University of Alberta

Redefining the Role of Sco1p in the Assembly Pathway of Cytochrome c Oxidase

By

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Abstract

Sco1p is a protein required for cytochrome *c* oxidase (COX) assembly. Sco1p is thought to deliver copper to COXII. Analysis of the crystal structure of hSco1p showed no copper. Based on the structure similarities of Sco1p with peroxiredoxins (Prxs), *Sco1* has been proposed to function as a redox-sensing protein. In this thesis *sco1* mutants were tested for hydrogen peroxide (H_2O_2) sensitivity and respiration phenotypes. *sco1* mutants showed two separable phenotypes, respiration competence (EG^+) and partial sensitivity to H_2O_2. These results support the theory of Sco1p as a mitochondrial redox-sensing molecule. Affinity purified COX and inner mitochondrial membranes from *sco1* mutants were analyzed by electron paramagnetic resonance (EPR). EPR analysis on the wild-type strain allowed detection of copper at g=2.08. We conclude that Sco1p could be a bifunctional protein that perhaps uses the thioredoxin-like function to reduce the copper binding cysteines, and delivers copper to the Cu_A site in COXII.

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Table of contents

List of Tables

List of Figures

List of Abbreviations

Å	Angstrom
ADP	Adenosine di-phosphate
ATP	Adenosine tri-phosphate
CPEO	Chronic progressive external ophtalmoplegia
DOC	Deoxycholate
EG	Ethanol/Glycerol
ETC	Electron transport chain
$FADH_2$	Flavin adenine dinucleotide
H_2O	Water
H_2O_2	Hydrogen peroxide
IMM	Inner mitochondrial membrane
IMS	Intermembrane space
kDa	Kilodalton
KSS	Kearns-Sayre syndrome
LSFC	Leigh syndrome French Canadian variant
MELAS	Mitochondrial encephalomyopathy, lactic acidosis and stroke-like episodes
Mg^{2+}	Magnesium ion
mtDNA	Mitochondrial DNA
NAD	Nicotinamide adenine dinucleotide
nDNA	Nuclear DNA

O_2	Molecular oxygen
OMM	Outer mitochondrial membrane
P*i*	Inorganic phosphate
Prx	Peroxiredoxin
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
TIM	Translocase of the inner membrane
TOM	Translocase of the outer membrane
Trx	Thioredoxin
WO	Minimal glucose medium
YPD	Yeast peptone dextrose, rich glucose medium
Zn^{2+}	Zinc ion

Chapter 1: Introduction

Mitochondria, derived from the Greek words *mitos*, thread, and *chondros*, granule, are one of the most important complex organelles of the eukaryotic cell. They generate most of the cell's supply of chemical energy in the form of ATP. Mitochondria are composed of two membranes: an external lipid bilayer that is smooth (outer membrane, OMM) and an inner membrane that is folded inside the mitochondrion forming roughened cristae (inner mitochondrial membrane, IMM). The OMM is permeable to small molecules and ions and contains porin channels, while the IMM is impermeable to almost all small molecules, ions and protons, although it is permeable to oxygen, carbon dioxide and water. There is a space between these two membranes called the intermembrane space (IMS) (Fig. 1-1). The chamber enclosed by the inner membrane is called the matrix and it contains a number of enzymes that carry out the oxidation of pyruvate and fatty acids through the citric acid cycle and oxidative phosphorylation (Frey and Manella, 2000). The mitochondrial matrix also houses the mitochondrial DNA (mtDNA), which encodes proteins that form part of the respiratory chain, as well as the ribosomal RNAs and transfer RNAs required for transcription, translation, and RNA maturation of the mtDNA (Burger et al., 2003).

Due to a number of fundamental similarities between mitochondria and bacteria, Lynn Margulis (1981) proposed the Endosymbiont Theory, which states that modern mitochondria originated from the introduction of aerobic bacteria into the early eukaryotic cell (Margulis, 1981). Mitochondria contain DNA that is different from that of the cell nucleus and similar to that of bacteria, in being circular and with respect to its size. The membrane system composition of mitochondria is like that of a prokaryotic cell membrane, and mitochondria and bacteria both have ribosomes. There are two

endosymbiont theories: primary endosymbiosis requires a free living organism to ingest bacteria, while secondary endosymbiosis is thought to happen when the product of primary endosymbiosis is itself ingested and retained by another eukaryote (Zauner et al., 2006).

Oxidative Phosphorylation and the Electron Transport Chain

In 1949, Lehninger and Kennedy described the mitochondrion as the organelle responsible for oxidative phosphorylation, a fundamental discovery that was a breakthrough in cell biology and metabolism (Kennedy and Lehninger, 1950). Electrons flow from cofactor substrates to molecular oxygen (O_2) in a series of protein complexes in the inner mitochondrial membrane; these linked sets of enzymes are organized into an electron transport chain (ETC), which links the reducing equivalents of nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide ($FADH_2$) to the production of ATP. The mechanism for ATP synthesis was first correctly described by Mitchell in 1961: he proposed that when electrons flow through the electron transport chain, energy changes occur and this energy is used to pump protons out of the matrix, producing an electrical potential and a pH gradient across the inner membrane. This proton motive force promotes the synthesis of ATP from ADP and inorganic phosphate (P_i) (Sherratt, 1991).

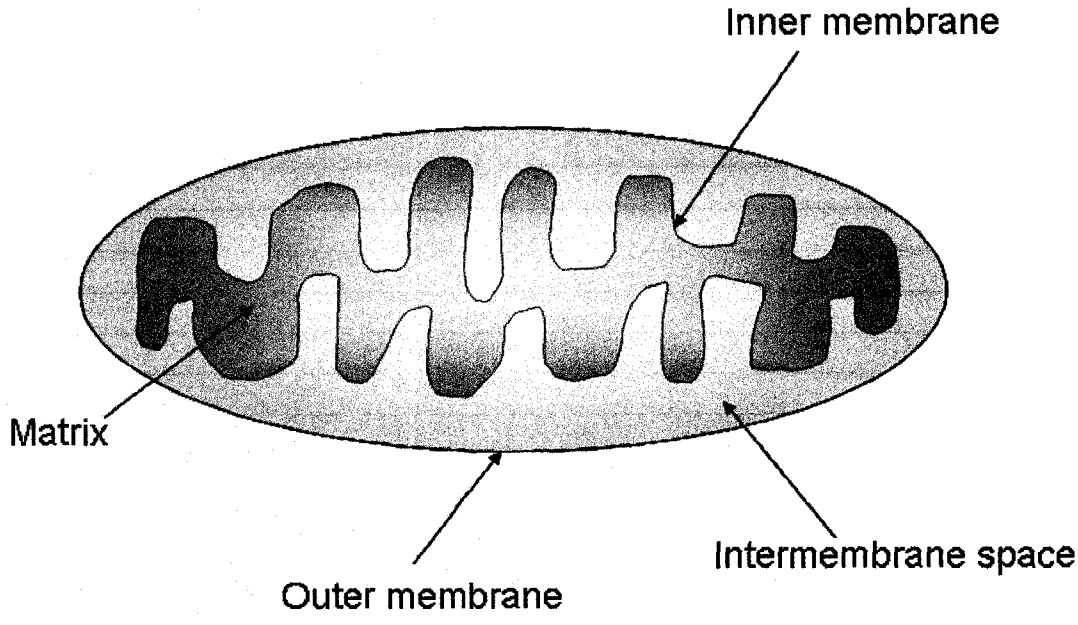


Figure 1-1. Schematic Representation of a Mitochondrion.

The complexes involved in the ETC are: Complex I (NADH dehydrogenase), Complex II (succinate dehydrogenase), Complex III (cytochrome bc_1) and Complex IV (cytochrome *c* oxidase); other intermediary molecules involved in the ETC are the electron carriers coenzyme Q (ubiquinone) and cytochrome *c*.

The energy obtained from the transfer of electrons is used to pump protons from the matrix to the intermembrane space, generating a proton gradient across the mitochondrial inner membrane. This gradient allows the ATP synthase (Complex V) to produce ATP (Fig. 1-2). Complex I accepts electrons from the NADH generated in the Krebs's cycle and passes them to Coenzyme Q, which also receives electrons from Complex II and $FADH_2$. Coenzyme Q then passes the electrons to Complex III, and Complex III passes them to cytochrome *c*. Cytochrome *c* transfers the electrons to Complex IV, which uses these electrons to reduce oxygen to water.

Cytochrome *c* oxidase, or Complex IV

Cytochrome *c* oxidase (COX) is a large transmembrane protein, the terminal enzyme of the ETC in eukaryotes and some aerobic prokaryotes. It catalyzes the transfer of four electrons from ferrocytochrome *c* to molecular oxygen while pumping protons across the mitochondrial inner membrane. It contains four redox-active metal centers: heme A, heme A_3, Cu_A, and Cu_B in a complex formed by multiple subunits (11 in yeast, 13 in mammals) (Table 1-1). The core of the enzyme is formed by three highly conserved subunits (COXI, II and III) that are encoded by mtDNA and synthesized on ribosomes within the mitochondrial matrix.

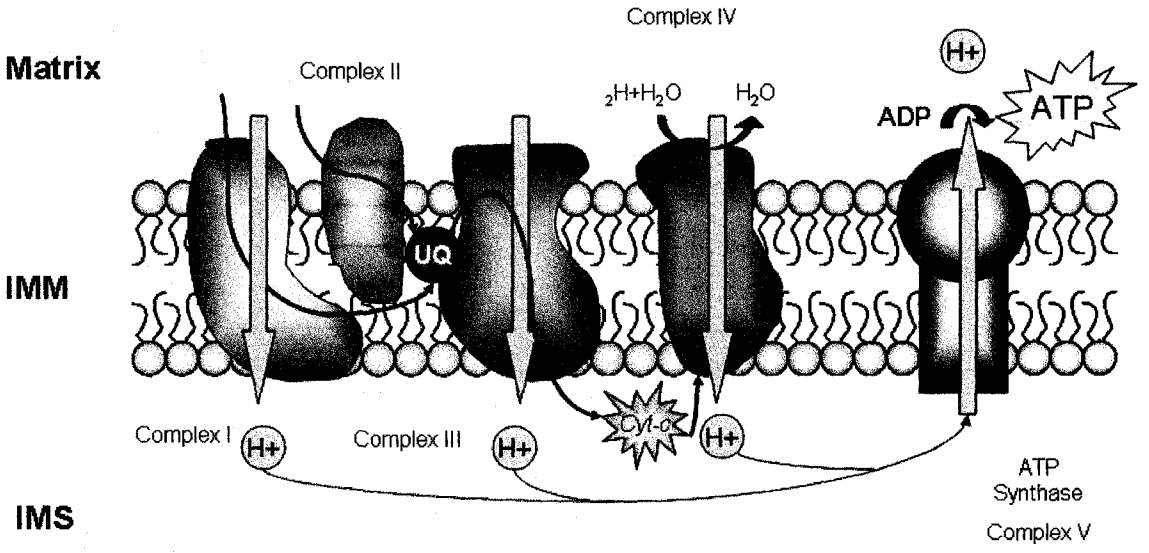


Figure 1-2. Schematic Representation of the Electron Transfer Chain. Electrons flow through the complexes I to IV resulting in the generation of a proton gradient. Complex IV reduces molecular oxygen to water and Complex V produces ATP. UQ refers to ubiquinone and *Cyt*-c to cytochrome c.

Mammals	Yeast	Subunit Features
CoxI	Cox1p	Heme a, heme a_3- Cu_B
CoxII	Cox2p	Cu_A
CoxIII	Cox3p	Phospholipids
CoxVb	Cox4p	Zn_{2+}
CoxIVp	Cox5p	
CoxVa	Cox6p	
CoxVIa	Cox6ap	
CoxVIb	Cox6bp	
CoxVIIa	Cox7p	
CoxVIc	Cox7ap	
CoxVIIc	Cox8p	
CoxVIIb	-	
CoxVIII	-	

Table 1-1. Species-specific COX Subunits. (Tsukihara et al., 1995; Khalimonchuk and Rodel, 2005; Capaldi, 1996)

COX I is the biggest subunit (55-57 kDa) and is highly conserved, binds the heme A, heme A_3 and Cu_B redox centers, and also ligates a sodium ion. Cox1p is a key subunit for both assembly and function of COX. COXII is the smallest subunit of the catalytic core (26-27 kDa), binds the Cu_A that is exposed to the IMS and participates in cytochrome *c* binding. In human COX, both subunits I and II are necessary for the binding of heme A_3 (Rahman et al., 1999). In yeast *cox2* mutants, the presence of Cox3p and Cox4p is dependant on Cox2p (Lemaire et al., 1998). COXIII (30 kDa) is thought to modulate the functions of both subunits I and II. It has also been proposed that Cox3p has a role in the folding and stabilization of COX. The remaining COX subunits are encoded by nuclear DNA and synthesized in the cytoplasm and imported into mitochondria by a protein complex known as the translocase of the outer membrane or TOM complex. The proteins pass through the intermembrane space and then to the inner mitochondrial membrane where they move into the matrix via the translocase of the inner mitochondrial membrane or TIM complex. These proteins are thought to function in the regulation of the catalytic activity or assembly of the holoenzyme (Poyton et al., 1995).

The crystal structure of bovine heart COX showed that the enzyme is a dimer, with a cytosolic domain extended 35 Å above the membrane surface (Fig.1-3). The central part of the enzyme is a 45 to 50 Å transmembranal region. The enzyme consists of 28 α-helices per monomer. Monomers are separated into two regions M_1, containing subunits I and II, and M_2 encompassing most of the subunit III and the α- helix of subunit VIa. COX extends for 30 to 35 Å into the matrix (Tsukihara et al., 1995). Subunits I and III do not interact directly with each other but rather interact through direct contact with subunit II.

Seven of the nuclear encoded subunits are located in the transmembrane domain surrounding the subunit I and II core complex. The C-terminal part of COXII is on the cytosolic side of the IMM, attached to the surface of subunit I and contains the Cu center, Cu_A, which contains two copper ions. A magnesium (Mg^{2+}) ion is located between the C-terminal domain of subunit II and subunit I, but its function is not clear. Subunit VIb is part of the C domain (cytosolic domain of COX, located at the upper part of the molecule in Figure 1-3), and comprising most of the matrix domain are subunits Va and Vb. The remaining nuclear-encoded subunits are single transmembrane α-helices linked to the core complex. There is still no complete understanding of the function of these subunits, but it is thought that they could work as regulators of COX activity (Tsukihara et al., 1996; Capaldi 1996).

The redox centers involved in the electron transfer from cytochrome *c* to the active site are two hemes A (a and a_3). As mentioned before, subunit I contains two heme A molecules, including one at the binuclear center, heme A_3-Cu_B, where oxygen is reduced to water (Fig. 1-4). The C-terminal domain of subunit II contains the Cu_A site, the primary electron acceptor from cytochrome *c* (Taanman, 1997). Electrons flow from cytochrome *c* to Cu_A and then to heme A before going in to the heme A_3-Cu_B center (the oxygen binding site). The rate limiting step is the electron transfer from heme A to heme A_3, but why the electron transfer from Cu_A to heme A and then to heme A_3 is the preferred pathway, rather than from Cu_A directly to heme A_3, is not well understood (Capaldi, 1996).

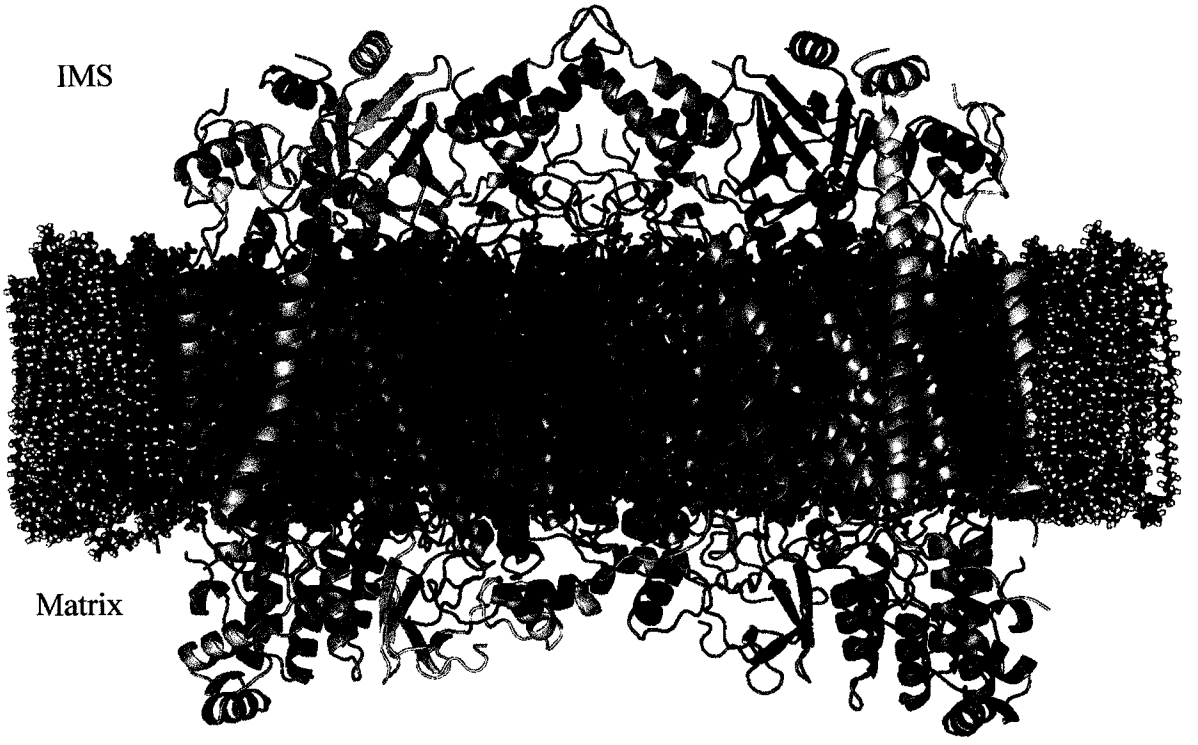


Figure 1-3. Crystal Structure of Bovine Heart Cytochrome *c* Oxidase in a Phospholipid Bilayer. (Image taken from Tsukihara et al., 1995).

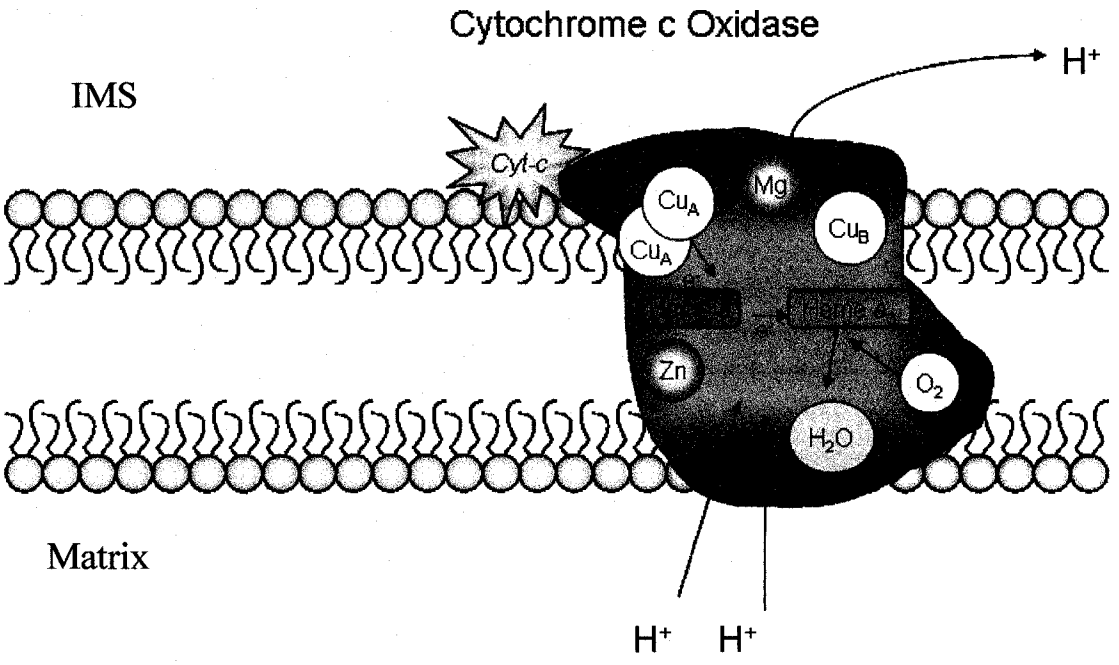


Figure 1-4. Representation of Complex IV Cytochrome *c* Oxidase. Detailed schematic showing the location of the copper centers, Mg and Zn ions as well as the hemes A and A_3.

The Assembly of Complex IV

COX formation requires many additional proteins, apart from the structural subunits, to facilitate the proper assembly of the enzyme. These nuclear-encoded assembly factors are necessary for formation and insertion of cofactors, also functioning as chaperones that facilitate delivery and stabilization of immature COX complexes (Khalimonchuk and Rodel, 2005). The assembly of COX therefore depends on the coordination of both nuclear- and mitochondrial-encoded proteins. Over 30 accessory proteins are thought to be necessary for COX assembly in yeast, and their functions can be classified in four main categories: assembly of COX subunits, formation and insertion of heme A, delivery and insertion of metal ions and maturation of the complex (Table 1-2).

pet mutants (resulting from mutations in *Petite* genes) can result from deficiencies in any of the protein complexes involved in oxidative phosphorylation, giving rise to a variety of phenotypes including COX deficiency. A large number of mutants with defects in COX have been identified in several different *pet* collections. These large numbers of mutants have shown that there are many assembly factors for COX. For a subset of these assembly factors, little is known about how they work. When mutated, these assembly factors do not show abnormalities in the expression of nuclear-or mitochondrial-encoded subunits, heme A biosynthesis or copper transport (Punter and Glerum, 2004). Such genes include the nuclear genes *PET117* and *PET191* (McEwen et al., 1993), *COX14,* which provides an important function at a late stage of COX assembly (Glerum et al., 1995), and *COX16,* another nuclear gene of unknown function (Carlson et al., 2003).

Pet100p is a protein thought to have a role as an assembly facilitator; it does not affect the localization of cytochrome *c* oxidase subunits to the inner mitochondrial membrane but instead functions after they have arrived at the inner membrane. Pet100p has been localized to the inner mitochondrial membrane, where it is present in a subassembly complex (Complex A) with cytochrome *c* oxidase subunits VII, VIIa, and VIII. Pet100p is thought to facilitate the interaction(s) between Complex A and other COX subassemblies and subunits (Church et al., 2005). Another gene, *SHY1* (*SURF1* homolog in yeast), encodes Shy1p and is thought to have a role in promoting the formation of an assembly intermediate in which Cox1 is one of the partners (Barrientos et al., 2002).

For the assembly of COX, the nuclear-encoded subunits need to be imported to the inside of the mitochondrion. The three core mitochondrially-encoded subunits need several processing steps. Cox2p is anchored to the inner membrane by two transmembrane helices so that the 15 N-terminal and 137 C-terminal residues protrude into the IMS and it requires two translocation events to extrude the residues to the IMM. Oxa1p and Cox18p are involved in this process. Yeast Cox2 precursor protein is synthesized and subsequently cleaved by a protease in the IMS. Cox20p facilitates this cleavage by supporting proteolysis (Carr and Winge, 2005). In mammals, Cox4p is found in an assembly intermediate, in association with COXI. The process of assembly is very complicated and the biosynthesis of COX is thought to occur mostly in the inner boundary of the IMM so the assembly point is located near the site where nuclear-encoded subunits are forced though the translocases of the outer and inner membranes (TOM/TIM) (Carr and Winge, 2005).

In the process of heme A formation and insertion, Cox10p (farnesyl transferase) first converts heme B to its intermediate heme O, (Nobrega et al., 1990; Mogi et al. 1994). The second step is the oxidation of the C8 pyrrole methyl moiety in two monooxygenase steps presumably catalyzed by Cox15p (putative heme A synthase), together with mitochondrial ferredoxin (Yah1p) and ferredoxin reductase (Arh1p) (Barros et al., 2001) (Fig. 1-5). The insertion of heme A into Cox1p has not yet been described (Carr and Winge, 2005).

Copper Acquisition in COX assembly

There are many accessory proteins that participate in the delivery of metal ions to COX. We are developing a better understanding of how copper is provided as discussed below, although little is currently known about how the Zn^{2+} and Mg^{2+} ions are inserted and delivered into COX. Although many proteins have been associated with copper transport, only three proteins have been directly implicated in copper delivery and insertion: Cox11p, Cox17p and Sco1p. Cox17p has been described as a copper shuttle, while Cox11p and Sco1p function downstream in the Cu insertion to Cox1p and Cox2p, respectively (Fig. 1-6). Yeast cells lacking Cox17p are respiratory deficient and not able to grow on non-fermentable carbon sources such as ethanol and glycerol (EG).

Assembly factor	Function	Localization	Human homolog	Reference
Translational regulators				
Pet309p	Cox1p translation	Matrix	LRPPRC	(Manthey and McEwen ,1995)
Mss51p	Regulation of Cox1p translation	Matrix		(Barrientos et al., 2004)
Cox14p	Regulation of Cox1p translation	IMM		(Barrientos et al., 2004)
Pet111p	Cox2p translation	Matrix		(Mulero et al., 1994)
Pet54p	Cox3p translation	Matrix		(Brown et al., 1994)
Pet122p	Cox3p translation	Matrix		(Brown et al., 1994)
Pet494p	Cox3p translation	Matrix		(Brown et al., 1994)
Membrane insertion				
Oxa1p	Insertion of charged domains	IMM	OXA1	(Hell et al.,1998)
Mba1p	Insertion of uncharged domains	IMM		(Ott et al., 2006)
Cox18p	Cox2p C-terminus insertion	IMM		(Saracco et al., 2002)
Mss2p	Cox2p C-terminus insertion	IMM		(Saracco et al., 2002)
Pnt1p	Cox2p C-terminus insertion	IMM		(Saracco et al., 2002)
Subunit specific chaperones				
Cox20p	Cox2p chaperone	IMM	COX20	(Hell et al., 2000)
Copper insertion				
Cox17p	Copper chaperone	Cytosol/IMS	COX17	(Glerum et al., 1996)
Cox11p	Copper insertion into Cox1p	IMM	COX11	(Carr and Winge, 2005)
Sco1p	Copper insertion into Cox2p	IMM	SCO1-SCO2	(Dickinson et al., 2000)
Heme biosynthesis				
Cox10p	Heme B to heme O conversion	IMM	COX10	(Tzagoloff et al., 1993)
Cox15p	Heme O to heme A conversion	IMM	COX15	(Barros et al., 2001)
Assembly chaperones				
Shy1p	Subcomplex assembly	IMM	SURF1	(Mashkevich et al.,1997)
Pet100p	Nuclear-encoded subunit assembly	IMM		(Church et al., 2005)
Unknown				
Cox19p	?	Cytosol/IMS	COX19	(Nobrega et al., 2002)
Cox23p	Copper chaperone?	Cytosol/IMS	COX23	(Barros et al., 2004)
Pet117p	?	?	?	(McEwen et al., 1993)
Pet191p	?	?	PET191	(McEwen et al., 1993)
Cox16p	?	IMM	COX16	(Carlson et al., 2003)

Table 1-2. Functional Classes of COX Assembly Factors (Zee and Glerum, 2006).

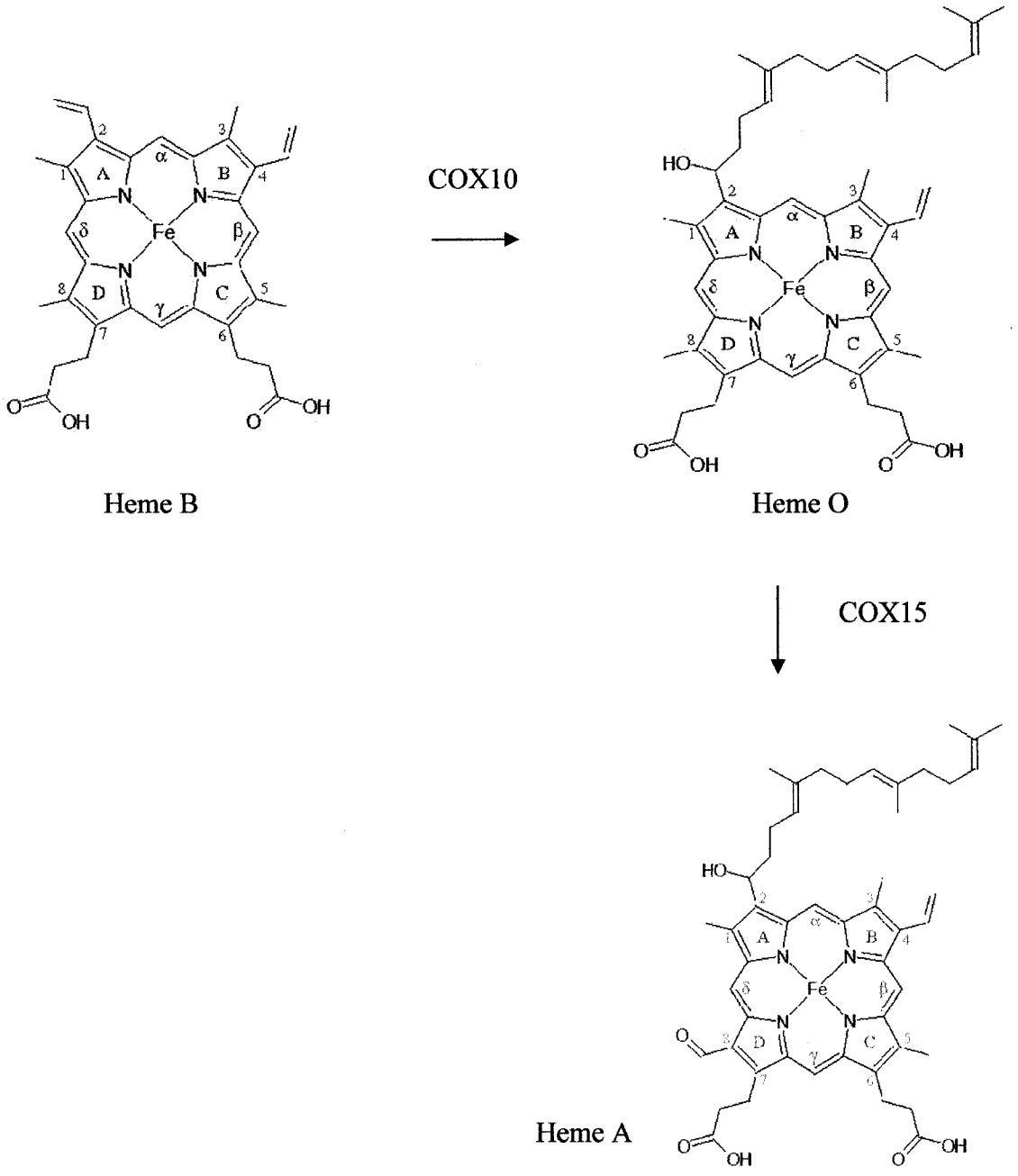


Figure 1-5. Heme A formation. Heme B is converted to the intermediate Heme O by Cox10p, which replaces the vinyl group of the porphyrin ring by a hydroxyethylfarnesyl chain. Heme A is produced by oxygenation of a methyl group of the porphyrin ring to a formyl group by Cox15p.

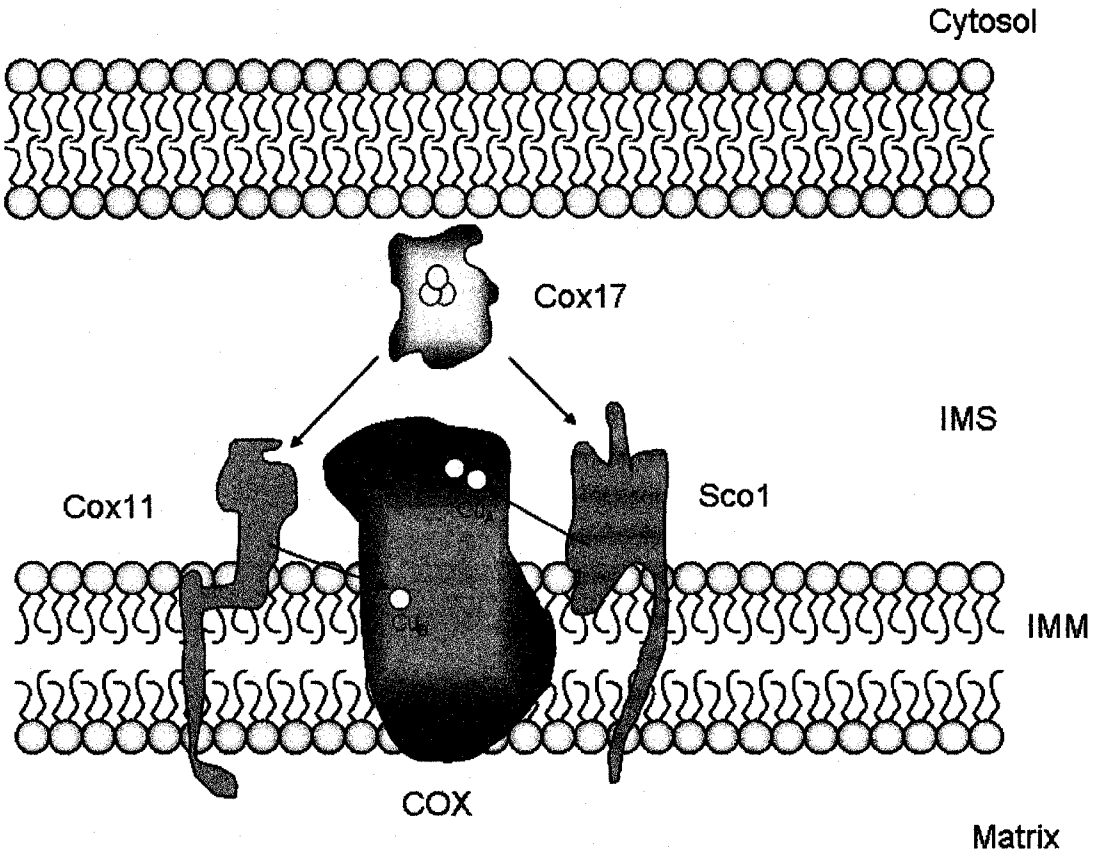


Figure 1-6. Scheme Representing Copper Transport to COX. Cox17p is capable of binding anywhere from zero up to four copper ions; it donates copper to Cox11p and Sco1p, which deliver copper to Cu_B (COXI) and Cu_A (COXII), respectively. The order of the copper delivery is unknown.

The respiratory phenotype of this knockout can be reversed by the addition of exogenous copper to the medium, which first led to the suggestion that Cox17p has a role in the delivery of copper to COX (Glerum et al., 1996; Punter and Glerum, 2003). Cox17p exists as a dimer/tetramer and has three conserved cysteines, two of them Cys(23) and Cys(26), are involved in copper binding at the CCxC motif (Abajian et al., 2004). Mutations in any of the three cysteines residues result in loss of function of the protein (Heaton et al., 2000). Cox17p has dual localization in the cytoplasm and the IMS, which led to the suggestion that Cox17p transports copper ions across the outer mitochondrial membrane (Beers et al., 1997). Although it is also possible that Cox17p may receive the copper within the IMS because *cox17*Δ cells are not deficient in mitochondrial Cu (Maxfield et al., 2004). Cox19p, a Cox17p homologue, is also important in COX assembly. It has a similar sequence, but without the copper-binding CCxC motif found in Cox17p, and it also has dual localization. Yeast *cox19*Δ cells are also respiratory deficient, but the phenotype can not be reversed by the addition of exogenous copper to the media, which suggests that Cox19 has a function in COX assembly that is independent of copper binding (Carr and Winge, 2005). Cox23p was recently identified as a soluble Cox17p-like protein, also localized in both the cytosol and the IMS. Although it does not share sequence similarities with Cox19p, it does share a similar sequence with the C-terminal portion of Cox17p. Yeast *cox23*Δ cells also show COX deficiency and mutants can be rescued by the addition of exogenous copper, but only together with the overexpression of *COX17* (Barros et al., 2004).

It was recently found that the yeast mitochondrial matrix has a copper pool that is distinct from the only two known mitochondrial copper-containing enzymes, Sod1p and COX. Most of the copper is found within the matrix as a soluble, anionic, low molecular weight complex. The mitochondrial copper pool was described as dynamic, because it responds to changes in the cytosolic copper level (Cobine et al., 2004). This copper pool could represent a storage source of copper available to an IMM transporter for copper delivery to the IMS, but the identity of such a transporter is still unclear.

Cox11p and Sco1p have been described as metallochaperones assisting the insertion of copper to COX. Cox17p has been shown to directly transfer copper to both Sco1p and Cox11p in *vitro* assays (Horng et al., 2004).The respiratory deficient phenotype of the *cox17-1* strain can be suppressed by Sco1p, or the homologous Sco2p, overexpression (Glerum et al., 1996). Sco2p is another member of the Sco family present in yeast, with 53% identity to Sco1p. The role of Sco2p is not clear. *SCO2* deletions do not affect the respiration phenotype and overexpression of *SCO2* does not substitute for the role of Sco1p, yet it can rescue the respiratory deficiency present in *cox17* mutants in the presence of higher concentrations of exogenous copper in the media (Glerum et al., 1996). Interestingly, a study suggested that Sco2p could have a role in the COX subunit stabilization; it was found that Sco2p is indispensable for the presence of residual levels of Cox2p in a *sco1*Δ strain. Most parts of Sco1p can be replaced by the homologous parts of Sco2p without loss of function. A short region of 13 amino acids, located adjacent to the transmembrane region, is crucial for Sco1p function and cannot be replaced by its Sco2p counterpart. It was proposed that this region is important for proper

spatial orientation of the protein. *In vitro* binding assays showed that Sco2p interacts with a portion of Cox2p, regardless of the presence or absence of Sco1p or the presence of bound copper ions (Lode et al., 2002).

In humans, mutations in *SCO2* are more common than in *SCO1*, and result in tissue-specific clinical features and selective degradation of COX mitochondrial subunits. It has been proposed that HsSCO1 and HsSCO2 have independent but cooperative functions in Cu delivery to COX (Leary et al., 2004). Mutations in either human *SCO* result in a cellular copper deficiency that is tissue- and allele-specific. The cellular copper deficiency phenotype can be suppressed by overexpression of *SCO2,* but not *SCO1* (Leary et al., 2007). Mutations in human *SCO2* were identified in infants with a fatal cardioencephalomyopathy and COX deficiency, with the enzymatic deficiency being ascribed to the loss of mtDNA-encoded COX subunits (Papadopoulou et al., 1999).

Cox11p is a 28 kDa copper-binding protein involved in the copper transfer pathway of COX that is highly conserved across eukaryotes and prokaryotes. It was first identified as a *PET* gene, on the basis of its homology to *ORF*3 in the *P. denitrificans* COX operon and its necessity for respiration and proper COX activity (Tzagoloff et al., 1990). Cox11p is associated with the IMM but is not a component of the purified cytochrome oxidase. Cox11p is similar in topology to Sco1p, with a single transmembrane helix downstream of the N-terminal mitochondrial target sequence. It has a soluble domain which contains an N-terminal fragment that anchors the protein to the membrane. In a study using the Cox11p homolog from *S. meliloti,* an anchor-free construct of 164 amino acids was used

to obtain an NMR structure, the first reported structure of this class of proteins (Banci et al., 2004). The copper binding motif is composed of two conserved cysteines, located on one side of the beta-barrel structure. When mutated at any of these Cys residues, the copper binding is reduced and the strains become respiratory deficient (Carr et al., 2002). In studies performed in *R. sphaeroides*, *cox11* null mutants lack Cu_B but not Cu_A. Additionally, the heme A_3, but not heme A, environment is disturbed. These findings lead to the conclusion that Cox11p is involved in the formation of the Cu_B site (Hiser et al., 2000; Khalimonchuk et al., 2005). Although Cox11p and Sco1p seem to have similar functions, there has been no interaction demonstrated between them. In a recent study of yeast Cox11p, random and site-directed mutants were tested in order to better understand the role of Cox11p. Some of the *cox11* mutants displayed very particular phenotypes such as a misassembled or partially assembled COX, with reduction in the levels of Cox1p. It was also shown that only *cox11* and *sco1* knockouts have a high sensitivity to an acute exposure to hydrogen peroxide (H_2O_2). These results suggest that Cox11p may have a role in the response to H_2O_2 exposure (Banting and Glerum, 2006). In addition, recent work in the Glerum Lab has identified differential phenotypes of *cox11* mutants that display respiratory competence and yet are sensitive to H_2O_2 exposure, as well as evidence of a peroxidase-like activity associated with Cox11p (Lari et al., manuscript in preparation).

Sco1p: Function and Structure

In the yeast *Saccaromyces cerevisiae,* copper is transported across the plasma membrane by Ctr1p and eventually transferred to small cytosolic copper-binding proteins such as

Cox17p. The binding of copper to its final acceptor protein requires specific mediator proteins (Yuan et al., 1997). Sco1p is a candidate mediator for copper attachment to COX. Sco1p (acronym for synthesis of cytochrome *c* oxidase) is a 28.7 kDa IMM protein with an amino acid sequence that is highly conserved among a wide variety of different organisms (Fig. 1-7). Sco1p is anchored to the inner mitochondrial membrane by a single trans-membrane segment of 17 amino acids. It has a copper binding domain, CxxxC, in the C-terminal portion of the protein, which is very similar to the Cox2p copper binding site (Glerum et al., 1996). Mutations in the conserved cysteines result in respiratory deficiency (Rentzsch et al., 1999). By using *in vitro* assays with purified proteins, it was demonstrated that Cox17p is a specific copper donor to both Sco1p and Cox11p (Horng et al., 2004). Immunoblot analysis using *sco1* mutants revealed a specific absence of subunit 2 from COX, suggesting that Sco1p is specifically involved in the formation of the Cu_A site in Cox2p (Dickinson and Glerum, 2000).

Based partially on the discovery of the structure of SCO1, another model for the role of Sco1p is its possible involvement in the reduction of the CxxxC cysteines in Cox2p, thereby allowing copper insertion. Analysis of the crystal structure of human SCO1 revealed similarity to the redox active proteins thioredoxins (Trx) and peroxiredoxins (Prx), which are oxidoreductases that can reduce a wide range of substrates, including hydrogen peroxide, several organic peroxides, and disulfide bonds in proteins and low molecular weight compounds (Figures 1-8 and 1-9) (Chinenov, 2000). Thioredoxin folds are common to enzymes that catalyze disulfide bond formation and isomerization. These proteins act as antioxidants by facilitating the reduction of other proteins by cysteine

thiol-disulfide exchange. Trxs share a common active site sequence, with two reactive cysteine residues: CXXC. Peroxiredoxins belong to the ubiquitous family of antioxidant enzymes that control cytokine-induced peroxide levels, which mediate signal transduction in mammalian cells. Prxs can be regulated by changes in phosphorylation, redox and possibly oligomerization states. The active-site cysteine is oxidized to a sulfenic acid by the peroxide substrate (Wood et al., 2003).

$$\text{Donor} + H_2O_2 \longrightarrow \text{Oxidized donor} + H_2O$$

(Equation 1)

The conserved catalytic residues in Trx and Prx share similarity with the proposed Sco1p copper-binding site. Surprisingly, there was no copper found in the hSCO1 crystal structure and the configuration of the residues implicated in copper binding was not optimal for trigonal Cu(I) binding. These unexpected findings suggested a potential role for Sco1p in oxidative stress and led to the discovery of a specific sensitivity to hydrogen peroxide exposure in the *sco1* null mutant, thus further supporting the possibility that Sco1p could have a role in peroxide sensing or mitochondrial redox signaling (Williams et al., 2005).

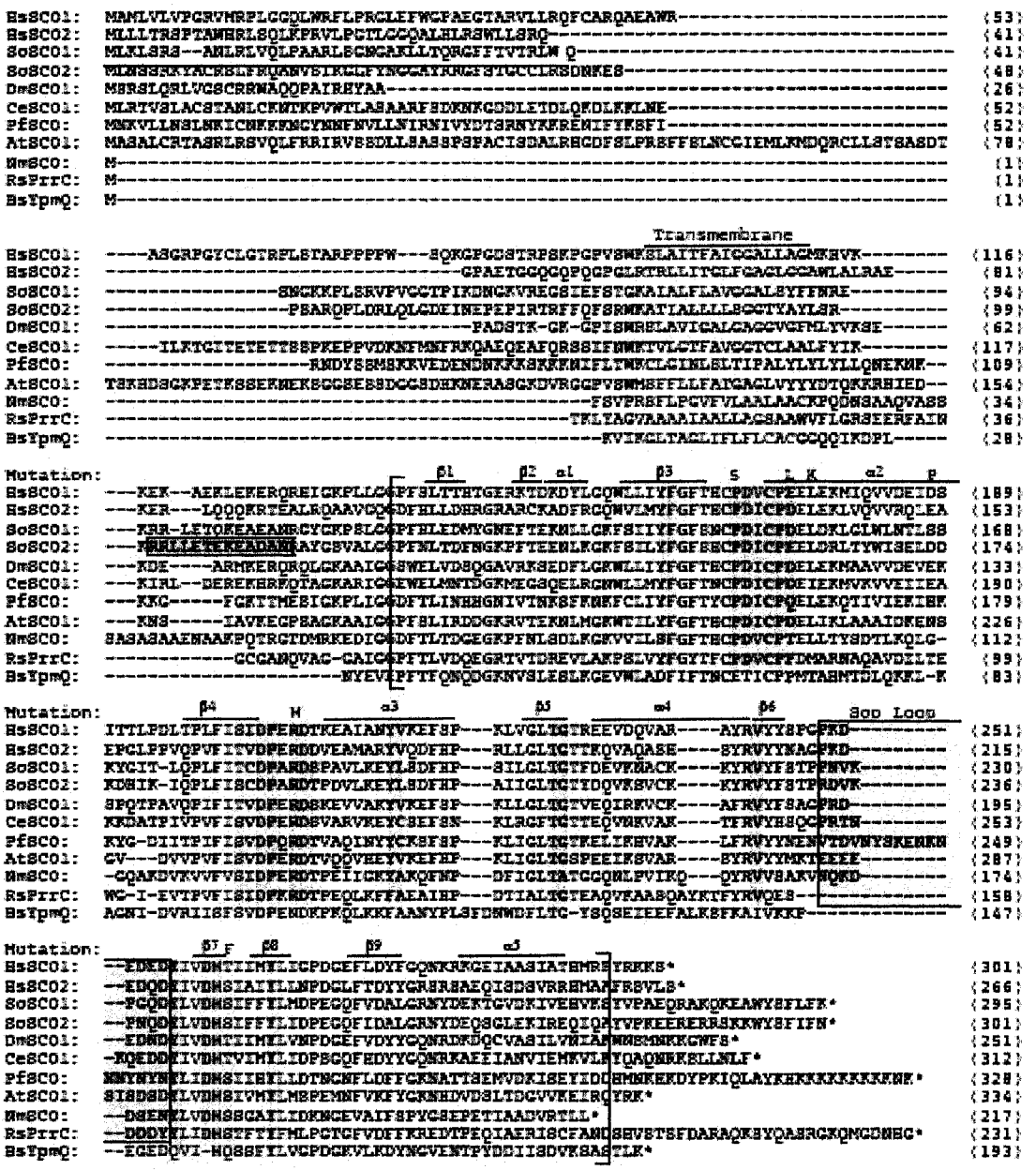


Figure 1-7. *SCO1* Sequences are Highly Conserved between Organisms. Transmembrane domain is shown in green, highly conserved amino acids are highlighted in yellow, Sco loop is shown in pink, α and β above the sequence indicates the helices and sheets of the structure, letters in red represent mutations that cause human COX deficiencies. (Human and yeast *SCO1* and *SCO2* are listed in the first four lines). Figure Taken from Williams et al., 2005.

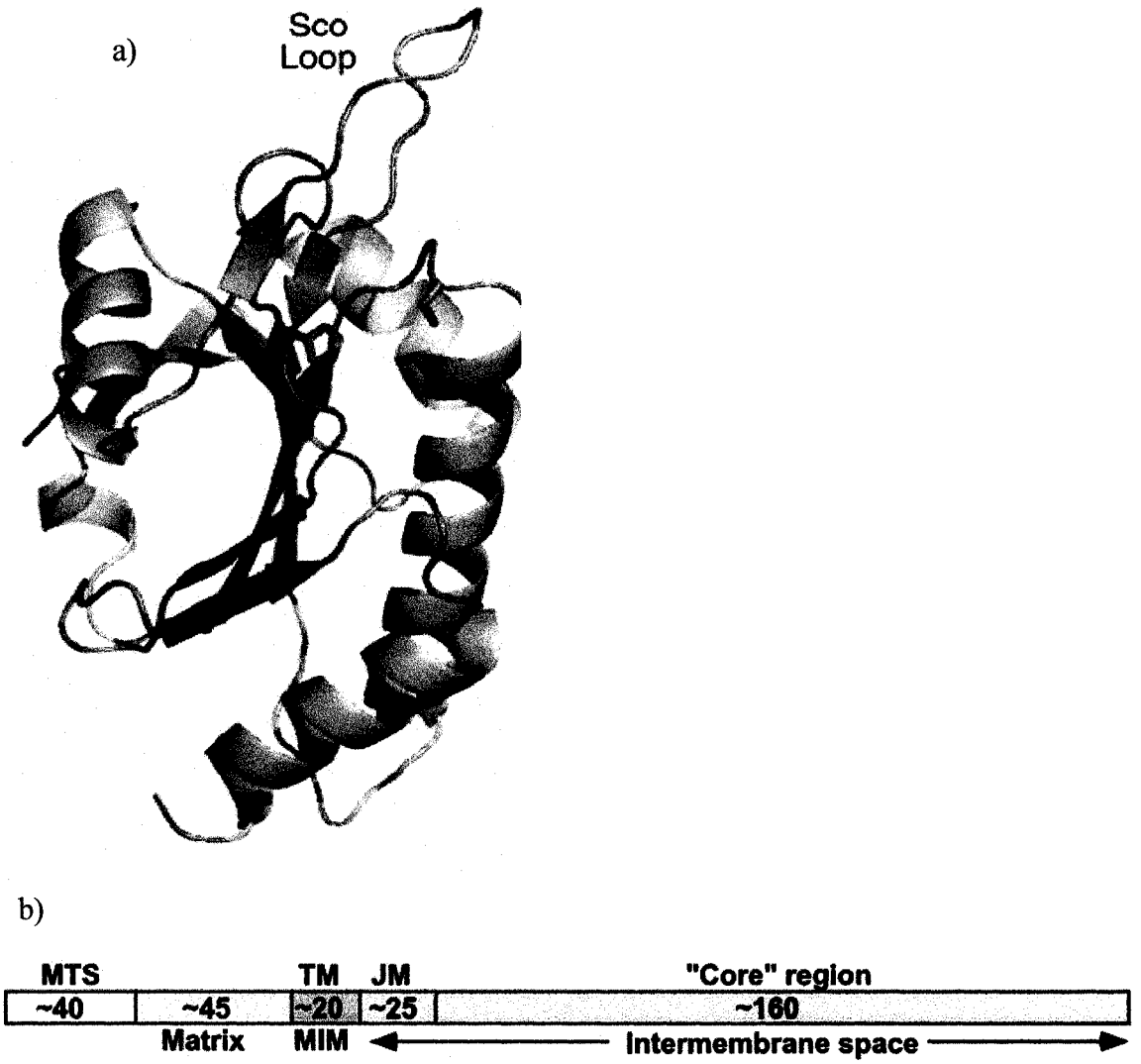


Figure 1-8. a) Ribbon Diagram of the Folded Core of hSco1. Helices are cyan and strands and loops are purple. b) hSCO1 MTS: mitochondrial targeting sequence, TM: transmembrane region, MIM: mitochondrial inner membrane, JM: "juxtamembrane" region. Numbers in the colored boxes represent amino acids (Williams et al., 2005).

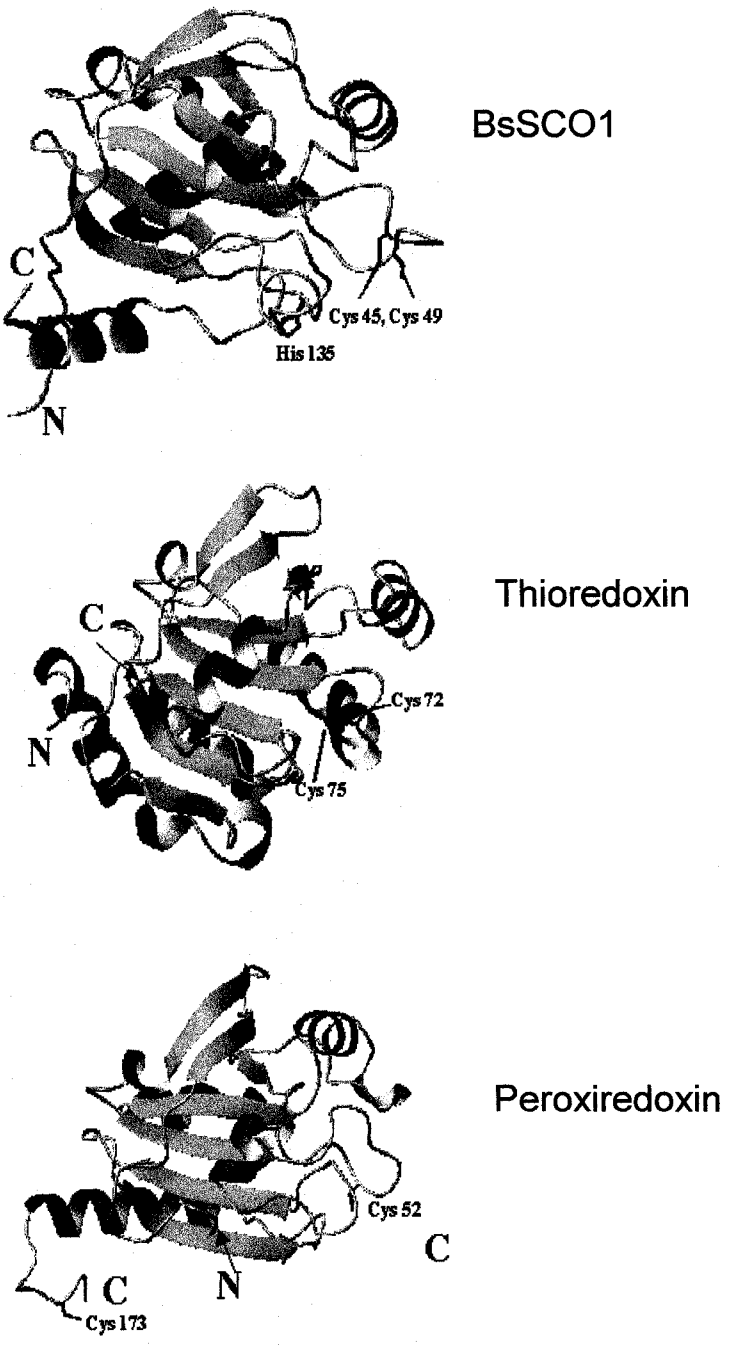


Figure 1-9. Similarities of the Structures of *Bacillus subtilis* Sco1, Thioredoxin and Peroxiredoxin. (Taken from Balatri et al., 2003).

In recent work, the crystal structure of yeast Sco1p in the presence and absence of copper ions (Cu-ySco1) was determined. The yeast Sco1p showed a fold very similar to that of thioredoxins, in concordance with what was seen with human SCO1. By soaking the Sco1p crystals in copper ions, a Cu-ySco1 structure was obtained, showing what the authors describe as an unexpected copper-binding site involving "other" cysteines: Cys181 and Cys216, which are present in yeast Sco1p but not in other Sco homologs (Abajian and Rosenzweig, 2006). There had been many previous attempts to obtain a crystal structure of Sco1p with copper bound, with no success. Given the methods used by Abajian et al., to obtain a Sco1p crystal bound with copper, it seems likely that this approach forced adventitious copper-binding at non-conserved cysteines.

Even though there is an increasing amount of data regarding the Sco1p role in COX assembly, its exact role is still not clear but it seems increasingly likely that Sco1p may play both a redox role (as a thiol-disulfide oxidoreductase) and a metal chaperone role (as a copper transferase).

Mitochondrial Diseases

Mitochondrial diseases are a clinically heterogeneous group of disorders that arise as a result of the dysfunction of mitochondrial enzymes and proteins, including those of the mitochondrial respiratory chain. They can be caused by mutations in nuclear DNA or mtDNA (Moslemi and Darin, 2007). Some mitochondrial disorders only affect a single organ, but many involve multiple organs and they may present at any age (Leonard and Schapira, 2000[1 and 2]). The affected individuals display a group of clinical features that fall into several definable clinical syndromes; however, clinical variability exists and many

individuals do not fit into one particular category (DiMauro and Schon, 2001). The clinical features of mitochondrial diseases include ptosis (drooping of the eye lids), external ophthalmoplegia (paralysis or weakness of the muscles that control eye movement), proximal myopathy (weakness of the muscles) and exercise intolerance, cardiomyopathy, deafness, optic atrophy, pigmentary retinopathy, and diabetes. Some central nervous system symptoms include encephalopathy, seizures, dementia, migraine, stroke-like episodes, ataxia, and spasticity (muscle overactivity) (Wallace, 1999).

Despite the small size of the mitochondrial genome, mtDNA mutations are a major cause of inherited diseases. In recent years, scientists have been able to understand basic mitochondrial genetics and the relationship between inherited mutations and disease phenotypes and progress has been made to identify acquired mtDNA mutations in both ageing and cancer. Until recently, mitochondrial diseases that are due to defects in mtDNA were considered to be very rare. It is difficult to estimate the true prevalence of mtDNA disease because of its very variable clinical presentations and the many causative mutations. The prevalence of mtDNA diseases fluctuates between populations, from 1 in 6,000 (on a single-point mutation in Finland) to 1 in 3,500 (British population) (Schaefer et al., 2004). These numbers are high, and could be higher if we take into account the recent apparent association of mtDNA mutations with common clinical features such as hypertension (Taylor and Turnbull, 2005).

Cytochrome *c* oxidase deficiency affects the terminal oxidation in respiration and blocks the energy flow pathway in the mitochondria, so there is no clinical presentation of a

complete COX deficiency, but there are many reports of partial COX deficiency. The wide variety of presentations of COX deficiencies can lead to congenital, late-onset, tissue-specific or multi-system disorders (DiMauro et al., 1990). Incorrectly assembled COX forms a significant subset of the mitochondrial diseases (Table 1-3). Leigh syndrome is the most common clinical presentation in COX deficiencies. It is a rare inherited neuro-metabolic disorder characterized by degeneration of the central nervous system and it usually affects infants and more rarely teenagers and adults (Lombes et al., 1991). COX deficiency resulting from nDNA mutations causes a variety of clinical features. COX deficiency-causing mutations have been identified in the following COX assembly factors: *SURF1*, *SCO1*, *SCO2*, *COX10* and *COX15* and LRPPRC.

A wide variety of mutations have been detected in the mtDNA encoded COX subunits (deletions, missense mutations, nonsense mutations and frameshift mutations). These disorders can have clinical features of weakness, exercise intolerance, hearing loss, seizures, ataxia, short stature, dementia, neuropathy, or strokes. Some mitochondrial disorders caused by deletions in mitochondrial DNA include chronic progressive external ophthalmoplegia (CPEO), Kearns-Sayre syndrome and mitochondrial encephalomyopathy with lactic acidosis and stroke-like episodes (MELAS). CPEO is a maternally-inherited disorder characterized by progressive external ophthalmoplegia, atypical pigmentary retinal degeneration, cardiac conduction block, dementia, ataxia, and short stature. Kearns-Sayre syndrome (KSS) is a rare neuromuscular disorder with onset usually before the age of 20. KSS is also characterized by eye pathologies, among a host of other symptoms (Filosto et al., 2007). MELAS is a condition that affects many of the

body's systems, particularly the brain and nervous system and muscles. The repeated stroke-like episodes can progressively damage the brain, leading to vision loss, problems with movement, and a loss of intellectual function. A variety of mtDNA mutations have been linked to (MELAS) (Filosto et al., 2007).

COX deficiencies of adolescent or adult onset are often associated with point mutations in tRNA genes in the mitochondrial DNA. These mutations are often associated with deficiencies of other enzymatic complexes as well, which also contain mtDNA-encoded subunits. Mutations in COXI have been found in patients with COX deficiency and motor neuron disease (Comi et al., 1998). Missense mutations in COXII and COXIII have also been found in patients with MELAS (Manfredi et al., 1995). Mutations in mtDNA accumulate over time and symptoms usually progress. Point mtDNA mutations often show maternal inheritance, while mtDNA rearrangements are sporadic. Another form of partial COX deficiency is presented in infants with mtDNA depletion, which is limited to skeletal muscle and liver or kidney (Taanman et al., 1997).

Gene	Clinical features	Reference
Mitochondrially encoded COX subunits		
COXI	Sideroplastic anemia Motor neuron-like degeneration Multisystemic disorder Myoglobinuria	(Gattermann et al., 1997; Comi et al., 1998; Bruno et al., 1999; Karadimas et al., 2000)
COXII	Encephalomyopathy Myopathy	(Clark et al., 1999; Rahman et al., 1999)
COXIII	MELAS Myoglobinuria Encephalomyopathy Leigh-like syndrome	(Manfredi et al., 1995; Keightley et al., 1996; Hanna et al., 1998; Tiranti et al., 2000)
Heme A biosynthesis		
COX10	Ataxia, tubulopathy	(Valnot et al., 2000[1])
COX15	Fatal infantile hypertrophic cardiomyopathy	
Copper metabolism		
SCO1	Hepatic failure and neonatal encephalopathy	(Valnot et al., 2000[2])
SCO2	Fatal infantile cardio-encephalomyopathy	(Papadopoulou et al., 1999)
COX assembly		
SURF1	Leigh's syndrome	(Tiranti et al., 1998)
LRPPRC	Leigh's syndrome French Canadian Type	(Mootha et al., 2003)

Table 1-3. Genetic and Clinical Heterogeneity with COX Deficiencies (modified from (Barrientos et al., 2002[2])

COX Deficiencies Due to Assembly Defects

Biochemical analyses of cultured fibroblasts from patients with COX defects revealed that most COX deficiencies arise from low enzyme stability and/or failure to complete assembly of the enzyme (Glerum et al., 1988). Using yeast as a model organism for understanding COX assembly, research has focused on the identification and characterization of homologous genes in yeast and humans, in order to correlate findings with yeast mutants to human COX deficiencies.

SURF1 is a gene that encodes a protein localized to the inner mitochondrial membrane and is thought to be involved in the biogenesis of the cytochrome *c* oxidase complex. The protein is a member of the *SURF* family, which includes the related yeast protein, Shy1p and the rickettsial protein Rp733p. Defects in this gene are a major cause of Leigh syndrome (Teraoka et al., 1999). The first nuclear gene mutation underlying human COX deficiency was described in this gene (Pequignot et al., 2001; Zhu et al., 1998). *SURF1* shares similarity with *SHY1*. Mutations in *SHY1* result in multiple phenotypic effects in yeast but *SURF1* mutations in humans are associated with an isolated COX deficiency. Analysis of human cell lines in experiments using blue native two-dimensional electrophoresis indicated that assembly of COX in *Surf1* null mutants is blocked at an early step (Tiranti et al., 1999). Patients with mutations in *SURF1* typically show a clinical presentation of Leigh syndrome, have no detectable SURF1 protein, a generalized COX deficiency and low levels of COX subunits.

As in yeast, a subset of human COX deficiencies is due to problems with mitochondrial copper transport. Failures in mitochondrial copper transport have been found to cause a number of different COX deficiencies and a variety of clinical symptoms. As discussed earlier, there are two *SCO* homologs (paralogs) in humans: *SCO1* and *SCO2*. Mutations in human *SCO2* have been found to underlie a subset of infantile cardiomyopathies: Q53X, E140K and S225F were all found in compound heterozygotes (two different mutant alleles at a particular gene locus, one on each chromosome of a pair) (Papadopoulou et al., 1999). In a study published by Dickinson *et al.*, the yeast *sco1* mutant E155K (analogous to E140K) was analyzed and was shown to be respiratory competent, while the S240F (analogous to S225F) (Fig. 1-10) mutant is respiratory deficient and showed partial assembly of COX, as the analysis of the COX spectrum revealed that the aa_3 peak is shifted to the blue (Dickinson and Glerum, 2000). The blue shift in the aa_3 peak is characteristic of hemes A and A_3 when they are present in a misfolded environment (Tzagoloff et al., 1974). When culturing human myoblasts from *SCO2*-deficient patients, supplementing the media with copper-histidine was found to lead to higher levels of COX activity, as well as increased levels of COXII. This finding suggested a possible therapy for the early treatment of this fatal infantile disease (Jaksch et al., 2001[1]). Mutations in *SCO2* can also cause hypertrophic cardiomyopathy (Jaksch et al., 2001[2]) and spinal muscle atrophy has been found in four cases of children with mutations in the *SCO2* gene (Tarnopolsky et al., 2004). In contrast, mutations in *SCO1* cause different clinical features and have been found in patients with neonatal onset hepatic failure and encephalomyopathy (Valnot et al., 2000[2]). The patients show COX deficiency in liver, skeletal muscle and lymphocytes, but not in the heart, suggesting that

there are tissue-specific consequences of the different *SCO* mutations. Although there are other very important proteins involved in copper transport to COX, no mutations in COX deficient patients have been reported in the *COX17* or *COX11* genes.

Some COX deficiencies are caused by defects in the biosynthesis of heme A. The human homolog for Cox10p is a IMM protein essential for the catalytic conversion of heme B to heme O. A missense mutation has been identified and associated with COX deficiency in a clinical presentation of tubulopathy and leukodystrophy. It is thought that this COX deficiency is caused by the lack of heme A incorporation into Cox1p (Valnot et al., 2000[1]). Cox15p is a membrane protein with seven or eight transmembrane domains. The yeast gene *COX15* has been shown to be essential for expression of COX (Glerum et al., 1997). Mutations in *COX15* in association with human COX deficiencies have been found in patients with an early-onset fatal hypertrophic cardiomyopathy. Overexpression of *COX15* has been shown to complement the isolated COX deficiency in fibroblasts from a patient with fatal, infantile hypertrophic cardiomyopathy. A missense mutation on one allele, resulting in an introduction of a premature stop codon, was also identified. COX assembly was reduced and mitochondrial heme A content was decreased while levels of heme O were increased in the patient's heart (Antonicka et al., 2003).

The gene defect underlying the French Canadian presentation of Leigh syndrome (LSFC) was found in the nuclear *LRPPRC* gene, which is ubiquitously expressed. *LRPPRC* mutations result in low levels of the product protein, resulting in reduced mRNA transcripts in the mitochondria. Like Pet309p, the *LRPPRC* product has a role in the

translation or stability of the mRNA for mitochondrially encoded COX subunits (Xu et al., 2004).

In this thesis, I present the results of experiments designed to test the hypothesis that Sco1p is a bifunctional protein, with both copper transfer and redox metabolism functions. Based on our findings that the absence of Sco1p confers sensitivity to hydrogen peroxide, we generated a quantitative strategy to analyze several *sco1* mutants in the presence of hydrogen peroxide. We also designed an experimental approach to study the proposed end-point of the copper transfer pathway of Sco1p, namely COX itself. This approach was selected to answer the question: what is the role of Sco1p in the COX assembly pathway? Since no one has been able to provide direct evidence of Sco1p as a copper chaperone, another question needs to be solved: Is Sco1p in fact delivering copper to the Cu_A site in COXII? This work has brought us closer to answering these questions and contribute to a better understanding of the role of Sco1p in the assembly pathway of COX.

Figure 1-10. *SCO* Alignment of Human and Yeast *SCO1* and *SCO2*. The transmembrane domain is highlighted in blue, the conserved cysteines of the copper-binding domain are highlighted in green, the *SCO* loop is highlighted in yellow, and the pink squares represent the sites of the *sco1* mutants Y142F, Y222F, R220A and S240F.

Chapter 2: Materials and Methods

Media

This study was carried out using yeast (S. *cerevisiae*) cultures. All media were prepared and autoclaved at least one day prior to use. Yeast was typically grown in rich media YPD plates (1% yeast extract, 2% peptone, 2% glucose, 2% agar). Minimal media (WO 0.067% nitrogen base lacking amino acids, 2% glucose, 2% agar) was supplemented with the necessary amino acids for phototrophic selection. Ethanol-glycerol (non-fermentable carbon source) medium (EG; 1% yeast extract, 1% peptone, 2% ethanol, 2% glycerol, 2% agar) was used to determine the respiratory phenotype of the different strains. Liquid GAL media (1% yeast extract, 1% peptone, 2% galactose) was used to grow yeast for mitochondrial isolation.

Strains

The work presented in this thesis can be divided into three main lines of investigation and main protocols: (1) hydrogen peroxide assays, (2) COX affinity column purification, and (3) mitoplast and inner mitochondrial membrane extraction.

1. For peroxide sensitivity assays, the following strains were used:
    - Wild-type strain, aW303 (*a ade2-1 his 3-11, 15 leu2-3, 112 trp1-1 ura 3-1*), respiratory competent (EG^+).
    - *sco1* knockout, aW303ΔSCO1(*a ade2-1 his 3-11, 15 leu2-3, 112 trp1-1 ura 3-, sco1::URA3*), respiration deficient (EG-).

- *sco1* knockout transformed with *SCO1* expressed from a plasmid, pSco1/ST33 aW303ΔSCO1 (*a ade2-1 his 3-11, 15 leu2-3, 112 trp1-1 ura 3-, sco1::URA3*)+ *pSco1/ST 33(TRP)*), respiratory competent (EG^+).

sco1 point mutants were generated previously by Dr. Moira Glerum and Dr. Graham Banting using site directed mutagenesis at highly conserved amino acids in the *SCO1* sequence:

- aW303ΔSCO1/pSco1/ST40, Y142F mutant in YCplac 22 (TRP, CEN), respiratory competent (EG^+).
- aW303ΔSCO1/pSco1/ST41, R220A mutant in YCplac 22 (TRP, CEN), respiratory competent (EG^+).
- aW303ΔSCO1/pSco1/ST42, Y222F mutant in YCplac 22 (TRP, CEN), respiratory competent (EG^+).
- aW303ΔSCO1/pSco1/ST16, S240F mutant in YCplac 22 (TRP, CEN), respiration deficient (EG^-).

2. For affinity column purification of COX, the following strains were used:

Biotinylated strains were generated by crossing aW303ΔSCO1 (HIS) with αW303ΔCox6 Int (URA/LEU), a strain expressing a biotinylated subunit 6, and this cross generated diploids on sporulation media, which is comprised of WO + potassium acetate (KAc) plus the necessary amino acids. Plates were incubated for seven to ten days on the bench. An inoculate of 1 ml of sterile distilled water with a loop full of cells in a 15 ml conical tube was incubated for 15 minutes at room temperature with 15 µl of glusulase, then 5 ml of water were added and the sample was centrifuged at 3,000 rpm. The pellet was washed

with 5 ml of water and resuspended in 1 ml of sterile water. A dilution of 1:100 was plated using 15 µl of suspended cells and tested for respiratory deficiency and auxotrophies. After isolation of spores, we generated both a and α colonies and they were tested by making small patches and crossing with the parental strains aCB11 and αKL14 (strains with no mitochondrial DNA). The obtained biotinylated strain was named aPRBio6.

- PRBio6-33: PRBio6 was transformed with pSco1/ST33, which contains the 1.9 kb *Eco*R1- HindIII fragment of wild- type *SCO1* cloned into YCplac 22 (TRP, CEN), respiratory competent (EG^+).
- PRBio6-35: PRBio6 was transformed with pSCO1/ST35, which consists of the 1.9 kb *Eco*R1- *Hind*III fragment containing the S240F mutation cloned into YCplac 22 (TRP, CEN), respiration deficient (EG^-).

A similar procedure was used to generate strains carrying the S240F mutation with a biotinylated subunit 5 (Elizabeth Dickinson, Glerum Lab). Briefly, aEKD1-33 was created by crossing aW303ΔSCO1(URA) with a wild-type strain with biotinylated subunit 5, αW303ΔCOX5Int-L (HIS, LEU), random spores were obtained and tested for respiratory deficiency and TRP and ADE auxotrophies.

- aEKD1-33 was created by transforming aEKD1 with pSCO1/ST33, which expresses 1-5 copies of wild-type *SCO1*, respiratory competent (EG^+).
- aEKD1-35 was created by transforming aEKD1 with pSCO1/ST35, which expresses 1-5 copies of the S240F *sco1* mutant, respiration deficient (EG^-).

3. For mitoplast and inner mitochondrial membrane extractions, the following strains were used (the origin and genotypes of most of the strains listed in this section are described above):
    - aW303
    - aW303ΔSCO1
    - aW303ΔCOX11 (*a ade2-1 his 3-11, 15 leu2-3, 112 trp1-1 ura 3-1, cox11::HIS3)*, respiratory deficient (EG^-).
    - aW303ΔSCO1/ ST 42
    - PRBio6-33
    - PRBio6-35

Biochemical Techniques

Hydrogen peroxide sensitivity assay

A small inoculate of yeast cells was grown overnight in a 50 ml conical tube, in 10 ml of YPD medium, incubating at 30°C with shaking at 225 rpm. The next day, 50 µl of the culture was diluted with 1 ml of PBS (140 mM NaCl, 10 mM NaPi, pH 7.5). Optical density (OD) was measured at 600 nm and cells were diluted to an OD of 0.1 in a final volume of 5 ml of PBS. Five aliquots of 1 ml each were placed in plastic tubes and 3% H_2O_2 was added to each tube to a final concentration of 0, 1, 2, 5, and 10 mM. Cultures were incubated for 30 minutes, shaking at 30°C. Cells were diluted with 1 ml of PBS and 1:100 dilutions were plated on YPD plates in duplicate. Plates were incubated at 30°C for two nights until colonies were visible. Colonies were counted and used to determine

percentage of cell survival in the presence of H_2O_2, comparing with the cells that were left untreated.

Yeast Mitochondrial Purification by Zymolyase

A small inoculate of yeast was incubated in 10 ml of liquid GAL medium, in a 50 ml conical tube, overnight at 30°C, with shaking at 225 rpm. The next day, 2 ml of the culture was added to 100 ml of liquid GAL medium in a 250 ml sterile glass flask and incubated overnight under the same conditions as for the 10 ml culture. Subsequently, 33 ml of the culture were made in 800 ml of GAL media in 2 L sterile glass flasks and incubated overnight in a bigger shaker, using the same growth conditions. The overnight culture was transferred to plastic Beckman centrifuge bottles and centrifuged for 10 minutes at 2,000 rpm at 4°C (Beckman J6-MI Centrifuge). After discarding the supernatant, cells were resuspended in 150 ml of 1.2 M sorbitol and transferred to 250 ml centrifuge tubes. Cells were rinsed twice by spinning at 6,000 rpm for 10 minutes (Sorvall RC 5B Plus, SLA-1500 rotor) and discarding the supernatant. At all times, the plastic bottles containing the cells were kept on ice buckets in between steps. After removing the supernatant, after the second wash cells were weighted in order to calculate the amount of digestion buffer required, according to the following chart:

2 M sorbitol	30 ml
NaPi pH 7.5	7.5 ml
0.5 M EDTA	0.1 ml
β-mercaptoethanol	0.5 ml
doubly-distilled water	12 ml
Zymolyase 20,000	22.5 mg

Cells were resuspended in 3 ml of digestion buffer per gram of wet weight of cells. Cells were incubated at 37°C until most cells were converted into spheroplasts (cell wall is absent or deficient, causing them to have a spherical form). Depending on the respiratory phenotype of the strains, cells were incubated for 60 to 90 minutes for respiration deficient or for 2 to 3 hours for respiratory competent strains. After incubation with digestion buffer, 1.2 M sorbitol was added to a final volume of 200 ml and the cells were centrifuged at 6,000 rpm for 10 min (at 4°C), repeating this step twice to wash the spheroplasts. Spheroplasts were resuspended in STE buffer (0.5 M sorbitol, 20 mM Tris pH 7.5, 0.5 M EDTA), using the same volume as the digestion buffer, and homogenized using a cold Waring blender for 20 seconds. 25 µl of PMSF were added at this point to prevent protein degradation, followed by spinning of the homogenate for 10 minutes at 2,500 rpm (4°C). Supernatant was collected for a second spin, to remove remaining cellular debris. The supernatant was then transferred to a fresh 40 ml Nalgene tube and centrifuged at 13,000 rpm in a SA-600 rotor for 15 minutes to sediment the mitochondria. A small aliquot of the supernatant was kept, which served as representative of the post-mitochondrial supernatant. The pellet was washed twice with STE buffer, followed by spinning at 12,000 rpm for 15 minutes. The final precipitate was resuspended in 1 ml of 20 mM Tris, followed by the addition of 30 µl of PMSF and kept at -80°C until needed.

Mitoplasts and Inner Mitochondrial Membrane Extraction

Using the same procedure used for mitochondrial extraction, the final pellet was washed with 20 mM HEPES, 0.6 M sorbitol pH 7.4, instead of STE buffer, and resuspended in a small volume of the same buffer in preparation for mitoplast isolation. After determining

the protein concentration by the Folin procedure, the sample was diluted with 7 volumes of 20 mM HEPES pH 7.4 and incubated on ice for 5 minutes. The diluted sample was centrifuged at 40,000 rpm in a TLA-110 rotor at 4°C for 20 minutes, in Beckman (13x51 mm) ultracentrifuge tubes. A small aliquot of mitoplasts was saved for future comparisons by Western blot. Supernatant fraction was discarded and the pellet was resuspended in the original volume of 20 mM HEPES pH 7.4 followed by a second protein determination by the Folin method. The sample of mitoplasts was diluted to 10 mg/ml and sonicated twice for 10 seconds, resting the tube on ice in between sonications. Sonicated samples were washed twice by spinning at 40,000 rpm for 30 minutes (4°C) and resuspended in the smallest volume possible of 20 mM HEPES pH 7.4, which thus contained a concentrated suspension of inner mitochondrial membrane. A third protein determination was made at the end of the procedure.

Folin Procedure for Protein Determination

This method was used to determine the protein concentrations of the isolated mitochondria, mitoplasts and inner mitochondrial membranes and is based on the Lowry protocol (Lowry et al., 1951). Using 1.5 x 15 cm test tubes, 5 µl of the sample was diluted with 600 µl of ddH_2O. All samples were tested in duplicate. Samples were incubated with 3 ml of copper reagent solution; which consists of: 0.5 ml of 1% $CuSO_4$, 0.5 ml of 2% 2% NaK tartrate and 49 ml of 2% Na_2CO_3 in 0.1 N NaOH.

After incubating for 10 minutes at room temperature, a volume of 0.3 ml of Folin reagent (1:1 dilution of stock) was added and the solution was mixed vigorously. After mixing the samples were placed at 90°C for two minutes and then cooled on ice to equilibrate to

room temperature. Absorbance was measured at 750 nm versus a reagent blank (using 600 µl of water or a specific buffer plus the rest of the cocktail), using a Shimadzu UV-1601PC spectrophotometer.

Western Blotting

Proteins were prepared at desired concentrations in loading buffer (4x stock: 0.19 M Tris-Cl, pH 6.8, 3.8% SDS, 38.5% glycerol, 3.85% β-mercaptoethanol, 0.02-0.05% bromophenol blue w/v). Proteins were separated by 12% SDS-PAGE (150 V for 75 minutes) using a running buffer of 25 mM Tris, 190 mM glycine, 1% SDS. When the run was completed (bromophenol dye reached the bottom of the gel), the proteins were transferred to a nitrocellulose membrane (using a Bio-Rad system) in cold transfer buffer (200 mM glycine, 25 mM Tris-Cl, 20% methanol) for 30 minutes at 100 V. After transfer was completed, proteins were observed by adding 15 ml of Ponceau red stain (2% Ponceau S, 30% trichloroacetic acid, 30% sulfosalicylic acid) and incubating for 30 seconds. The membrane was washed with water until bands were visible and the molecular standard bands were marked with a pen in order to have a reference of sizes on the developed film at the end. The membrane was incubated with a blocking solution of 3% skimmed milk powder (w/v) dissolved in rinse buffer (10 mM Tris-Cl pH 8.0, 1 mM EDTA, 150 mM NaCl, 1% TritonX-100) overnight at 4°C or at least 60 minutes at room temperature on a rocking plate. The membrane was washed three times for 10 minutes in rinse buffer and then incubated overnight on a rocking plate at 4°C with the primary antibody (Table 2-1) diluted in blocking buffer. The primary antibody was removed and the blot was washed three times for 10 minutes with rinse buffer.

The blot was incubated with the secondary antibody, diluted in blocking buffer, for at least 60 minutes with rocking at room temperature. The blot was washed three times for 10 minutes with rinse buffer followed by exposure to 1:1 (same volume of solution A plus solution B) with Western Chemiluminescent HRP Substrate (Millipore) detection reagents and exposed for 3 minutes. The blot was exposed to film (Super RX- Fujifilm) and developed with a Kodak processor (M35A X-OMAT).

Primary antibody (Molecular Probes)	**Dilution**	**Secondary antibody (BD Pharmingen)**	**Dilution**
Yeast CoxI	1:5,000	Anti-mouse	1:7,500
Yeast CoxII	1:5,000	Anti-mouse	1:7,500
Yeast CoxIII	1:5,000	Anti-mouse	1:7,500
Porin	1:10,000	Anti-mouse	1:7,500

Table 2-1. Antibody Concentrations for Western Blot Analysis of Proteins. Concentrations listed for Immobilion Western Chemiluminescent HRP Substrate (Millipore)

Spectral Analysis of Mitochondria

Isolated mitochondria (13 mg of protein) were solubilized in a buffer containing 1% deoxycholate (DOC), 50 mM Tris pH 8.0 and 80 mg of KCl, in a total volume of 2 ml, in a Beckman (13x52 mm) ultracentrifuge tube. The samples were centrifuged at 40,000 rpm for 20 minutes at 4°C in a TLA-110 rotor. The supernatant was transferred to a glass test tube and cholate was added to a final concentration of 1%. The solution was divided into two cuvettes (one milliliter each). Each cuvette was placed in the spectrophotometer rear and front holders, respectively. The cuvette placed in the rear holder was oxidized with potassium-ferricyanide (KFeCN) and the one placed in the front was reduced with sodium dithionite ($Na_2S_2O_4$). Reduced minus oxidized spectra were measured at 0.2 nm intervals from 650 to 450 nm.

COX Activity Assay

10 µl of isolated mitochondria were diluted with 10 µl of 20 mM Tris pH 7.5, (only if mitochondrial protein concentration was 10 mg/ml or more). A 10 µl portion of the previous dilution was mixed with 10 µl of 0.5% DOC. After mixing with the pipette tip, 5 µl of the sample were added to a cuvette containing 920 µl of 10 mM sodium phosphate (KPi) pH 7.0 and 80 µl of 1% reduced cytochrome *c* (with $Na_2S_2O_4$) and placed in the front cuvette holder. In the rear holder, a cuvette containing 920 µl of 10 mM KPi pH 7 and 80 µl of 1% oxidized cytochrome *c* (with KFeCN) was placed and the COX activity was measured for 2 minutes at 550 nm and reaction rates determined using the kinetics function of the UV probe software.

Column Purification of Biotinylated COX (Homemade Column)

The affinity of avidin for biotin provided the basis for purification of biotinylated COX from various yeast strains. One gram of cyanogen bromide-activated Sepharose (CNBr-sepharose) was hydrated with 20 ml of 1 mM HCl in a small beaker. In a 15 ml conical tube, 15 mg of tetrameric avidin was dissolved in 5 ml of Coupling buffer (0.1 mM $NaHCO_3$, pH 8.3, containing 0.5 mM NaCl) at a concentration of 3 mg/ml. The optical density (O.D.) was measured at 280 nm. The avidin solution was mixed with the swollen sepharose (2-3 ml), and incubated in a rocker at room temperature for two hours. The suspension was vacuum-filtered and the filtrate collected to measure OD_{280} nm to determine the coupling efficiency. The gel was filtered again and washed with five gel volumes of coupling buffer. The gel was incubated with 5 ml of Blocking agent (0.1 M Tris-Cl pH 8.0) for two hours and washed with 0.1 M Tris-Cl acetate buffer (18 ml 0.1M NaOAc, 82 ml 0.1 M HOAc, 0.5 M NaCl), pH 4.0. The gel was washed with five gel volumes of 0.1 M Tris-Cl pH 8.0, 0.5 M NaCl, followed by a rinse with five gel volumes of PBS buffer (0.1 M sodium phosphate, 0.15 M NaCl, pH 7.0).

At this point, one can continue with the protocol or stop and store at 4°C. The conversion of tetravalent to monovalent sepharose-coupled avidin was needed in order to facilitate elution of a native COX complex. A 5 ml column was created by placing a cotton wool plug in the bottom of a 5 ml syringe barrel, followed by Whatman filter paper cut to size. The column was placed in a universal support system and appropriate tubing connected the tip of the syringe to a peristaltic pump, set at a flow rate of 50 ml per hour. The monomerization process was carried out at room temperature. The tetrameric avidin gel

was poured into the column and sequentially washed with 5 column volumes of 6 mM guanidine hydrochloride (GnHCl) in 0.2 M KCl, pH 1.5, 5 column volumes of 10 mM NaPi, pH 7.0, five column volumes of 3 M guanidine thiocyanate (GnSCN) in 0.2 M KCl, pH 1.5, five column volumes of 10 mM NaPi, pH 7.0 and five column volumes of PBS. To block the non-exchangeable biotin binding sites, the column was charged with a solution of 2 mM biotin, pH 7.0 (in PBS) and was incubated for 30 minutes. Then the column was washed with four column volumes of 0.1 M glycine in 0.2 M KCl, pH 1.5 to remove free biotin from the gel. The gel was washed with six column volumes of PBS and stored at 4°C.

The small-scale adsorption of biotinylated proteins on monomeric avidin was accomplished as follows: A mitochondrial extract was obtained by mixing isolated mitochondria to a final concentration of 3 or 9 mg in 0.3 ml or 0.9 ml, with 1% DOC, 0.5% NaCl and the necessary amount of 20 mM Tris, pH 7.5 to reach desired volume. Mitochondrial extract sample was centrifuged at 4°C for 20 minutes at 40,000 rpm. The supernatant which contains the solubilized mitochondrial extract was collected and used in the following steps. A column with a 300 µl bed volume was made in a 1 ml syringe barrel and connected to a peristaltic pump and placed at 4°C. The column was equilibrated with six column volumes of PBS containing (0.05%, 0.1% or 1%) TritonX-100 (TX-100), and let set for 1 minute, then it ran at 14 ml/ hour, until the liquid had passed through. A 0.3 ml aliquot of isolated mitochondrial extract was run into the column and allowed to equilibrate for 15 minutes. A volume of 1.8 ml of PBS/TX-100 was added, and the column was run at a rate of 1.4 ml/h. The first fraction, corresponding

to the dead volume (430 µl) of the tubing, was discarded. The unbound fraction was collected in a volume of 900 µl. The column was washed with 1.8 ml of PBS/TX-100 and then chased with 300 µl of 2 mM biotin in PBS/TX-100, pH 7.0. The column was incubated for 15 minutes and then washed at 14 ml/hour with 2 mM biotin in PBS/TX-100 to collect the eluted protein (in the first 1.2 ml). The eluted fractions were collected in 300 µl aliquots, with 5 µl of PMSF (17.5 mg/ml stock). The column was regenerated by washing sequentially at 1 ml/minute with six column volumes of PBS, six column volumes of 0.1 M glycine in 0.2 M KCl, pH 1.5 and six volumes of PBS. The column was stored at 4°C and reused no more than five times. Samples were kept at -85°C .

In developing a protocol that would provide a sample suitable for EPR analysis, the buffers PBS or MOPS buffer (100 mM MOPS, 5 mM EDTA pH 7.0) were adjusted, as were the percentage of detergent TX-100 (0.05%, 0.1% and 1%), the amount of mitochondrial extract, the size of the column, flow rate, times of equilibration and the addition of an extra salt (NaCl) wash.

Column Purification of Biotinylated COX (Kit)

A Pierce ImmunoPure immobilized monomeric avidin gel kit (pre-packed column) was used with a settled gel volume of 2 ml (all steps were done at 4°C). The gravity-flow column was equilibrated with 8 ml of MOPS buffer (100 mM MOPS, 5 mM EDTA, 50 mM NaCl, 1% $C_{12}E_9$). To block non-reversible biotin binding sites, 6 ml of biotin blocking and elution buffer (2 mM biotin in MOPS buffer) were added to the column. The column was washed with 12 ml of regeneration buffer (0.1 M glycine pH 2.8). The

column was placed on top of a receptacle tube and the mitochondrial extract (2 ml final volume) was placed on the center of the column's disk platform. The flow was interrupted when the sample had passed through the column and the flow was stopped by placing the bottom cap, then the top cap to let incubate for 15 minutes. The top cap was removed first to avoid introducing air bubbles to the gel matrix, 2 ml of MOPS buffer was then added six times and six samples of unbound fraction were collected. OD was measured at 280 nm, using MOPS buffer as a blank, in order to monitor protein in each fraction. When absorbance values returned to baseline the non-bound protein had been removed. 2 ml of biotin blocking elution buffer (2 mM biotin in PBS/TX-100) was added to the column and the column was allowed to sit for 15 minutes. New collecting tubes were placed below the column and eluted biotinylated protein was collected in six fractions of 2 ml each, by adding 2 ml of elution buffer at a time. 5 µl PMSF (17.5 mg/ml stock) was added to all eluted fractions to avoid protein degradation. OD at 280 nm was measured to assure that all the protein was being eluted from the column. The column was regenerated by washing with 4 ml of regeneration buffer, followed by a wash with 5 ml of MOPS buffer. Caps were placed leaving some buffer above the column's disk. The column was stored at 4°C and reused four times. Samples were kept at -85°C.

Protein Concentration

After column purification, it was sometimes necessary to concentrate the protein to a smaller volume. This was accomplished by using Centricon devices. Solution containing eluate was added to the sample reservoir in a 2 ml volume and devices were sealed with the retention vial. The covered device was placed into the centrifuge and balanced with a

similar device. Spinning at 5,000 rpm (at 4°C) for 30 minutes or until reaching desired final volume, samples were concentrated to a final volume of approximately 150 µl. After re-suspending carefully with a pipette, devices were inverted and placed into the filtrate vial to collect the concentrated protein by a two minutes spin at 1,000 rpm at 4°C. Samples were pooled and kept at - 85°C.

Electron Paramagnetic Resonance (EPR)

Electron paramagnetic resonance (EPR) or electron spin resonance (ESR) is a technique used for the study of chemical species that have one or more unpaired electrons, such as organic and inorganic free radicals, and compounds which have a transition metal in their structure. Spectroscopy is a branch of science that enables us to gaze into the inner properties of molecules, to identify their component parts, follow their changing form, measure and recognize them. Electrons and the nuclei of many atoms behave like tiny magnets. When placed in a magnetic field, the atomic and electron magnets try to align themselves in the field. The measurement of this effect is called electron magnetic resonance or nuclear magnetic resonance, depending on whether the electron or the nucleus is being observed. For EPR the electrons behave as if they are spinning on an axis, and since they have positive and negative charges, this provides them with the property of a little magnetic field. The unpaired electron moves between the two energy levels by either absorbing or emitting electromagnetic radiation of energy, and it is this absorption which is monitored and converted into a spectrum (Nokhrin et al., 2005). EPR is a sensitive, specific method used for studying radicals formed in chemical reactions and identifying the reactions themselves. Organic and inorganic radicals can be detected

in electrochemical systems and in materials exposed to UV light. In medical and biological applications of EPR, the radicals are very reactive and normally do not occur in high concentrations. EPR is also used to detect metal centers, in our case, detecting copper in purified cytochrome *c* oxidase or in inner mitochondrial membranes isolated from yeast strains containing *sco1* and *cox11* mutations, and comparing the EPR spectra with the wild-type strain, aW303. Two approaches were followed to get EPR analysis of different *sco1* strains: One using purified COX and the second using isolated inner mitochondrial membranes. EPR analysis was performed by Dr. Richard Rothery, Department of Biochemistry, University of Alberta.

Chapter 3: Results

sco1 Mutants Show Two Distinctive and Separable Phenotypes: Respiration Deficiency and Hydrogen Peroxide Sensitivity.

As mentioned in the Introduction, the role of Sco1p is still under investigation and two roles have been proposed for this protein: a role as a copper chaperone and a role as a redox sensing protein. Based on the similarities that Sco1p shares with the Trx and Prx families of proteins, and on the finding that the yeast strain, aW303ΔSCO1 showed a high sensitivity to H_2O_2, we wanted to ask whether different *sco1* mutants displayed differential sensitivities to H_2O_2. Site directed mutagenesis was used to generate three *sco1* mutants, two of them in suggested phosphorylation sites (Y142F and Y222F), and one in a hydrogen bonding site (R220A). Phosphorylation is a common mechanism, by which many enzymes are switched on and become activated, and hydrogen bonding is responsible for the secondary, tertiary, and quaternary structures of proteins and nucleic acids; therefore we thought mutations in these sites would generate important changes in the protein. Control strains were also tested, along with the point mutants: (aW303, aW303Δ*SCO1* and aW303Δ*SCO1*+p*SCO1*). Wild-type (aW303) was selected as a control because it is respiratory competent (EG^+), it has the highest level of COX activity, a normal COX spectrum and high steady state levels of the three core COX subunits. The knockout strain (aW303ΔSCO1), was chosen as a negative control: it is respiration deficient (EG^-), it shows no COX activity, abnormal COX spectrum (lacking heme A peak) and low or no expression of COX core subunits. The strain aW303Δ*SCO1*+p*SCO1* was chosen as a positive control, to show that by the addition of the p*SCO1* plasmid into the knockout, the wild-type phenotype was rescued. All strains were tested for their respiratory phenotype by growing a culture of cells on YPD plates,

after two nights of growth, the plates were replicated to EG plates (Fig. 3-1) using sterilized velvets as copy stencils for the impression of yeast colonies. The colonies printed in the velvet were transferred to supplemented WO plates as exact copies of the original plate. Following one night of incubation at 30°C, the following growth phenotypes were observed (Table 3-1):

Strain	**Growth on ethanol-glycerol medium (EG)**
aw303	+
aw303Δ*SCO1*	-
aw303Δ*SCO1*+ p*SCO1*	+
aw303Δ*SCO1* (Y142F)	+
aw303Δ*SCO* (R220A)	+
aw303Δ*SCO1* (Y222F)	+

Table 3-1. Growth of *sco1* Mutants and *SCO1* Wild-type Strains on Non-Fermentable Carbon Sources. Symbols: (+) means the strains grow on ethanol-glycerol medium, (-) means the strains do not grow on ethanol-glycerol medium.

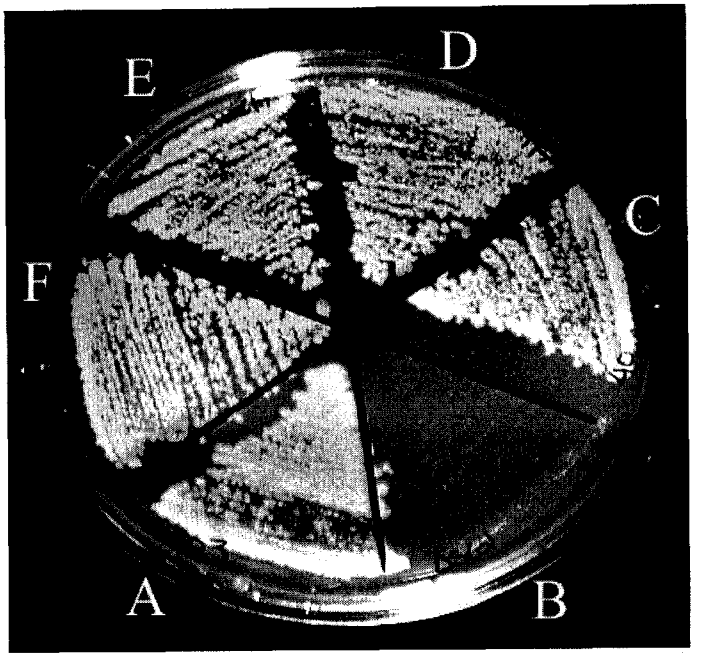


Figure 3-1. Respiratory Phenotype of *SCO1* Strains. An ethanol/glycerol (EG) plate showing growth of *sco1* mutant strains after one night of incubation at 30°C. (A -aW303, C -aW303Δ*SCO1* (Y142F), D -aW303Δ*SCO1* (R220A), E- aW303Δ*SCO1* (Y222F) and F- aW303Δ*SCO1*+p*SCO1* are all respiratory competent (EG^+), while B- aW303Δ*SCO1* is respiration deficient (EG^-) and fails to grow.

It was very interesting to observe that the three *sco1* mutants are able to grow on EG over-night, just like the wild-type strain. We also tested the respiration phenotype of the *sco1* mutant S240F and as described before, it was respiration deficient (EG^-).

The surprising finding that none of our three mutants appeared to have any defect in COX assembly prompted us to investigate whether any of the characteristics of *sco1* mutants could be identified in these strains. COX assembly mutants are typically EG^-, have a shifted COX spectrum or absence of the heme A peak, have very low levels of COX activity and display a loss or very low expression of COXII. The strains were subjected to Western blotting analysis (Fig. 3-2) to analyze the steady state levels of the three core COX subunits (COXI, COXII and COXIII). 13 μg of isolated mitochondria from the control strains, as well as the mutants (Y142F, R220A and Y222F), were loaded onto a 12% SDS polyacrylamide gel. The *sco1* mutants showed reduced levels of the three COX subunits, with a more obvious decrease in COXI and COXIII. COXII antibody has always displayed a stronger signal compared to the other COX subunits, but still there is a visible reduction of expression when compared to the wild-type, aW303 (lane 1) and the knockout transformed with p*Sco1* (lane 3). Surprisingly, in contrast to what has been described before for *sco1* mutants, our mutants show a dramatic decrease in COXI and COXIII expression. Although COXII expression is lower than in the wild-type, it is not completely missing. This result can be attributed to the fact that COXII antibody is the strongest of the three and the film had to be developed for one or two seconds only. Had the film been developed longer for the other two antibodies, the signal would have been stronger too. Although two Western blots were done, clearly this needs to be tested a few more times to have a definitive result.

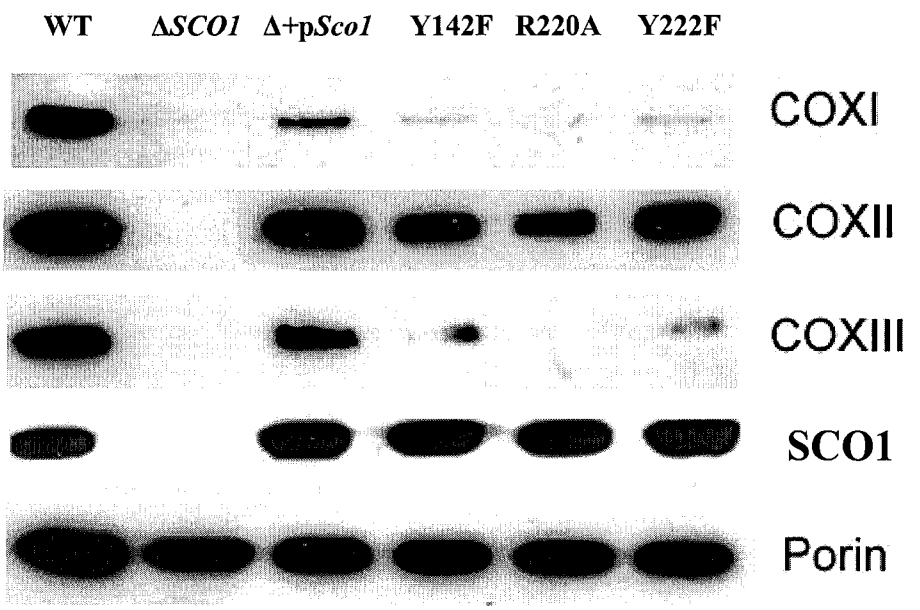


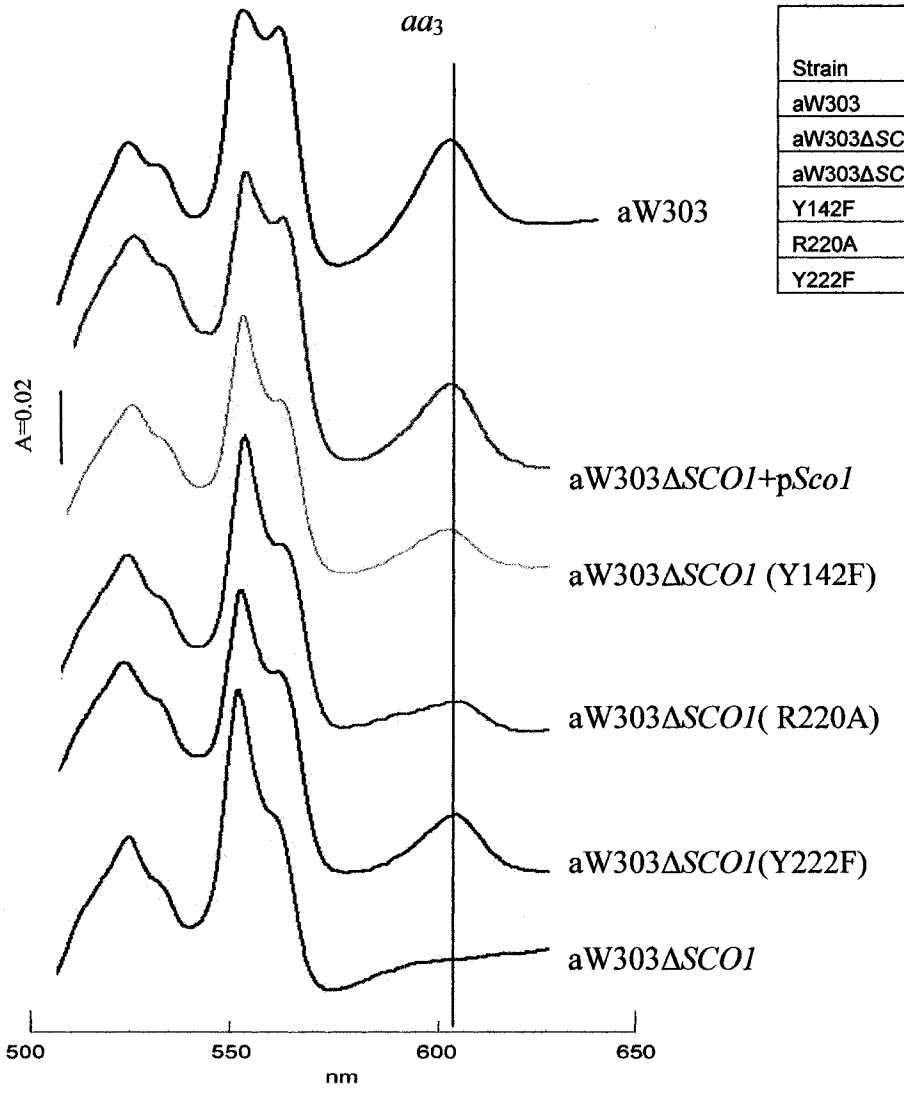
Figure 3-2. Steady state level of COX Subunits in *sco1* Strains-1. Western blot illustrating the steady state levels of the COX catalytic subunits in *sco1* mutants, loading 13μg of WT- aW303, aW303Δ*SCO1*, aW303Δ*SCO1*+pSco1, aW303Δ*SCO1* (Y142F), aW303 Δ*SCO1* (R220A) and aW303Δ*SCO1* (Y222F) probed with monoclonal antibodies against COXI, COXII and COXIII. Anti-porin antibody was used as a loading control (detects outer mitochondrial membrane). This result represents one of two experiments.

Spectral analysis (Fig. 3-3) and COX activity (Fig. 3-4) were also tested to further investigate the status of COX assembly of the different strains. As described above, generally *sco1* mutants display a characteristic COX spectrum. The wild-type strain has a normal spectrum; it displays a typical high and clear peak representing heme A (603 nm). In contrast, the knockout strain has no heme A peak. The *sco1* mutants display a smaller heme A peak and it also seems shifted (note the differences in wavelengths shown on the chart in figure 3-3). Shifting either to the blue or red in this peak is related to misassembly or partial assembly of COX. This finding is interesting because it is telling us that the *sco1* mutants have partial COX assembly, and this is consistent to the levels of COX subunits we observed in the Western blot; but somehow, the levels of COX assembly, and expression of COX subunits is enough to allow them to use EG as a non fermentable carbon source medium.

Given these somewhat contradictory findings, I next tested the COX activity in *sco1* strains and compared to the results to those obtained with wild-type, knockout, and knockout transformed with p*Sco1*. Following the protocol described in Materials and Methods, COX activity was assessed (Fig. 3-4) Taking the wild-type activity as 100%, calculations were made for the rest of the strains. The knockout strain shows almost no COX activity (2.40%) and the knockout when transformed with p*Sco1* has 82.3% activity. The Y142F mutant shows 40.7 % activity, the R220A 36.6% and the Y222F 75.9%. This result is important because the mutants have intermediate COX activity values, which is not very common in mutant strains. The result is consistent with the fact that they are EG^+, they have loss of expression of the core COX subunits, and the shifted

heme A peak in COX spectral analysis. Looking again at the COX spectrum (Fig. 3-3) of the three mutants, we can correlate the higher heme A peak of one of the *sco1* mutants, Y222F, which is also the one with the higher COX activity.

Having done the past biochemical tests we decided to test the sensitivity of these *sco1* point mutants to H_2O_2, the strains were treated with different concentrations of H_2O_2 as described in the Materials and Methods section. Data was collected from three independent experiments and standard deviations were calculated (seen as error bars in Fig. 3-5).



Strain	aa_3 peak wavelength (nm)
aW303	602.8
aW303Δ*SCO1*	
aW303Δ*SCO1+pSco1*	602.8
Y142F	601.6
R220A	602.8
Y222F	602.4

Figure 3-3. Reduced Minus Oxidized Spectral Analysis of Mitochondrial Cytochromes in *sco1* Mutants and Controls. Cytochromes were extracted from 13 mg of isolated mitochondria. Position of the aa_3 is marked with a line to highlight the shifts that are present in *sco1* mutants (wavelength values of aa_3 peak are shown in the chart), suggesting a missasembly of COX.

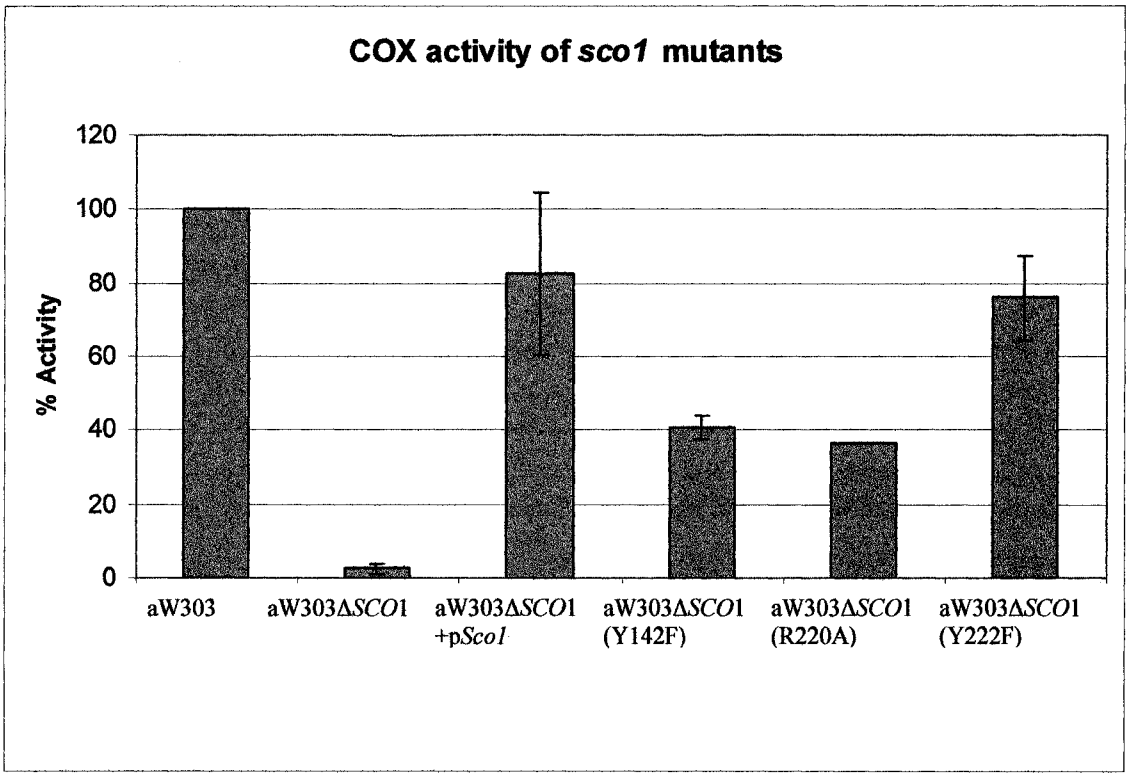


Figure 3-4. COX Activity of *sco1* Strains expressed as a Percentage of Wild-type COX Activity. The three *sco1* mutants show an intermediate level of COX activity. Data collected from three independent experiments, error bars represent the standard deviation.

Strain	COX activity expressed in $\mu mol/min^{-1}/mg^{-1}$	COX activity expressed in %	Standard deviation of the average
aW303	3.3	100 %	± 0.0
aW303Δ*sco1*	0.14	2.40 %	± 1.30
aW303Δ*sco1*+p*SCO1*	3.7	82.3 %	± 22.0
aW303Δ*sco1*/ST 40 (Y142F)	1.2	40.7 %	± 3.0
aW303Δ*sco1*/ST 41 (R220A)	1.2	36.6 %	± 0.08
aW303Δ*sco1*/ST 42 (Y222F)	3.0	75.9 %	± 11.4

Table 3-2. Levels of COX Activity of *sco1* Mutants. Values are expressed in $\mu mol/min^{-1}/mg^{-1}$ and in % of activity compared to the wild-type strain. COX activity and percentages were calculated from the average taken from three independent experiments.

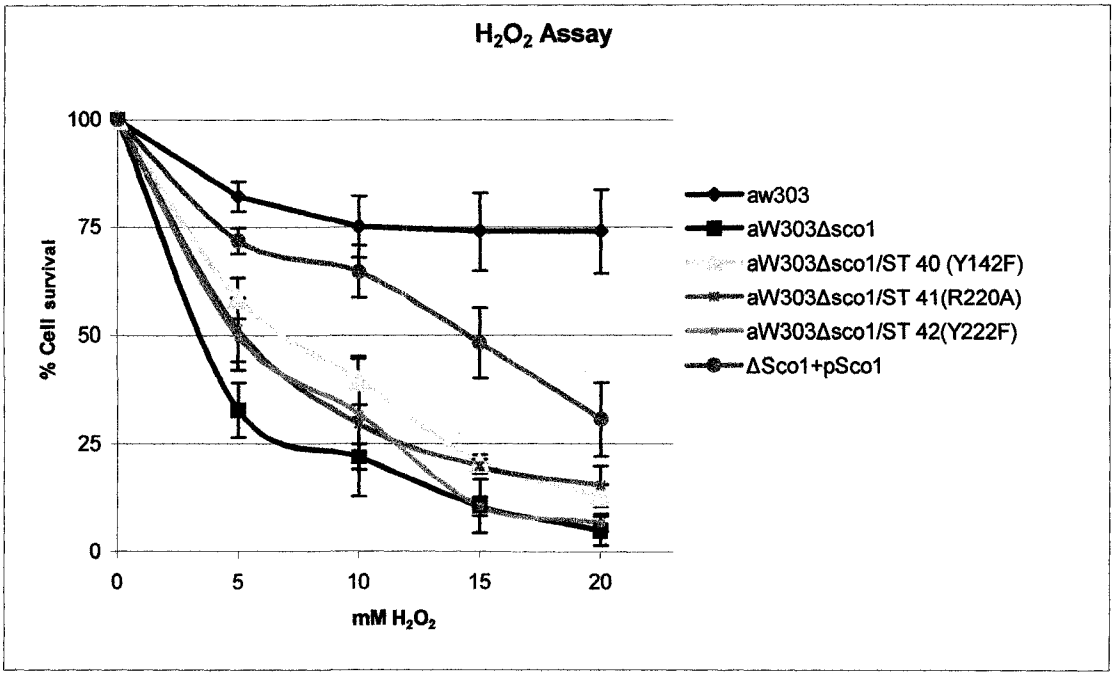


Figure 3-5. *sco1* Mutants show Sensitivity to H_2O_2. Strains were incubated with H_2O_2 for 30 minutes and cell survival was measured after two days of incubation at 30°C. The *sco1* mutants show similar survival to that of the knockout strain.

Strain	EG	H_2O_2 resistance	% Cell survival at 10 mM H_2O_2
aW303	+	++	75%
aw303Δ*SCO1*	-	--	22%
aw303Δ *SCO1*+p*Sco1*	+	++	65%
aw303Δ *SCO1* (S240F)	-	+/-	48%
aw303Δ *SCO1*/ST40 (Y142F)	+	+/-	40%
aw303Δ *SCO1*/ST41 (R220A)	+	+/-	29%
aw303Δ *SCO1*/ST42 (Y222F)	+	+/-	31%

Table 3-3. Summary of Respiration Phenotype, H_2O_2 Resistance and % Cell Survival at 10 mM H_2O_2 of *sco1* Mutants. Respiration phenotype is described as their ability to use ethanol/glycerol (EG) as a non-fermentable carbon source. H_2O_2 resistance is given as (++), the strain is resistant; (--), sensitive; and (+/-), partially resistant. Percentages are expressed as the average taken from three independent experiments; each experiment was made by duplicate.

The three *sco1* mutants studied show two distinguishable phenotypes. The mutants are respiratory proficient, although they also have only an intermediate level of COX activity. The chart below (Table 3-2) indicates the specific activity of COX found in these strains. The level of activity in the knockout strain is almost zero, while, when transformed with p*SCO1*, the activity of the enzyme is partially restored. COX activities in the *sco1* mutants fall between 1.19 and 3 $\mu mol/min^{-1}/mg^{-1}$. The other phenotype these *sco1* mutants showed is their partial sensitivity to H_2O_2. At 10 mM H_2O_2, the wild-type strain demonstrates 75% survival (Table 3-3), while the knockout strain, in contrast, demonstrates only 22% survival and can therefore be described as being highly sensitive to hydrogen peroxide. The knockout transformed with the *SCO1* plasmid shows 65% survival to H_2O_2 damage. Interestingly, the three *sco1* point mutants show high sensitivity to H_2O_2; aW303Δ*sco1* Y142F is 40% resistant, aW303Δ*sco1* R220A is 29% resistant and aW303Δ*sco1* Y222F is 31% resistant. Thus, with respect to H_2O_2 sensitivity, these *sco1* mutants behave more like the knockout strain even though they are respiratory competent, contrasting with the knockout which is respiration deficient. These results suggest that Sco1p has an additional role beside that of conferring proper COX assembly by functioning as a copper transporter, namely a role perhaps as a peroxide sensing molecule. This is the first time that the H_2O_2 sensitivity of *sco1* mutants has been demonstrated in a quantitative manner. These results also demonstrate that these two functions are separable and distinct.

The S240F *sco1* mutant was shown to allow a partial but incorrect assembly of COX in previous work in our lab. Given that characterization of this mutant had first led to the

proposal that Sco1p acted exclusively on the Cu_A site, we wanted to analyze the impact of the S240F mutation on the ability of the strain to resist a challenge with hydrogen peroxide (up to 10 mM) (Fig. 3-6). S240F also shows partial sensitivity to H_2O_2. In this test, we decided to treat the cells with lower concentrations of hydrogen peroxide, which seemed more physiologically relevant.

Hydrogen peroxide is a harmful byproduct of many metabolic processes and to prevent damage to the cell, it must be quickly converted into other, less dangerous substances. Catalase is used by cells to catalyze the decomposition of hydrogen peroxide into less reactive gaseous oxygen and water molecules. Cytosolic catalase T (Ctt1p) is known to perform this protective role to shield yeast cells from H_2O_2 damage. The *sco1* mutants were transformed with the gene encoding cytosolic catalase T (*CTT1*), which was isolated as a multi-copy suppressor of the *cox11* and *sco1* knockouts (G. Banting, unpublished results). The three *sco1* mutants transformed with *CTT1* show a small amount of protection from damage by H_2O_2, which supports the theory that Sco1p has a role either in protecting from damage by H_2O_2 or in redox sensing (Fig. 3-7).

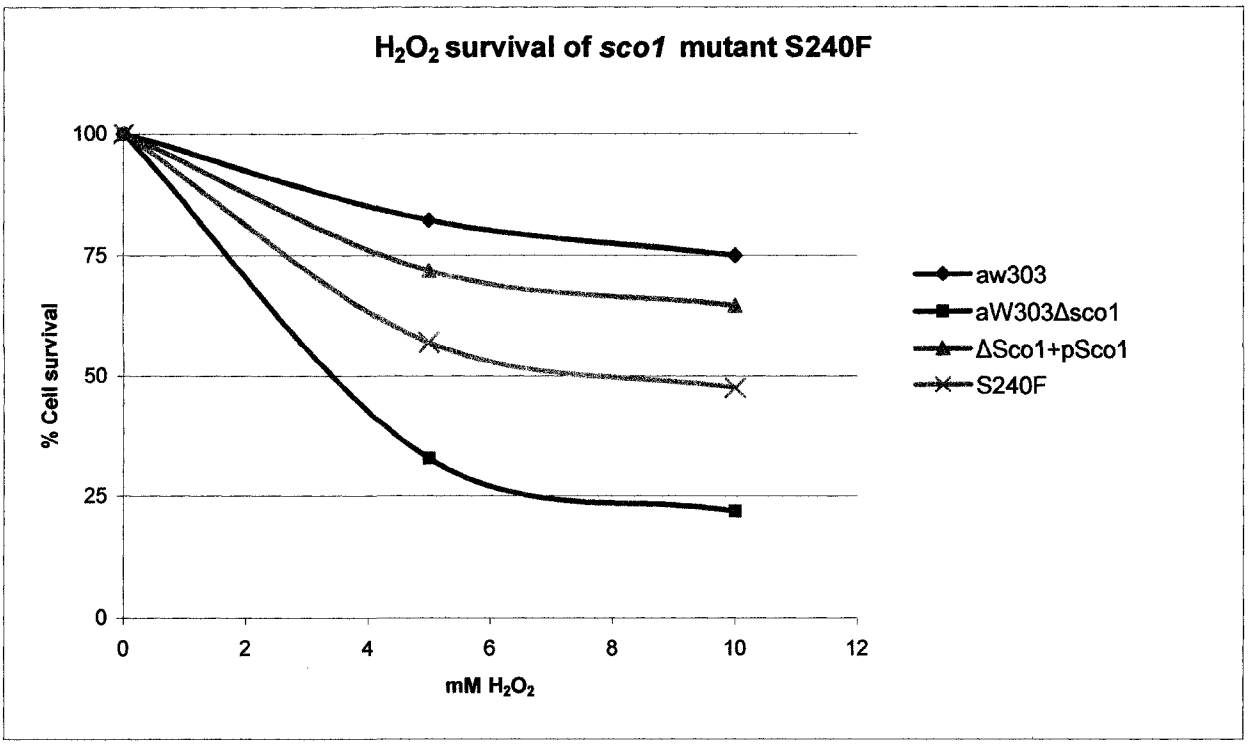


Fig 3-6. *sco1* Mutant S240F Shows a Partial Sensitivity to H_2O_2. This graphic shows results under treatment of 0, 5 and 10 mM H_2O_2 only. H_2O_2 assay for the *sco1* strain S240F, was performed only once.

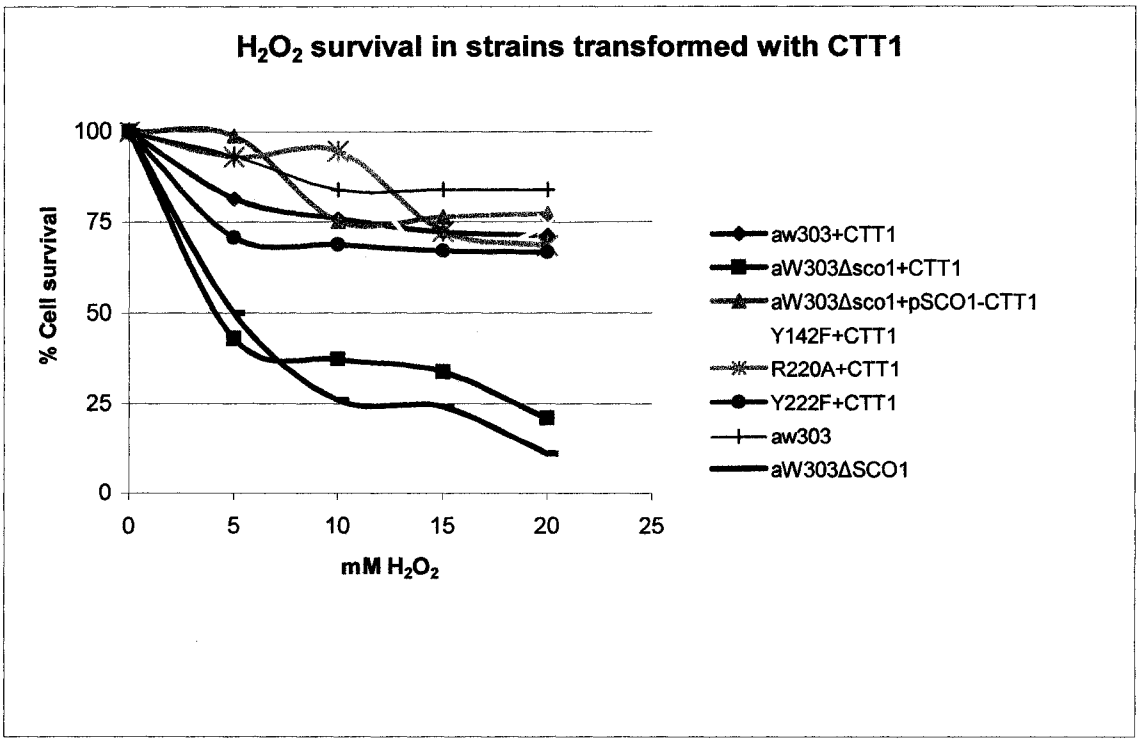


Figure 3-7. *sco1* Mutants Show some Protection from H_2O_2 Sensitivity when Transformed with *CTT1*. These results were taken from a single experiment made by duplicate.

Developing COX Purification Approaches for EPR Analysis of COX Metal Centers

Membrane proteins are known to present significant challenges in purification for a number of reasons: membrane proteins are insoluble, there are several steps involved in the process, resulting in significant manipulation of the proteins, the yield is generally low, cost is high, biological activity must be maintained, purity level must be high, it requires polishing steps (such as addition of salt washes and concentration of the eluted protein), it is time consuming (from initial mitochondrial isolation, to analysis of the eluted protein by Western blotting takes approximately two weeks) and detergent solubilization represents a risk while permeabilizing the membranes, because high concentrations could degrade the protein. In the course of my research in the laboratory, I optimized a technique for affinity column purification of COX in yeast strains expressing a biotinylated COX subunit. The initial attempts involved a sepharose-coupled avidin column that was generated as described in the Materials and Methods section. My initial purification experiments were carried out using several strains expressing a biotinylated subunit five (Cox5p-Bio), which were generated previously in the Glerum lab (EKD1-33 and EKD1-35). The EKD1-33 was chosen for column purification due to its similar phenotype to the wild-type strain and it was used in our lab for a few attempts of column purification in the past. We decided to take this strain and start the column purification with what was available. In the beginning, the size of the column and the amount of mitochondrial extract was changed after observing that most of the protein was not binding to the column. Thinking that the column might have been overcharged with protein, we decided to load a less concentrated extract on a bigger column, which led to a more efficient binding of COX (Fig. 3-8). Western blot analysis after a 0.3 ml column

purification of aEKD1-33 (3 mg extract), run with 0.1% PBS/TX-100, was carried out using COXII antibody. Lanes represent the extract, unbound and elutions 1-4, and in E1 and E2, the eluted protein is marked with an arrow. It was difficult to estimate the concentration of the eluted protein, because the sample was diluted in four fractions of 300 µl each, so the amount of protein could not be determined by the Folin method. Attempts were made to read absorbance of the eluted fraction at 280 nm, however the Triton used in the buffer caused interference at this wavelength. For EPR analysis, MOPS buffer would be ideal and we would need a sample of at least 15 µM COX (a total of 2.6 µg of COX), in 1 ml of buffer, for reliable EPR analysis. For those two reasons, we decided to increase the size of the column to a 0.9 ml column. We also changed the concentration of the detergent (1% TX-100) to improve the permeabilization of the membranes and get more eluted fraction. The buffer was exchanged to MOPS by using PD-10 desalting columns, but the process requires elution with 3.5 ml of buffer, ending up with 2.5 ml of each eluted fraction. Although this step was successful in exchanging the buffers, the samples needed to be diluted, therefore we decided to run a column using MOPS buffer from the start. MOPS buffer is more appropriate for the EPR analysis because its pH is more stable when freezing the samples. Using 100 mM MOPS to replace the 0.1 M PBS, in the running and elution buffers, we compared the COX purification side-by-side, by Western blot analysis of the relevant fractions (Fig. 3-9), and observed that the elution pattern was practically not affected by these adjustments.

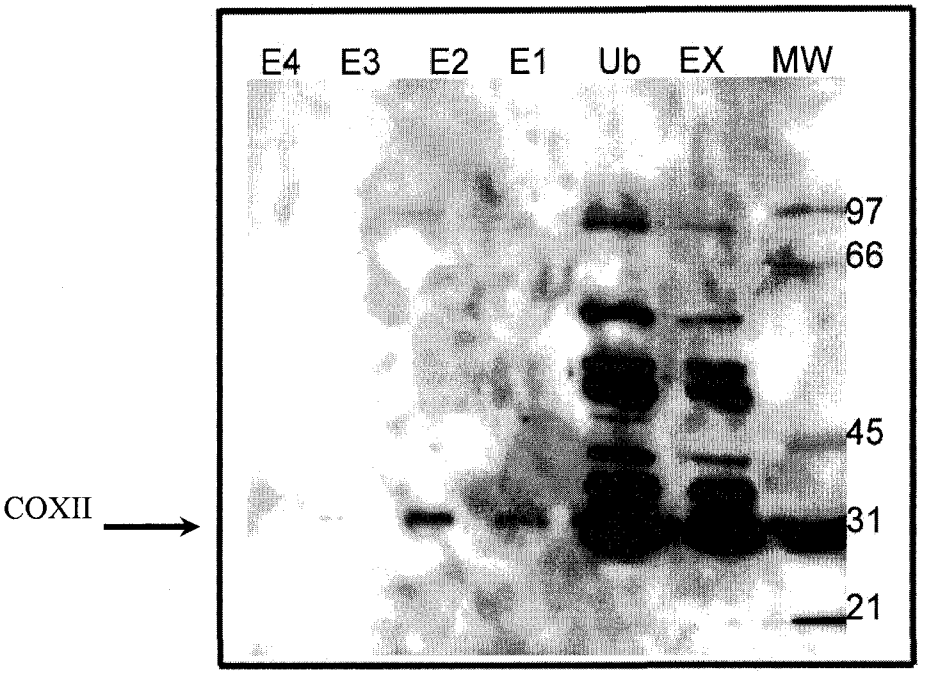


Figure 3-8. COX Purification of a Biotinylated Strain. A 0.3 ml column purification using 3 mg of mitochondrial extract of aEKD1-33 using 0.1% of TritonX-100. Lanes show molecular marker (MW), extract (EX), unbound (Ub) and elutions 1 to 4 (E1-E4). This Western blot was probed with antibody to COXII.

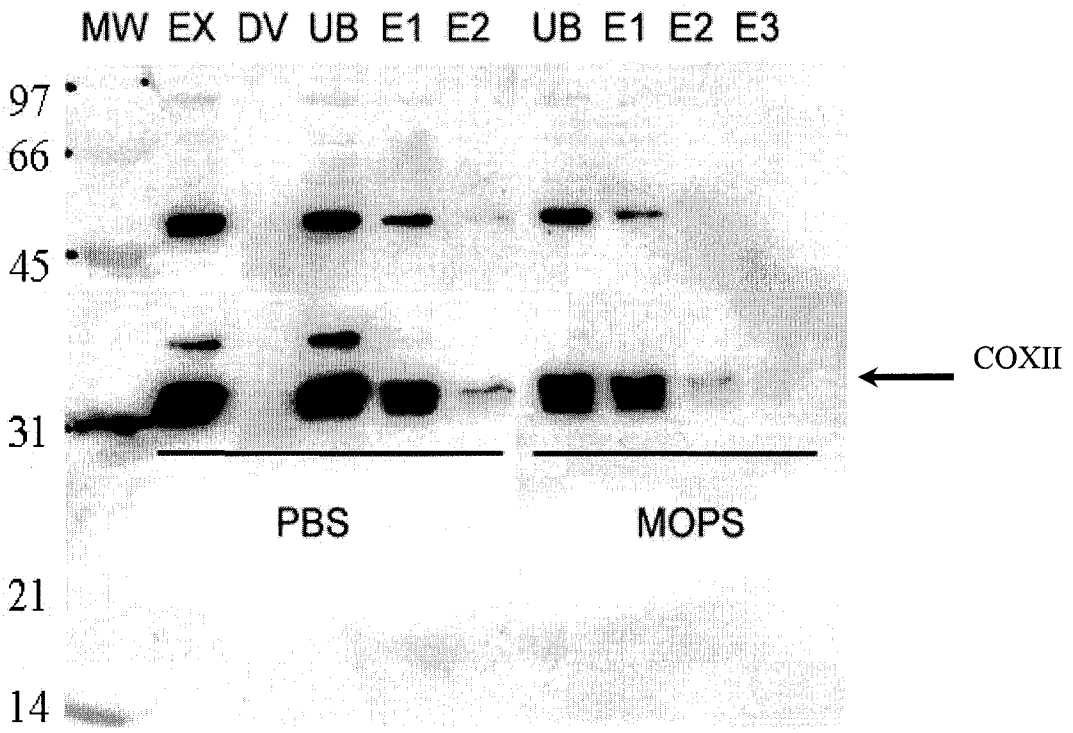


Figure 3-9. Comparison of COX Purification of Biotinylated Strain Using PBS and MOPS Buffer. A 0.9 ml column purification of aEKD1-33 PBS and MOPS. Lanes show abbreviations for molecular weight marker (MW), extract (EX), dead volume (DV), unbound (UB) and eluted fractions (E1 and E2). This Western blot was probed with COXII antibody.

Because it is difficult to know the effect of the biotin on the structure of the protein, we decided to generate another set of strains containing the biotin on subunit six (Cox6p-Bio) and then compare the column purification results. In order to assess subunit composition in these new strains, the steady state level of expression of the three core COX subunits was tested by Western blot (Fig. 3-10). At first glance, the PRBio6 strains seemed like good candidates for the column purification because these strains appeared to have a good level of expression of COXI, II and III and also showed a normal COX spectrum and high COX activity (data not shown). The use of PRBio6-33 strain in the column purification was successful, as we observed a better binding and a larger amount of COX in the eluted fractions in our experiments. To reduce the non-specific binding, we decided to incorporate an additional salt wash step in the protocol. The result was a cleaner band in the elution shown by Western blot, less nonspecific binding and more eluted protein (Fig. 3-11). For this specific blot the COXI antibody was used, because its signal is cleaner and less intense than the COXII signal, although to monitor the elution of purified COX, antibodies to any of the three mitochondrially encoded COX subunits could be used.

As mentioned before, one of the challenges of purifying a membrane protein is the final yield of the eluted protein. In order to address this issue, from a practical point of view, we decided to incorporate a concentration step after the column purification. The eluted fractions were pooled into one sample and placed in Centricon devices. These devices allow concentration of the protein by filtering the sample while they are centrifuged, as described in Materials and Methods. The method was successful and more concentrated

samples were obtained. However, in spite of repeated efforts, I was unable to scale up the protocol without loosing a large percentage of eluted protein and we therefore decided to try the 2 ml ImmunoPure immobilized monomeric avidin pre-packed column kit available from Pierce. With this column, we were able to load a bigger mitochondrial extract (20 mg). The column was run as described in Materials and Methods, using gravity flow and reading the OD at 280 nm to monitor the eluted fractions. After the column purification, a small aliquot of eluted protein was kept before proceeding with the concentration process using the Centricon devices. Western blot analysis and Ponceau staining of membranes was carried out in order to monitor the amount of protein present in the elutions (Fig. 3-12). The Ponceau stained membrane shows the band pattern of proteins from a PRBio6-33 column purification, before and after concentrating the samples. The improvement in the yield of eluted protein after the concentration process is noticeable when stained with Ponceau red (Fig. 3-12), but the eluted fraction appeared to be not quite pure, judging by the number of bands still present after purification. The increased yield of eluted fraction is even more noticeable in the developed film from the membrane probed with COXII antibody (Fig. 3-13). After making all the before mentioned improvements to the technique, I was successful in obtaining a sample of virtually purified COX from PRbio6-33 (2.45 mg) and PRbio6-35 (1.27 mg) for EPR analysis.

Inner Mitochondrial Membranes for EPR Analysis

Analyzing inner mitochondrial membranes for EPR is the most common approach to study metal centers in bacterial membranes, therefore we decided to take an alternative

approach to get EPR analysis without having to go through as many steps and manipulations. Several strains were used to isolate mitoplasts (mitochondria that have had their outer membrane removed by osmotic shock) and mitochondrial inner membranes (separating them by sonication and centrifugation), as described in the Materials and Methods section. Several strains were used in this experiment: the wild-type, aW303, was used as a control strain because we know it is completely functional and copper should be found at the Cu_A site in this strain. PRBio6-33 was also used as a control as it has the wild-type *SCO1*on CEN plasmid, along with the PRBio6-35 (S240F mutant) strain, because these are the strains used in the affinity column purification and we can correlate the results of both approaches in the future. PRBio6-35 is one of the most important strains to test, because it has the *sco1* mutant S240F, which has been shown to cause COX deficiency and we wanted to determine if this strain has copper at the Cu_A site. The knockout strain, aW303Δ*SCO1,* was used as a negative control as it is COX deficient and we should expect no copper in the structure. The mutant, aW303Δ*SCO1* (Y222F), was chosen for inclusion as it is one of the *sco1* mutants that show the two distinctive and separable phenotypes (respiratory competence and partial peroxide sensitivity) we could assess whether the copper binding is affected or not. Finally the aW303Δ*COX11* strain was selected because it is proposed that Cox11p delivers copper to the Cu_B site and thus removing Cox11p eliminates the presence of copper in the Cu_B site, but not in the Cu_A site. The starting yield of isolated mitochondria was around 50 mg/ml. After isolation of mitoplasts, the final IMM samples had a protein concentration ranging from 9 to 28 mg/ml, suspended in HEPES buffer. We used Western blot analysis and stained it with Ponceau red staining. This staining detects

protein and it allowed us to monitor the isolation of inner mitochondrial membranes, although the number of bands between lanes remained stable (Fig. 3-14). In spite of using the most common protocol for generating mitoplasts, when we analyzed our samples, probing for IMM and OMM (a test for matrix proteins and IMS remains to be completed), to our surprise, we found that even those samples we considered to contain only inner mitochondrial membranes were positive for porin (OMM) (Fig. 3-15). The presence of porin, which is a marker characteristic of the outer mitochondrial membrane, indicates that we failed to completely remove the outer membrane in our preparations. As expected, when probing for COXII, the strains PRBio6-35, aW303Δ*SCO1* and aW303Δ*COX11* have a very weak expression or are missing the subunit II of COX. But it is important to note that in lanes corresponding to mitochondria (M), mitoplast (P) and IMM (I) of PRBio6-35, the signal for COXII gets stronger for IMM, while the porin control remains the same. This means that regardless of the lack of refinement of the technique, we are still able to concentrate IMM. In the case of aW303Δ*SCO1*, we can see that the signal for mitoplasts is very low for COXII and very weak for porin, meaning that there is less outer membranes, whereas in lane (I), the porin signal is high again, suggesting that when concentrating the IMM, we are also concentrating remnants of outer membrane too. The issue of having outer membranes remainings in the IMM samples represents a problem of contamination and the protocol clearly requires considerable optimization and refinement. Nevertheless, the samples obtained by this process were used for preliminary EPR analysis.

Preliminary EPR Analysis of Wild-type IMM Allows Detection of Copper

Two separate samples of IMM isolated from the respiratory competent aW303 were analyzed by EPR and allowed preliminary assignment of the copper in the sample. The obtained spectrum reveals two peaks in the characteristic position of the hemes A and A_3 (Fig. 3-16) and a noticeably pronounced peak that could be aconitase, which is an enzyme that catalyses the stereo-specific isomerization of citrate to isocitrate in the tricarboxylic acid cycle. This enzyme has an active iron Fe_4S_4 cluster. The suggestion of this peak corresponding to aconitase is a surprising result, because aconitase is a soluble protein that is located in the matrix, and it should not be in a properly isolated sample of IMM, however as we realized from the previous Western blot analysis (Figures 3-14 and 3-15), some contamination could be happening. A magnification of the EPR spectrum demonstrates the presence of a peak, likely corresponding to copper, in the expected position (at g= 2.08, highlighted in orange) (Fig. 3-17). This preliminary result shows, as expected, the presence of copper in COX of the wild-type strain, aW303. By comparing the knockout *sco1* and *cox11* strains, as well as some *sco1* point mutants, we will be able to elucidate if in fact Sco1p is delivering copper to the Cu_A site in COXII. This approach (of using EPR) should prove the theory of Sco1p function in the role of copper transfer to COX.

In summary, in the course of my thesis research, I have carried out experiments designed to test the hypothesis that Sco1p is a bifunctional protein. A quantitative strategy was designed to analyze several *sco1* mutants in the presence of hydrogen peroxide. The analysis of three *sco1* point mutants (Y142F, R220A and Y222F) revealed that although

they show partial sensitivity to hydrogen peroxide, they present a respiratory competent phenotype, thus possessing two distinctive phenotypes and suggesting two possible roles for Sco1p: copper transfer and redox metabolism functions.

I have done work to standardize a protein purification method that allows us to efficiently obtain a good yield and achieve the purest possible elution of COX by this method. Further experiments need to be done to standardize the IMM extraction so that the samples contain only inner mitochondrial membranes, without any contaminants from the outer membrane, although these samples were of sufficient quality to allow for a preliminary EPR analysis. With this type of analysis we will be certain of the endpoint of the Sco1p model of copper transport.

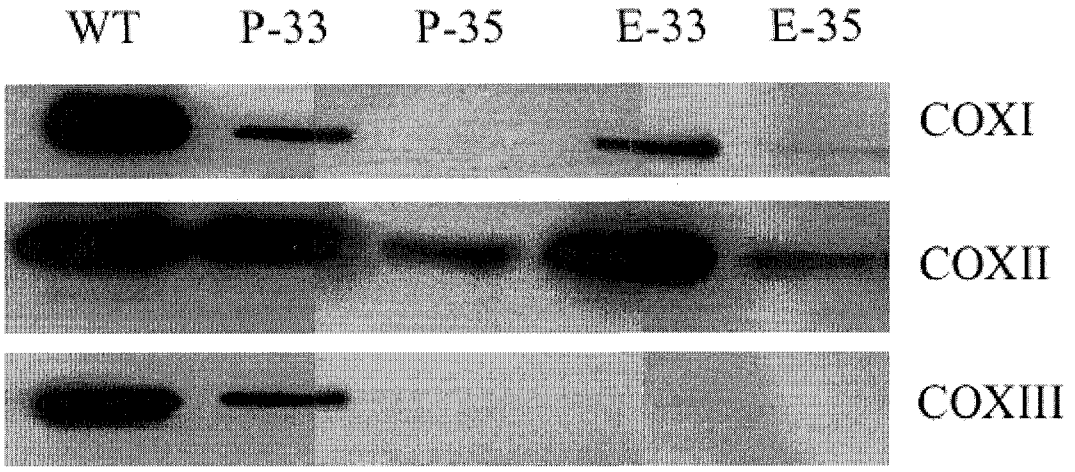


Figure 3-10. Steady State Levels of COX Subunits in *sco1* Strains-2. Expression of COX subunits in biotinylated strains, loading 13 μg of aW303, PRbio6-33 (P-33), PRbio6-35 (P-35), EKD1-33 (E-33) and EKD1-35 (E-35). The blot was probed with porin antibody as a loading control.

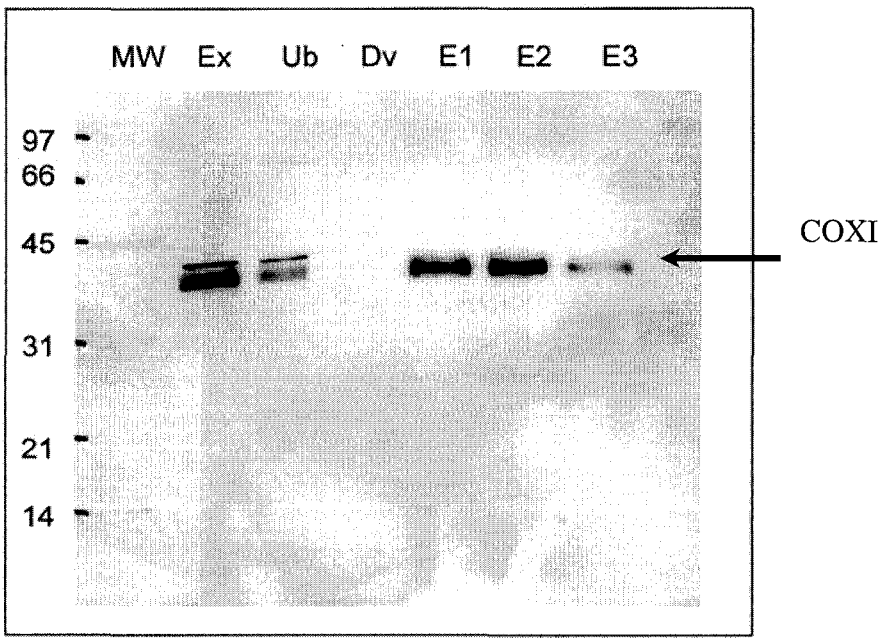


Figure 3-11. COX Purification of a Biotinylated Strain Using MOPS Buffer. A 0.9 ml column purification of the strain PRBio6-33 (biotinylated subunit six) using MOPS buffer and an additional salt wash to reduce nonspecific binding. Lanes show molecular weight markers (MW), extract (Ex), unbound (Ub), dead volume (Dv) and eluted fractions (E1-3). The Western blot was probed using COXI antibody.

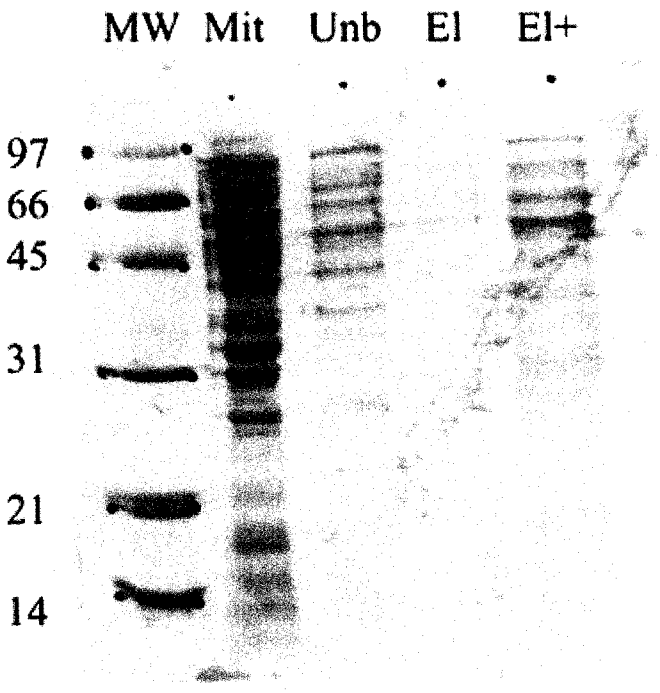


Figure 3-12. Protein Profiles of COX Purification from PRBio6-33. Ponceau red stained Western blot after a 2 ml (20 mg) column purification using the Pierce kit and concentration by centricon devices. Lanes show molecular weight markers (MW), mitochondrial extract (Mit), unbound (Unb) and eluted fractions before (El) and after concentration (El+)

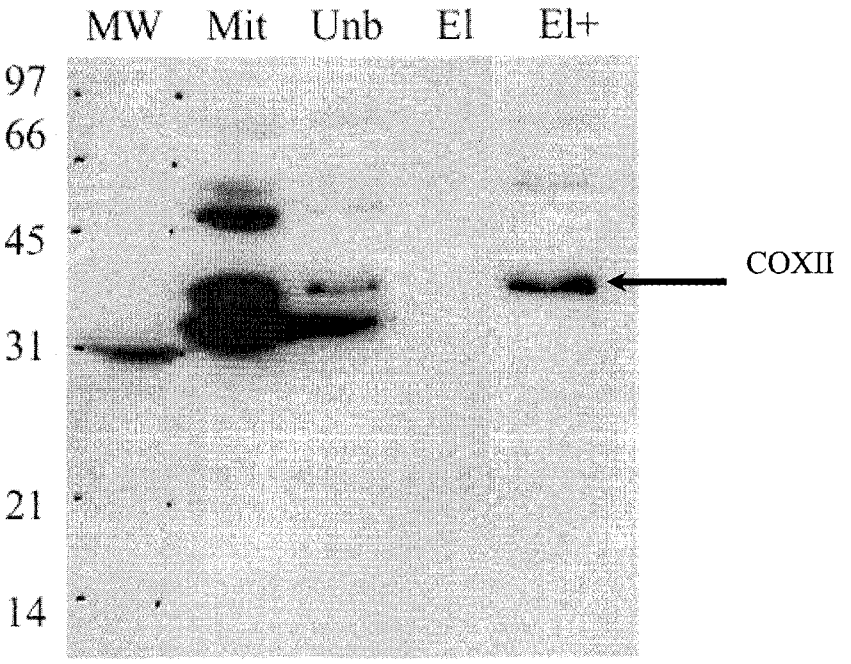


Figure 3-13. Purified COX from PRBio6-33 Before and After Concentrating Samples. The same Western blot as shown in figure 3-12 after probing with COXII antibody. Lanes show molecular weight markers (MW), mitochondrial extract (Mit), unbound (Unb) and eluted fractions before (El) and after (El+) concentration. The band below COXII could be a product of proteolitic degradation of subunit II or a nonspecific reaction band.

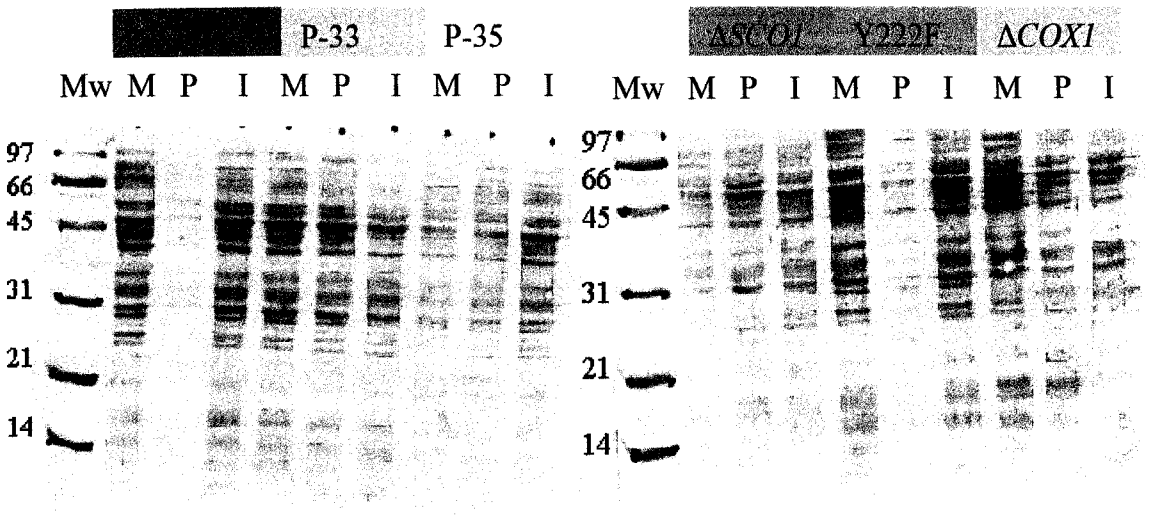


Figure 3-14. Ponceau Red Stained Western blot of Isolated IMM Samples. Mitochondria (M), Mitoplasts (P) and Inner mitochondrial membranes (I). On colored boxes strains aW303, PRBio-33, PRBio-35, aW303Δ*SCO1*, aW303Δ*SCO1* (Y222F) and aW303Δ*COX11*.

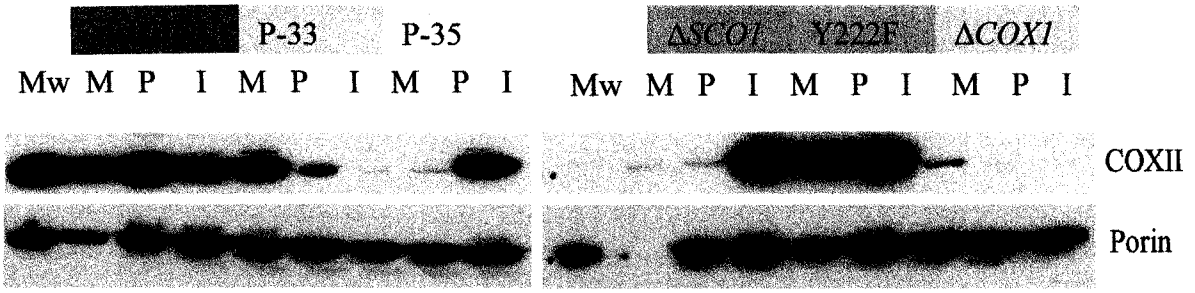


Figure 3-15. Expression of COXII and Porin in Isolated IMM Samples. The same Western blot as shown in figure 3-14. Mitochondria (M), Mitoplasts (P) and Inner mitochondrial membranes (I). On colored boxes strains aW303, PRBio-33, PRBio-35, aW303Δ*SCO1*, aW303Δ*SCO1* (Y222F) and aW303Δ*COX11*.

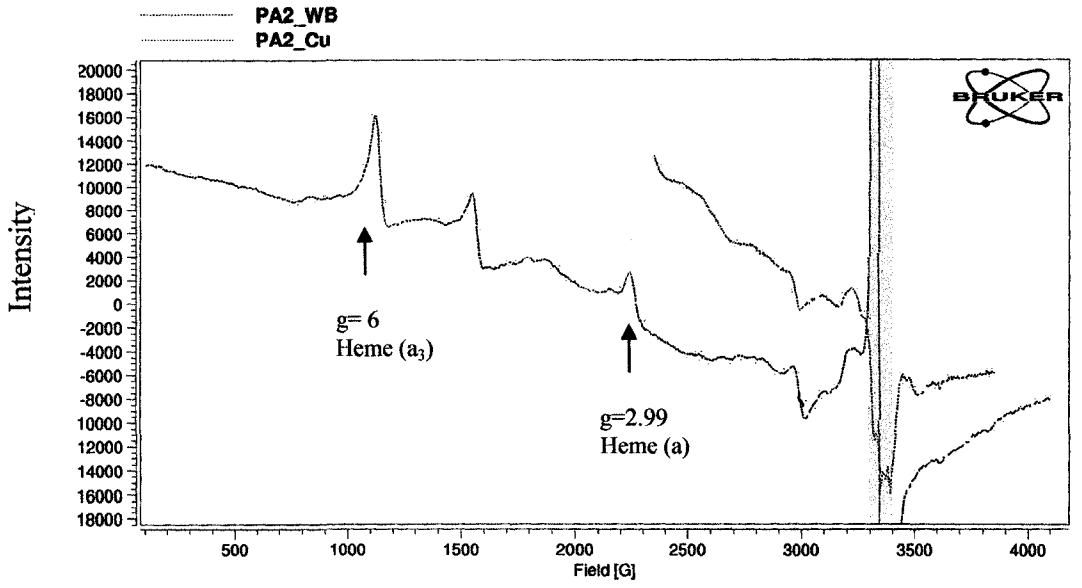


Figure 3-16. EPR Analysis of a Sample of Isolated IMM from Wild-type aW303 Sample. The zoomed-out view of the spectrum shows the heme A and A_3 peaks highlighted in yellow. Highlighted in blue is a big peak possibly from aconitase. (EPR obtained and annotated by Richard Rothery Biochemistry Department, University of Alberta).

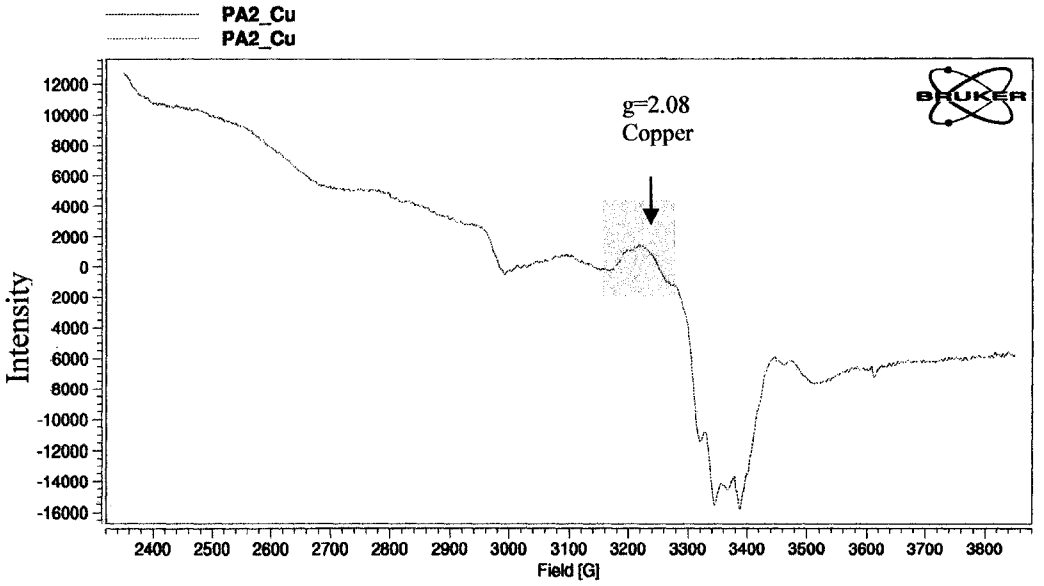


Figure 3-17. EPR Analysis of Wild-type Strain Detects the Cu_A Copper. A close up view of the spectrum reveals copper at the 2.08 position, highlighted in orange. (EPR obtained and annotated by Richard Rothery Biochemistry Department, University of Alberta).

Chapter 4: Discussion

Sco1p as a Bifunctional Protein

Cytochrome *c* oxidase is the terminal enzyme of the ETC and it reduces molecular oxygen to water. Yeast COX is composed of three mitochondrial-encoded subunits and 10 nuclear-encoded subunits. The assembly of COX is crucial for its proper functioning and depends on the coordination of over 30 encoded accessory proteins in yeast. These so called assembly factors have different roles: assembly of COX subunits, formation and insertion of heme A, delivery and insertion of metal ions, and maturation of the complex. Absence of any of the assembly factors can result in a non functional and misassembled COX. Inherited COX deficiencies in humans can be caused by mutations in mtDNA or in nDNA. COX deficiency resulting from nDNA mutations causes different clinical features and some of these mutations have been identified in several COX assembly factors (*SURF1*, *SCO1*, *SCO2*, *COX10* and *COX15* and *LRPPRC*). Studies in yeast have been very helpful in identifying some homolog COX assembly factors. There are three important accessory proteins that are known to play roles in forming the Cu_A and Cu_B centers in mammals: Cox11p, Cox17p and Sco1p. The present thesis is focused on studying one of these accessory proteins, Sco1p. Sco1p has been shown to be essential for COX expression in yeast (Krummeck and Rodel, 1990) and it is thought that this protein binds copper and delivers it to the Cu_A center of COX (Dickinson and Glerum, 2000). Although the specific function of Sco1p remains unclear, two roles have been proposed for this protein: the transfer of copper to the Cu_A site and a mitochondrial redox sensing function. Sco1p is fixed to the IMM by a single trans-membrane segment of 17 amino acids. Its copper binding domain (CxxxC) is very similar to the Cox2p copper binding site (Glerum et al., 1996[2]). Mutations in these highly conserved cysteines result

in respiratory deficiency (Rentzsch et al., 1999). The discovery of SCO1 structure gave rise to a new model for the role of Sco1p. It is thought that Sco1p could be involved in the reduction of the CxxxC cysteines in Cox2p, allowing copper insertion. Analysis of the crystal structure of human SCO1 revealed no copper bound to the proposed copper binding site (Williams et al., 2005). In contrast, studies on the crystal structure of yeast Sco1p revealed the presence of copper in an alternative copper-binding site, which involves non-conserved cysteines (Abajian and Rosenzweig, 2006). The structure of Sco1p has been often compared to the one of redox active proteins (Trx and Prx), due to the similarity of their conserved catalytic residue, with the proposed Sco1p copper-binding site.

In this thesis, the main objective was to define the role of Sco1p, addressing the two proposed roles for this protein. To assess the redox-sensing role for Sco1p the research for this thesis began by choosing interesting previously generated *sco1* point mutants (Y142F, R220A and Y222F) in yeast. Y142F and Y222F *sco1* mutants caught our attention because Y142F and Y222F may be phosphorylation sites. Phosphorylation usually occurs on serine, threonine, and tyrosine residues in eukaryotic proteins and regulates the activity of enzymes and also changes the protein's hydrophobicity. The R220A residue is implicated in hydrogen bonding (hydrogen bonds are partly responsible for the secondary, tertiary, and quaternary structures of proteins). Changes in these important residues, such as R220A, are expected to cause important conformational changes in the structure of the protein. As described in the results, all three of these mutants were able to grow on EG media in a manner similar to the wild-type strain, but

surprisingly they also revealed partial sensitivity to H_2O_2 treatment. Spectral analyses of cytochrome aa_3 in these mutants indicate a shifted peak, suggesting that COX is misassembled, in concordance with a decreased level of expression of the three core subunits of COX and intermediate levels of COX activity. The respiration deficient (EG^-) *sco1* mutant (S240F), was also shown to be partially sensitive to H_2O_2. Because the Y142F, R220A and Y222F mutants display two distinctive and separable phenotypes; my results suggest that Sco1p has a redox-sensing role, in addition to or instead of, the previously proposed role as a copper chaperone. If Sco1p has both of these roles, it could be that it first uses the thioredoxin-like function to reduce the cysteines of the copper binding site, and then it can bind copper and deliver it to the Cu_A center in COXII. Sco1p could also be functioning as an antioxidant protecting from damage produced by peroxide.

The other part of the research done in this thesis was focused on testing the theory of Sco1p as a copper chaperone. Biotinylated strains were used in affinity chromatography purification for further EPR analysis of the metal centers of COX. This technique required optimization and adjustments in different areas: changes in the mitochondrial extract concentration, size of the column, type of buffer, concentration of detergent in the buffer, times of equilibration, additional salt washes and the use of a pre-packed avidin column kit. Purification of COX from strains expressing the biotinylated subunit 6 was successful, on a small (0.3 ml column with 3 mg extract) and bigger scale (2 ml column with 20 mg extract), nevertheless the level of purification was not high enough to detect fewer bands when staining with Ponceau red stain, which means that the avidin-biotin

purification technique, although it was modified to its higher efficiency, is very prone to unspecific binding. The final yield of purified protein was undetectable by the Folin method, therefore subsequent concentration of the samples was necessary to obtain 1 to 2.45 mg of purified COX from PRBio6-33 (2.45 mg) and PRBio6-35 (1.27 mg) for further EPR analysis. EPR is the most important spectroscopic technique that has been applied to mitochondrial samples (Hudder et al., 2007) and is used to detect metal centers, in this case, copper. Looking for the presence or absence of copper in different *sco1* mutants will prove whether Sco1p is in fact delivering copper to the Cu_A site in COXII. Another approach taken in this research, in order to facilitate EPR analysis, was the isolation of IMM from different strains (wild-type, *SCO1* knockout, *COX11* knockout, *sco1* mutant Y222F, and biotinylated strains PRBio6-33 and PRBio6-35). EPR analyses were carried out for all six strains, using both purified COX and IMM. Although most of the samples for EPR gave very weak spectra, a preliminary EPR analysis of IMM from the wild-type strain was obtained (from three independent IMM isolations). The EPR spectrum displayed the presence of a weak peak, likely corresponding to copper (at g= 2.08). It was surprising that the purified COX samples were not very good for the EPR analysis, suggesting that the biotin was somehow affecting the metallic centers of COX (data not shown). The rest of the strains analyzed by EPR all had weaker spectra, very close to the baseline signal (data not shown). At first glance it seemed like the *sco1* mutant samples had no copper, but it too early to make any conclusions yet, as further EPR analyses with better quality and more concentrated samples need to be done in order to be able to have a clearer, more representative spectrum and a reliable result.

The fact that there is contamination with outer membranes and possibly matrix proteins in the supposedly exclusive IMM samples, explains the presence of aconitase in the EPR analysis. It is clear that the most commonly used protocol for isolation of IMM is not very precise and several modifications to improve the protocol are currently being developed in the Glerum lab. Modifications in the mitoplast extraction had been implemented by diluting isolated mitochondria 20-fold with hypoosmotic buffer (150 mM KCl, 0.1 mM $CaCl_2$, 20 mM HEPES–KOH pH 7.4) and incubating 15 min on ice, followed by a 5 min incubation with 0.2% Digitonin. These modifications seem to eliminate the residual outer membrane contamination and allow a better extraction of IMM.

The use of EPR analysis of either purified COX or IMM from *sco1* mutants seems to be a very obvious and direct way to answer the question as to the role of Sco1p, however the methodology required to obtain a suitable EPR sample is very difficult. It seems that the most direct, and less complicated, approach to get EPR samples is to analyze more concentrated IMM instead of purified COX. Some future directions following the same objective are to optimize and scale up the IMM isolation protocol, by growing the yeast in five or 10 L fermenters.

Some future directions in the Sco1p project include assessing the potential catalase/peroxidase activity function of Sco1p by running a native PAGE and incubating the gel with the enzyme substrate (containing H_2O_2), as the activity of catalase can then be detected as a band when staining the gel. This approach will determine if Sco1p has a

catalase activity. The same type of experiment was carried out for Cox11p, revealing that Cox11p has some catalase activity (Lari et al., manuscript in progress). Another interesting direction for the Sco1p project is to look for phosphorylation sites in the yeast Sco1p sequence. The predicted phosphorylation sites can be chosen for generation of *sco1* point mutants to determine if the phosphorylation of this protein has a role in regulating copper binding, COX assembly and/or COX activity. This thesis has provided important methods that will contribute to a better understanding of the roles for Sco1p and therefore, a better understanding of the COX assembly pathway and mitochondrial redox metabolism.

Bibliography

Abajian C. and Rosenzweig A.C. (2006) Crystal structure of yeast Sco1. *J. Biol. Inorg. Chem.*, **11**, 459-466.

Abajian C., Yatsunyk L.A., Ramirez B.E. and Rosenzweig A.C. (2004) Yeast cox17 solution structure and copper(I) binding. *J. Biol. Chem.*, **279**, 53584-53592.

Antonicka H., Mattman A., Carlson C.G., Glerum D.M., Hoffbuhr K.C., Leary S.C., Kennaway N.G. and Shoubridge E.A. (2003) 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.

Banci L., Bertini I., Cantini F., Ciofi-Baffoni S., Gonnelli L. and Mangani S. (2004) Solution structure of Cox11, a novel type of beta-immunoglobulin-like fold involved in CuB site formation of cytochrome c oxidase. *J. Biol. Chem.*, **279**, 34833-34839.

Banting G.S. and Glerum D.M. (2006) Mutational analysis of the Saccaromyces cerevisiae cytochrome c oxidase assembly protein Cox11p. *Eukaryot. Cell.*, **5**, 568-578.

Balatri, E., Banci L., Bertini, I., Cantini F., Ciofi-Baffoni S. (2003) Solution structure of Sco1: a thioredoxin-like preotein involved in cytochrome *c* oxidase assembly. *Structure*, **279**-33, 34833-34839.

Barrientos A., Barros M.H., Valnot I., Rotig A., Rustin P. and Tzagoloff A. (2002) Cytochrome oxidase in health and disease. *Gene,* **286**, 53-63[2].

Barrientos A., Korr D. and Tzagoloff A. (2002) Shy1p is necessary for full expression of mitochondrial COX1 in the yeast model of leigh's syndrome. *EMBO J.*, **21**, 43-52.

Barrientos A., Zambrano A. and Tzagoloff A. (2004) Mss51p and Cox14p jointly regulate mitochondrial Cox1p expression in Saccaromyces cerevisiae. *EMBO J.*, **23**, 3472-3482.

Barros M.H., Carlson C.G., Glerum D.M. and Tzagoloff A. (2001) Involvement of mitochondrial ferredoxin and Cox15p in hydroxylation of heme O. *FEBS Lett.*, **492**, 133-138.

Barros M.H., Johnson A. and Tzagoloff A. (2004) COX23, a homologue of COX17, is required for cytochrome oxidase assembly. *J. Biol. Chem.*, **279**, 31943-31947.

Beers J., Glerum D.M. and Tzagoloff A. (1997) Purification, characterization, and localization of yeast Cox17p, a mitochondrial copper shuttle. *J. Biol. Chem.*, **272**, 33191-33196.

Brown N.G., Costanzo M.C. and Fox T.D. (1994) Interactions among three proteins that specifically activate translation of the mitochondrial COX3 mRNA in saccharomyces cerevisiae. *Mol. Cell. Biol.*, **14**, 1045-1053.

Bruno C., Martinuzzi A., Tang Y., Andreu A.L., Pallotti F., Bonilla E., Shanske S., Fu J., Sue C.M., Angelini C. *et al.* (1999) A stop-codon mutation in the human mtDNA cytochrome c oxidase I gene disrupts the functional structure of complex IV. *Am. J. Hum. Genet.*, **65**, 611-620.

Burger G., Gray M.W. and Lang B.F. (2003) Mitochondrial genomes: Anything goes. *TRENDS in genetics,* **19,** 709.

Capaldi R.A. (1996) The complexity of a respiratory complex. *Nat. Struct. Biol.,* **3,** 570-574.

Carlson C.G., Barrientos A., Tzagoloff A. and Glerum D.M. (2003) COX16 encodes a novel protein required for the assembly of cytochrome oxidase in saccharomyces cerevisiae. *J. Biol. Chem.,* **278,** 3770-3775.

Carr H.S. and Winge D.R. (2005) Assembly of cytochrome *c* oxidase within the mitochondrion. *Acc. Chem. Res.,* **36,** 309.

Carr H.S., George G.N. and Winge D.R. (2002) Yeast Cox11, a protein essential for cytochrome c oxidase assembly, is a cu(I)-binding protein. *J. Biol. Chem.,* **277,** 31237-31242.

Chinenov Y.V. (2000) Cytochrome c oxidase assembly factors with a thioredoxin fold are conserved among prokaryotes and eukaryotes. *J. Mol. Med.,* **78,** 239-242.

Church C., Goehring B., Forsha D., Wazny P. and Poyton R.O. (2005) A role for Pet100p in the assembly of yeast cytochrome c oxidase: Interaction with a subassembly that accumulates in a pet100 mutant. *J. Biol. Chem.,* **280,** 1854-1863.

Clark K.M., Taylor R.W., Johnson M.A., Chinnery P.F., Chrzanowska-Lightowlers Z.M., Andrews R.M., Nelson I.P., Wood N.W., Lamont P.J., Hanna M.G. *et al.* (1999) An mtDNA mutation in the initiation codon of the cytochrome C oxidase subunit II gene

results in lower levels of the protein and a mitochondrial encephalomyopathy. *Am. J. Hum. Genet.*, **64**, 1330-1339.

Cobine P.A., Ojeda L.D., Rigby K.M. and Winge D.R. (2004) Yeast contain a non-proteinaceous pool of copper in the mitochondrial matrix. *J. Biol. Chem.*, **279**, 14447-14455.

Comi G.P., Bordoni A., Salani S., Franceschina L., Sciacco M., Prelle A., Fortunato F., Zeviani M., Napoli L., Bresolin N. *et al.* (1998) Cytochrome c oxidase subunit I microdeletion in a patient with motor neuron disease. *Ann. Neurol.*, **43**, 110-116.

Dickinson E.K., Adams D.L., Schon E.A. and Glerum D.M. (2000) A human SCO2 mutation helps define the role of Sco1p in the cytochrome oxidase assembly pathway. *J. Biol. Chem.*, **275**, 26780-26785.

DiMauro S., Lombes A., Nakase H., Mita S., Fabrizi G.M., Tritschler H.J., Bonilla E., Miranda A.F., DeVivo D.C. and Schon E.A. (1990) Cytochrome c oxidase deficiency. *Pediatr. Res.*, **28**, 536-541.

DiMauro S. and Schon E.A. (2001) Mitochondrial DNA mutations in human disease. *Am. J. Med. Genet.*, **106**, 18-26.

Filosto M., Tomelleri G., Tonin P., Scarpelli M., Vattemi G., Rizzuto N., Padovani A. and Simonati A. (2007) Neuropathology of mitochondrial diseases. *Biosci. Rep.*, **27**, 23-30.

Frey T.G. and Manella C.A. (2000) The internal structure of mitochondria. *TIBS*, **25**, 319.

Gattermann N., Retzlaff S., Wang Y.L., Hofhaus G., Heinisch J., Aul C. and Schneider W. (1997) Heteroplasmic point mutations of mitochondrial DNA affecting subunit I of cytochrome c oxidase in two patients with acquired idiopathic sideroblastic anemia. *Blood,* **90**, 4961-4972.

Glerum D.M., Koerner T.J. and Tzagoloff A. (1995) Cloning and characterization of COX14, whose product is required for assembly of yeast cytochrome oxidase. *J. Biol. Chem.,* **270**, 15585-15590.

Glerum D.M., Muroff I., Jin C. and Tzagoloff A. (1997) COX15 codes for a mitochondrial protein essential for the assembly of yeast cytochrome oxidase. *J. Biol. Chem.,* **272**, 19088-19094.

Glerum D.M., Shtanko A. and Tzagoloff A. (1996) Characterization of COX17, a yeast gene involved in copper metabolism and assembly of cytochrome oxidase. *J. Biol. Chem.,* **271**, 14504-14509[1].

Glerum D.M., Shtanko A. and Tzagoloff A. (1996) SCO1 and SCO2 act as high copy suppressors of a mitochondrial copper recruitment defect in saccharomyces cerevisiae. *J. Biol. Chem.,* **271**, 20531-20535[2].

Glerum D.M., Yanamura W., Capaldi R.A. and Robinson B.H. (1988) Characterization of cytochrome-c oxidase mutants in human fibroblasts. *FEBS Lett.,* **236**, 100-104.

Hanna M.G., Nelson I.P., Morgan-Hughes J.A. and Wood N.W. (1998) MELAS: A new disease associated mitochondrial DNA mutation and evidence for further genetic heterogeneity. *J. Neurol. Neurosurg. Psychiatry.*, **65**, 512-517.

Heaton D., Nittis T., Srinivasan C. and Winge D.R. (2000) Mutational analysis of the mitochondrial copper metallochaperone Cox17. *J. Biol. Chem.*, **275**, 37582-37587.

Hell K., Herrmann J.M., Pratje E., Neupert W. and Stuart R.A. (1998) Oxa1p, an essential component of the N-tail protein export machinery in mitochondria. *Proc. Natl. Acad. Sci. U. S. A.*, **95**, 2250-2255.

Hell K., Tzagoloff A., Neupert W. and Stuart R.A. (2000) Identification of Cox20p, a novel protein involved in the maturation and assembly of cytochrome oxidase subunit 2. *J. Biol. Chem.*, **275**, 4571-4578.

Hiser L., Di Valentin M., Hamer A.G. and Hosler J.P. (2000) Cox11p is required for stable formation of the cu(B) and magnesium centers of cytochrome c oxidase. *J. Biol. Chem.*, **275**, 619-623.

Horng Y.C., Cobine P.A., Maxfield A.B., Carr H.S. and Winge D.R. (2004) 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.

Hudder B.N., Morales J.G., Stubna A., Munck E., Hendrich M.P. and Lindahl P.A. (2007) Electron paramagnetic resonance and mossbauer spectroscopy of intact

mitochondria from respiring saccharomyces cerevisiae. *J. Biol. Inorg. Chem.,* **12**, 1029-1053.

Jaksch M., Horvath R., Horn N., Auer D.P., Macmillan C., Peters J., Gerbitz K.D., Kraegeloh-Mann I., Muntau A., Karcagi V. *et al.* (2001) Homozygosity (E140K) in SCO2 causes delayed infantile onset of cardiomyopathy and neuropathy. *Neurology,* **57**, 1440-1446[1].

Jaksch M., Paret C., Stucka R., Horn N., Muller-Hocker J., Horvath R., Trepesch N., Stecker G., Freisinger P., Thirion C. *et al.* (2001) 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[2].

Karadimas C.L., Greenstein P., Sue C.M., Joseph J.T., Tanji K., Haller R.G., Taivassalo T., Davidson M.M., Shanske S., Bonilla E. *et al.* (2000) Recurrent myoglobinuria due to a nonsense mutation in the COX I gene of mitochondrial DNA. *Neurology,* **55**, 644-649.

Keightley J.A., Hoffbuhr K.C., Burton M.D., Salas V.M., Johnston W.S., Penn A.M., Buist N.R. and Kennaway N.G. (1996) A microdeletion in cytochrome c oxidase (COX) subunit III associated with COX deficiency and recurrent myoglobinuria. *Nat. Genet.,* **12**, 410-416.

Kennedy E. P and Lehninger A.L. (1950) The products of oxidation of fatty acids by isolated rat liver mitochondria. *J. Biol. Chem.,* **185**, 275-285.

Khalimonchuk O. and Rodel G. (2005) Biogenesis of cytochrome *c* oxidase. *Mitochondrion,* **xx**, 1.

Khalimonchuk O., Ostermann K. and Rodel G. (2005) Evidence for the association of yeast mitochondrial ribosomes with Cox11p, a protein required for the cu(B) site formation of cytochrome c oxidase. *Curr. Genet.,* **47**, 223-233.

Krummeck G. and Rodel G. (1990) Yeast SCO1 protein is required for a post-translational step in the accumulation of mitochondrial cytochrome c oxidase subunits I and II. *Curr. Genet.,* **18**, 13-15.

Leary S.C., Cobine P.A., Kaufman B.A., Guercin G.H., Mattman A., Palaty J., Lockitch G., Winge D.R., Rustin P., Horvath R. *et al.* (2007) The human cytochrome c oxidase assembly factors SCO1 and SCO2 have regulatory roles in the maintenance of cellular copper homeostasis. *Cell. Metab.,* **5**, 9-20.

Leary S.C., Kaufman B.A., Pellecchia G., Guercin G.H., Mattman A., Jaksch M. and Shoubridge E.A. (2004) Human SCO1 and SCO2 have independent, cooperative functions in copper delivery to cytochrome c oxidase. *Hum. Mol. Genet.,* **13**, 1839-1848.

Lemaire C., Robineau S. and Netter P. (1998) Molecular and biochemical analysis of saccharomyces cerevisiae cox1 mutants. *Curr. Genet.,* **34**, 138-145.

Leonard J.V. and Schapira A.H. (2000) Mitochondrial respiratory chain disorders I: Mitochondrial DNA defects. *Lancet,* **355**, 299-304[1].

Leonard J.V. and Schapira A.H. (2000) Mitochondrial respiratory chain disorders II: Neurodegenerative disorders and nuclear gene defects. *Lancet,* **355**, 389-394[2].

Lode A., Paret C. and Rodel G. (2002) Molecular characterization of saccharomyces cerevisiae Sco2p reveals a high degree of redundancy with Sco1p. *Yeast,* **19**, 909-922.

Lombes A., Nakase H., Tritschler H.J., Kadenbach B., Bonilla E., DeVivo D.C., Schon E.A. and DiMauro S. (1991) Biochemical and molecular analysis of cytochrome c oxidase deficiency in leigh's syndrome. *Neurology,* **41**, 491-498.

Lowry O.H., Rosebrough N.J., Farr A.L. and Randall R.J. (1951) Protein measurement with the folin phenol reagent. *J. Biol. Chem.,* **193**, 265-275.

Manfredi G., Schon E.A., Moraes C.T., Bonilla E., Berry G.T., Sladky J.T. and DiMauro S. (1995) A new mutation associated with MELAS is located in a mitochondrial DNA polypeptide-coding gene. *Neuromuscul. Disord.,* **5**, 391-398.

Manthey G.M. and McEwen J.E. (1995) The product of the nuclear gene PET309 is required for translation of mature mRNA and stability or production of intron-containing RNAs derived from the mitochondrial COX1 locus of saccharomyces cerevisiae. *EMBO J.,* **14**, 4031-4043.

Margulis L. (1981) Symbiosis in cell evolution. *L. S.,* **36**, 452.

Mashkevich G., Repetto B., Glerum D.M., Jin C. and Tzagoloff A. (1997) SHY1, the yeast homolog of the mammalian SURF-1 gene, encodes a mitochondrial protein required for respiration. *J. Biol. Chem.,* **272**, 14356-14364.

Maxfield A.B., Heaton D.N. and Winge D.R. (2004) Cox17 is functional when tethered to the mitochondrial inner membrane. *J. Biol. Chem.,* **279**, 5072-5080.

McEwen J.E., Hong K.H., Park S. and Preciado G.T. (1993) Sequence and chromosomal localization of two PET genes required for cytochrome c oxidase assembly in saccharomyces cerevisiae. *Curr. Genet.,* **23**, 9-14.

Mogi T., Saiki K. and Anraku Y. (1994) Biosynthesis and functional role of haem O and haem A. *Mol. Microbiol.,* **14**, 391-398.

Mootha V.K., Lepage P., Miller K., Bunkenborg J., Reich M., Hjerrild M., Delmonte T., Villeneuve A., Sladek R., Xu F. *et al.* (2003) Identification of a gene causing human cytochrome c oxidase deficiency by integrative genomics. *Proc. Natl. Acad. Sci. U. S. A.,* **100**, 605-610.

Moslemi A.R. and Darin N. (2007) Molecular genetic and clinical aspects of mitochondrial disorders in childhood. *Mitochondrion,* **7**, 241-252.

Mulero J.J., Rosenthal J.K. and Fox T.D. (1994) PET112, a saccharomyces cerevisiae nuclear gene required to maintain rho+ mitochondrial DNA. *Curr. Genet.,* **25**, 299-304.

Nobrega M.P., Bandeira S.C., Beers J. and Tzagoloff A. (2002) Characterization of COX19, a widely distributed gene required for expression of mitochondrial cytochrome oxidase. *J. Biol. Chem.,* **277**, 40206-40211.

Nobrega M.P., Nobrega F.G. and Tzagoloff A. (1990) COX10 codes for a protein homologous to the ORF1 product of paracoccus denitrificans and is required for the synthesis of yeast cytochrome oxidase. *J. Biol. Chem.,* **265**, 14220-14226.

Nokhrin S.M., Weil J.A. and Howarth D.F. (2005) Magnetic resonance in systems with equivalent spin-1/2 nuclides. part 1. *J. Magn. Reson.,* **174**, 209-218.

Ott M., Prestele M., Bauerschmitt H., Funes S., Bonnefoy N. and Herrmann J.M. (2006) Mba1, a membrane-associated ribosome receptor in mitochondria. *EMBO J.,* **25**, 1603-1610.

Papadopoulou L.C., Sue C.M., Davidson M.M., Tanji K., Nishino I., Sadlock J.E., Krishna S., Walker W., Selby J., Glerum D.M. *et al.* (1999) Fatal infantile cardioencephalomyopathy with COX deficiency and mutations in SCO2, a COX assembly gene. *Nat. Genet.,* **23**, 333-337.

Pequignot M.O., Dey R., Zeviani M., Tiranti V., Godinot C., Poyau A., Sue C., Di Mauro S., Abitbol M. and Marsac C. (2001) Mutations in the SURF1 gene associated with leigh syndrome and cytochrome C oxidase deficiency. *Hum. Mutat.,* **17**, 374-381.

Poyton R.O., Goehring B., Droste M., Sevarino K.A., Allen L.A. and Zhao X.J. (1995) Cytochrome-*c* Oxidase from *Saccaromyces Cerevisiae.* In *Methods in Enzymology.* Academic Press, Inc., Vol. 260, pp. 97.

Punter A.F. and Glerum D.M. (2004) Defects in Assembly of Cytochrome Oxidase: Roles in Mitochondrial Disease. In Koehler C.M. and Bauer M.F. (eds.), *Topics in Current Genetics.* Springer-Verlag, pp. 123-141.

Punter F.A. and Glerum D.M. (2003) Mutagenesis reveals a specific role for Cox17p in copper transport to cytochrome oxidase. *J. Biol. Chem.,* **278,** 30875-30880.

Rahman S., Taanman J.W., Cooper J.M., Nelson I., Hargreaves I., Meunier B., Hanna M.G., Garcia J.J., Capaldi R.A., Lake B.D. *et al.* (1999) A missense mutation of cytochrome oxidase subunit II causes defective assembly and myopathy. *Am. J. Hum. Genet.,* **65**, 1030-1039.

Rentzsch A., Krummeck-Weiss G., Hofer A., Bartuschka A., Ostermann K. and Rodel G. (1999) Mitochondrial copper metabolism in yeast: Mutational analysis of Sco1p involved in the biogenesis of cytochrome c oxidase. *Curr. Genet.,* **35**, 103-108.

Saracco S.A. and Fox T.D. (2002) 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.

Schaefer A.M., Taylor R.W., Turnbull D.M. and Chinnery P.F. (2004) The epidemiology of mitochondrial disorders--past, present and future. *Biochim. Biophys. Acta,* **1659**, 115-120.

Sherratt H.S.A. (1991) Mitochondria: Structure and function. *Rev. Neurol. (Paris),* **147**, 417.

Shoubridge E.A. (2001) Nuclear genetic defects of oxidative phosphorylation. *Hum. Mol. Genet.*, **10**, 2277-2284[1].

Shoubridge E.A. (2001) Nuclear gene defects in respiratory chain disorders. *Semin. Neurol.*, **21**, 261-267[2].

Shoubridge E.A. (2001) Cytochrome c oxidase deficiency. *Am. J. Med. Genet.*, **106**, 46-52[3].

Taanman J.W. (1997) Human cytochrome *c* oxidase: Structure, function and deficiency. *Journal of Bioenergetics and Biomembranes*, **29**, 151.

Tarnopolsky M.A., Bourgeois J.M., Fu M.H., Kataeva G., Shah J., Simon D.K., Mahoney D., Johns D., MacKay N. and Robinson B.H. (2004) Novel SCO2 mutation (G1521A) presenting as a spinal muscular atrophy type I phenotype. *Am. J. Med. Genet. A.*, **125**, 310-314.

Taylor R.W. and Turnbull D.M. (2005) Mitochondrial DNA mutations in human disease. *Nat. Rev. Genet.*, **6**, 389-402.

Teraoka M., Yokoyama Y., Ninomiya S., Inoue C., Yamashita S. and Seino Y. (1999) Two novel mutations of SURF1 in leigh syndrome with cytochrome c oxidase deficiency. *Hum. Genet.*, **105**, 560-563.

Tiranti V., Corona P., Greco M., Taanman J.W., Carrara F., Lamantea E., Nijtmans L., Uziel G. and Zeviani M. (2000) 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.

Tiranti V., Galimberti C., Nijtmans L., Bovolenta S., Perini M.P. and Zeviani M. (1999) Characterization of SURF-1 expression and surf-1p function in normal and disease conditions. *Hum. Mol. Genet.,* **8**, 2533-2540.

Tiranti V., Hoertnagel K., Carrozzo R., Galimberti C., Munaro M., Granatiero M., Zelante L., Gasparini P., Marzella R., Rocchi M. *et al.* (1998) Mutations of SURF-1 in leigh disease associated with cytochrome c oxidase deficiency. *Am. J. Hum. Genet.,* **63**, 1609-1621.

Tsukihara T., Aoyama H., Yamashita E., Tomizaki T., Yamaguchi H., Shinzawa-Itoh K., Nakashima R., Yaono R. and Yoshikawa S. (1995) Structures of metal sites of oxidized bovine heart cytochrome c oxidase at 2.8 A. *Science,* **269**, 1069-1074.

Tsukihara T., Itoh-Shinzawa K. and Yoshikawa S. (1996) Structures of metal centers of bovine heart cytochrome c oxidase. *Tanpakushitsu Kakusan Koso,* **41**, 1353-1362.

Tzagoloff A., Akai A. and Rubin M.S. (1974) In the biogenesis of mitochondria. *Academic Press, Inc., New York,* , 405- 421.

Tzagoloff A., Capitanio N., Nobrega M.P. and Gatti D. (1990) 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.

Tzagoloff A., Nobrega M., Gorman N. and Sinclair P. (1993) On the functions of the yeast COX10 and COX11 gene products. *Biochem. Mol. Biol. Int.,* **31**, 593-598.

Valnot I., Osmond S., Gigarel N., Mehaye B., Amiel J., Cormier-Daire V., Munnich A., Bonnefont J.P., Rustin P. and Rotig A. (2000) 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[2].

Valnot I., von Kleist-Retzow J.C., Barrientos A., Gorbatyuk M., Taanman J.W., Mehaye B., Rustin P., Tzagoloff A., Munnich A. and Rotig A. (2000) A mutation in the human heme A:Farnesyltransferase gene (COX10) causes cytochrome c oxidase deficiency. *Hum. Mol. Genet.,* **9**, 1245-1249[1].

Wallace D.C. (1999) Mitochondrial diseases in man and mouse. *Science,* **283**, 1482-1488.

Williams J.C., Sue C., Banting G.S., Yang H., Glerum D.M., Hendrickson W.A. and Schon E.A. (2005) Crystal structure of human SCO1: Implications for redox signaling by a mitochondrial cytochrome c oxidase "assembly" protein. *J. Biol. Chem.,* **280**, 15202-15211.

Wood Z.A., Schroder E., Harris J.R. and Poole L.B. (2003) Structure, mechanism and regulation of peroxiredoxins. *TRENDS in Biochemical Sciences,* **28**, 32.

Xu F., Morin C., Mitchell G., Ackerley C. and Robinson B.H. (2004) The role of the LRPPRC (leucine-rich pentatricopeptide repeat cassette) gene in cytochrome oxidase

assembly: Mutation causes lowered levels of COX (cytochrome c oxidase) I and COX III mRNA. *Biochem. J.*, **382**, 331-336.

Yuan D.S., Dancis A. and Klausner R.D. (1997) Restriction of copper export in saccharomyces cerevisiae to a late golgi or post-golgi compartment in the secretory pathway. *J. Biol. Chem.*, **272**, 25787-25793.

Zauner S., Lockhart P., Stoebe-Maier B., Gilson P., McFadden G.I. and Maier U.G. (2006) Differential gene transfers and gene duplications in primary and secondary endosymbioses. *BMC Evol. Biol.*, **6**, 38.

Zee J.M. and Glerum D.M. (2006) Defects in cytochrome oxidase assembly in humans: Lessons from yeast. *Biochem. Cell Biol.*, **84**, 859-869.

Zhu Z., Yao J., Johns T., Fu K., De Bie I., Macmillan C., Cuthbert A.P., Newbold R.F., Wang J., Chevrette M. *et al.* (1998) SURF1, encoding a factor involved in the biogenesis of cytochrome *c* oxidase, is mutated in leigh syndrome. *Nat. Genet.*, **20**, 337-343.