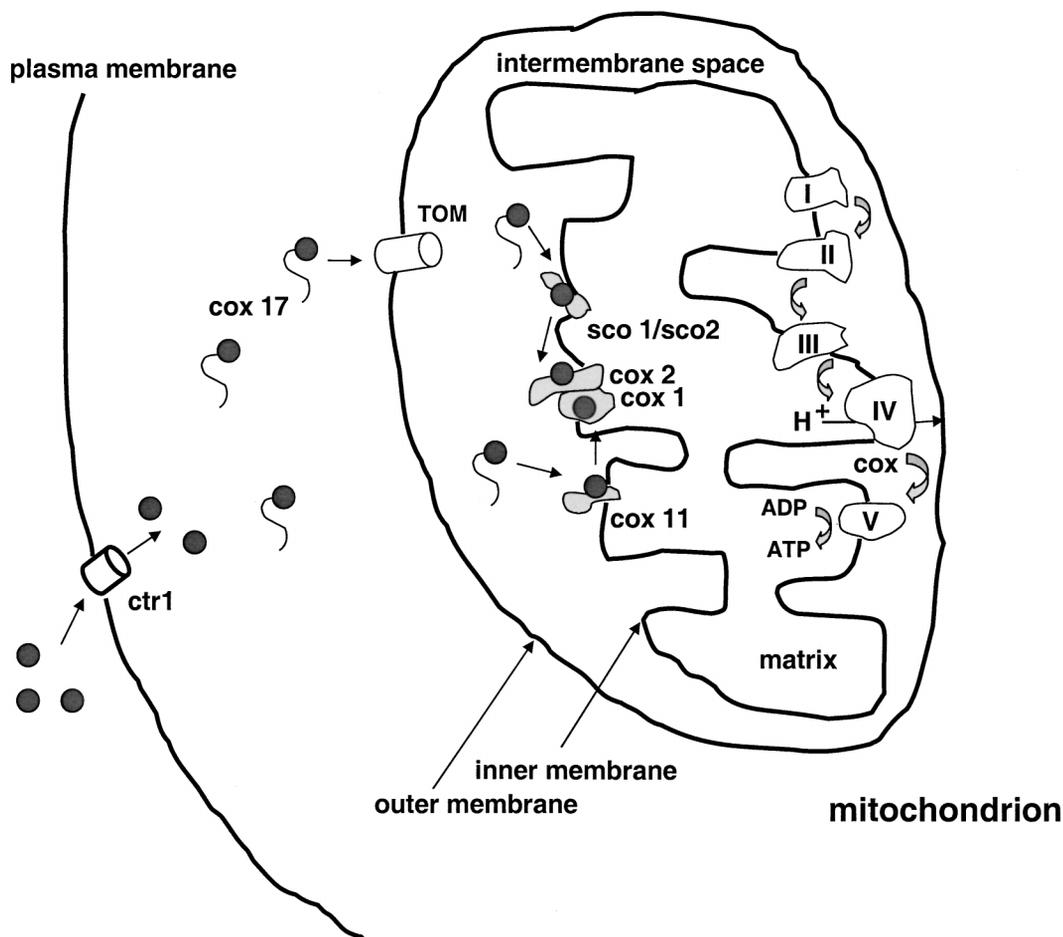
### Cox 17

Cox 17 was initially discovered in *S. cerevisiae* as a cysteine-rich 8kDa protein required for production of a functional cytochrome *c* oxidase complex (Glerum *et al.*, 1996a). Cox 17 $\Delta$  mutant strains synthesize all of the mitochondrial and nuclear encoded subunits yet display respiratory incompetence. This phenotype can be partially rescued by the addition of excess copper to the growth medium, a finding not observed in other cytochrome *c* oxidase mutants, suggesting that cox 17 may play a role in copper acquisition by cytochrome *c* oxidase (Glerum

*et al.*, 1996a). This protein was found to localize to both the intermembrane mitochondrial space and the cytoplasm consistent with a possible role in the delivery of copper to the apoenzyme complex (Beers *et al.*, 1997) (Fig. 3). Furthermore, this pathway was shown to be specific for cytochrome oxidase as other cuproenzymes were found to have normal activity in this cox17 $\Delta$  mutant. Cox17 is a copper-binding protein that binds Cu<sup>+</sup> in a labile, cuprous-thioalate polycopper cluster utilizing three cysteine residues in a CCXC motif that may also involve oligomerization of the protein (Heaton *et al.*, 2000, 2001; Srinivasan *et al.*, 1998). Single substitution of any of these cysteine residues results in a nonfunctional cytochrome *c* oxidase complex. Interestingly, mutation of these critical cysteine residues impairs copper-binding and function, but not mitochondrial uptake, suggesting that the copper-bound structure is not critical for this process (Heaton *et al.*, 2000). A carboxyl terminal C57Y cox 17 mutant, originally defined in cox17 $\Delta$  strains, does show impaired uptake into mitochondria, suggesting that this mutant results in instability of cox 17 as well as impaired interaction with sco1 (vide infra). The mammalian homologue of cox 17 has also been identified and found to be ubiquitously expressed in the mouse, a finding consistent with a critical role for this protein in mitochondrial respiration (Amaravadi *et al.*, 1997; Kako *et al.*, 2000). Human cox17 is encoded in a single gene on the long-arm of chromosome 3. A processed pseudogene is present on 13q14–21 and this sequence must be considered in any analysis for mutations in the *cox 17* gene (Horvath *et al.*, 2000; Punter *et al.*, 2000). Although cox 17 is a feasible candidate for cytochrome *c* oxidase deficiency, nucleotide sequence analysis of the *cox 17* gene in multiple families has thus far not revealed any abnormalities (Horvath *et al.*, 2000).

### SCO1 and SCO2

SCO1 was originally identified through yeast genetics as a mitochondrial protein required for cytochrome *c* oxidase assembly and function (Schulze and Rodel, 1988). Subsequently, sco1 and the homologous gene sco2 were identified as multicopy suppressors of mutant strains carrying a missense cox 17 mutation in yeast (Glerum *et al.*, 1996b). These data suggested that the proteins encoded at these two loci play a role in copper acquisition by cytochrome *c* oxidase and that this process is required for proper assembly of complex IV. Genetic experiments in yeast suggest that sco1 and sco2 have nonoverlapping roles in the pathway of copper delivery to cytochrome oxidase as sco1 $\Delta$  but not sco2 $\Delta$  mutants are respiratory



**Fig. 3.** Model of copper trafficking to the mitochondria. The pathways of copper incorporation into cytochrome *c* oxidase are illustrated as well as the flow of electrons along the entire respiratory chain.

insufficient (Glerum *et al.*, 1996b). Sco1 contains a CXXXC motif that binds copper via these cysteine residues and this copper binding is essential for the rescue of cytochrome *c* oxidase deficiency (Beers *et al.*, 2002; Nittis *et al.*, 2001; Rentzsch *et al.*, 1999). Although the precise role of sco1 in copper transfer remains unclear, biochemical and genetic analysis indicates interaction with the cytochrome oxidase catalytic subunit cox2, suggesting direct transfer from sco1 to the Cu<sub>A</sub> enzymatic site in this subunit (Dickinson *et al.*, 2000; Lode *et al.*, 2000) (Fig. 3). Recently, a role for sco1 in cysteine reduction of cox2 to allow for copper incorporation into this subunit has also been proposed (Chinenov, 2000).

The gene encoding human sco1 has been identified on chromosome 17p13.1 and the encoded protein shown to localize to the inner mitochondria membrane in HeLa cells (Horvath *et al.*, 2000; Paret *et al.*, 1999; Petruzzella *et al.*, 1998). Genomic mapping of a large kindred of neonates

with ketoacidosis and coma, in whom cytochrome oxidase deficiency had been established, localized the affected gene to chromosome 17, in a region encoding SCO1. Nucleotide sequence analysis of the sco1 gene revealed that all affected individuals were compound heterozygotes for a 2 bp frameshift deletion resulting in both a premature stop codon and a missense mutation (P174L) (Valnot *et al.*, 2000). This proline was found to be adjacent to the CXXXC copper-binding domain of SCO1 and therefore presumed to play a crucial role in sco1 copper-binding or transport. Consistent with this concept, analysis in yeast revealed that this mutation results in impairment in the assembly of cytochrome oxidase (Paret *et al.*, 2000). The clinical presentation of SCO1-deficient patients differs markedly from that of patients harboring mutations in other COX assembly genes. Affected children present in infancy with lactic acidosis and severe liver failure with no evidence of cardiac or neurological involvement. Human

sco1 does not compensate for sco1 deficiency in sco1 $\Delta$  yeast, but a chimeric molecule consisting of the amino-terminal domain of yeast sco1 and the carboxyl-terminal domain of human sco1 containing the CXXXC motif will correct the respiratory incompetence in this mutant strain (Paret *et al.*, 1999). Using this approach, the P147L patient mutation was found not to rescue the sco1 $\Delta$  mutant, supporting the proposed deleterious role of this mutation in humans (Paret *et al.*, 2000).

The functional relationship between human and yeast sco2 is currently unknown. Sco2 deficient yeast have no abnormalities of cytochrome *c* oxidase assembly or function and sco2 will not suppress the cytochrome *c* oxidase deficiency observed in sco1 $\Delta$  null mutants. It is possible that the function of these genes is redundant in yeast but not in humans, an explanation that would account for the observations noted above yet still allow for the same biochemical function of sco2 in both organisms. The human sco2 gene is located on chromosome 22 and encodes a 22 kDa protein with a putative CPDXC copper-binding motif (Jaksch *et al.*, 2001b). Direct evidence of a critical role for sco2 in cytochrome oxidase assembly has come from the identification of mutations in the sco2 gene in patients with autosomal recessive cytochrome *c* oxidase deficiency (Jaksch *et al.*, 2000; Papadopoulou *et al.*, 1999). These mutations were originally identified in a series of unrelated infants from several families with hypertrophic obstructive cardiomyopathy, severe muscle weakness, and encephalopathy. Consistent with the chromosomal location of sco2, the cytochrome *c* oxidase deficiency in patient fibroblasts is rescued by transferring chromosome 22 but not other chromosomes (Jaksch *et al.*, 2000). Although all such patients were compound heterozygotes, each carried on one allele a E140K missense mutation in a region immediately downstream from the copper-binding motif. Analysis of the homologous mutation in sco1 in yeast was not revealing for respiratory incompetence (Dickinson *et al.*, 2000). This suggests that this mutation may result in a less severe interruption of cytochrome *c* oxidase function and consistent with this, patients homozygous for this mutation present with delayed onset of symptoms. Nevertheless, these patients do have neurogenic muscular atrophy, cardiomyopathy, and neurodegeneration, confirming the pathogenic nature of this mutation in humans (Jaksch *et al.*, 2001a). Biochemical and histologic analysis of affected tissues indicates that loss-of-function of sco2 is associated with a more selective loss of the mitochondrial rather than nuclear encoded cox subunits, supporting a role for this protein in assembly of the copper-catalytic center (Jaksch *et al.*, 2001b; Sue *et al.*, 2000). The clinical features of patients with

loss-of-function of sco2 is significantly different from that of sco1 or other factors involved in cytochrome oxidase assembly (Sue *et al.*, 2000). These individuals do not have the typical neuropathology observed in Leigh syndrome or other forms of cytochrome *c* oxidase deficiency. Furthermore, the severity of the deficiency is highly tissue-specific with cardiac and skeletal muscle being predominantly affected. More recently, a report of an infant with sco2 mutations and anterior horn cell deterioration and spinal muscular atrophy further emphasizes the clinical variability in this disorder (Salviati *et al.*, 2002b).

Analysis of fibroblasts and myoblasts from sco2-deficient patients has revealed a marked increase in intracellular copper content secondary to increased copper uptake (Jaksch *et al.*, 2001a,b). These data suggest that mitochondria may regulate the set point for cellular copper uptake, depending upon the availability of and need for cytochrome *c* oxidase activity. Although this increased intracellular copper does not appear to alter enzyme activity in cells from affected patients, a further increase in the copper content in such cells using copper-histidine has been shown to restore cytochrome *c* oxidase activity as effectively as genetic replacement of sco2 (Jaksch *et al.*, 2001b; Salviati *et al.*, 2002a). As the E140K mutation does result in detectable levels of protein, it remains unclear if the copper treatment works through this residual sco2 or by binding directly to the catalytic center in the cox2 subunit. Consistent with previous observations on the role of copper in mammalian cells, this treatment was not found to alter the expression of cox17 or sco1 at the level of transcription (Jaksch *et al.*, 2001b). These studies reveal a fundamental mechanism for cellular copper uptake not previously appreciated and offer the possibility of early prenatal treatment for cytochrome *c* oxidase deficiency secondary to abnormalities in this protein.

### Cox11

Cox11 is a 28 kDa mitochondrial membrane polypeptide originally identified in yeast as essential for cytochrome *c* oxidase activity and heme *a* stability in subunit I (Tzagoloff *et al.*, 1990). More recently, it has been shown that cytochrome *c* oxidase isolated from *Rhodobacter* deficient in cox11 contains no Cu<sub>B</sub> site, suggesting a role of this protein in formation of the binuclear copper-heme center (Hiser *et al.*, 2000) (Fig. 3). Consistent with this, recent biochemical studies indicate that cox 11 is a copper-binding protein (Carr *et al.*, in press). Spectroscopic and mutagenesis studies indicate that the copper ion in cox 11 is ligated by three conserved cysteine residues. The C-terminal domain of this protein forms a dimer that

coordinates a single Cu<sup>+</sup> per monomer. While *cox11* was found to be a dimer under the conditions used in this study it remains possible that copper transfer to and from *cox11* occurs through heterodimeric interaction of a single *cox11* subunit with *cox17* and *cox1* respectively. As is the case with the role of *sco1/sco2* in assembly of the Cu<sub>A</sub> site, it is unknown if *cox 11* mediated copper incorporation occurs after cytochrome *c* oxidase assembly or co-translationally, prior to the association of *cox1* with other subunits. As *cox 11* is implicated in the assembly of the Cu<sub>B</sub> site of cytochrome *c* oxidase in yeast and a human homologue has been previously identified (Petruzzella *et al.*, 1998), this protein is a reasonable candidate for human cytochrome *c* oxidase deficiency secondary to impaired assembly of complex IV.

## CONCLUSION

The last several years have witnessed considerable advances in our understanding of the mechanisms of copper metallation of cytochrome *c* oxidase. As this oxidase is critical for cellular respiration and survival, it seems likely that the pathways for copper delivery to this protein within the mitochondria will be tightly regulated within the cell. The presence of partial loss-of-function in almost all cases of cytochrome *c* oxidase deficiency in humans taken together with the postnatal presentation of these disorders, suggests that the onset of respiration at birth places a considerable increase in the need for maximal activity of this enzyme. Nevertheless, it seems reasonable to assume that complete absence of cytochrome *c* oxidase activity would not be compatible with the survival of the mammalian embryo. This possibility would account for the early fetal demise observed in *ctr1* deficient embryos (Kuo *et al.*, 2001; Lee *et al.*, 2001) and may explain why no mutations in *cox 17* have been discovered. Although deficiency of *cox 17* in yeast can be partially compensated by increasing the copper content, the human fetus would have no inherent mechanism to allow for this and therefore the absence of this protein would likely result in complete cytochrome *c* oxidase deficiency.

A central role for mitochondrial copper homeostasis in mammalian cell function and survival is also suggested by observations in the filamentous ascomycete *Podospira anserina*. In this organism, the availability of mitochondrial copper is directly associated with lifespan control as revealed by the GRISEA mutant. In this case, loss of a nuclear encoded transcription factor results in impaired copper uptake, loss of complex IV activity, utilization of an alternate respiratory pathway, and decreased production of reactive oxygen species. In this mutant, molecular

damage, including age-related mitochondrial DNA mutations, is decreased, resulting in prolonged lifespan, revealing that copper and cytochrome *c* oxidase play a critical role in cellular respiration and survival in this organism (Osiewacz, 2002). As mitochondrial DNA mutations and oxidant injury are implicated in human aging and neurodegenerative disease (Melov, 2000), these findings suggest that further elucidation of the pathways and mechanisms of mitochondrial copper homeostasis may have important implications for human health and disease.

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