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The role of human Sco1, Sco2, Surf1 and Oxa1l in the biogenesis of the oxidative phosphorylation system

PhD Thesis

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ABBREVIATIONS

2D	two-dimensional
AAA	atpases associated with a variety of cellular activities
ADP	adenosine diphosphate
Alb3	ALBINO3
ATP	adenosine triphosphate
ATPase	adenosine triphosphatase
BN-PAGE	blue native polyacrylamide gel electrophoresis
bp	base pair
CcO	cytochrome *c* oxidase, complex IV
CNS	central nervous system
CO	carbon monoxide
Coa1/2	cytochrome oxidase assembly 1/2
CoQ	coenzyme Q
COX	cytochrome *c* oxidase, complex IV
CS	citrate synthase
CxxxC	cysteine-undetermined (3x)-cysteine
DNA	deoxyribonucleic acid
FAAS	flame atomic absorption spectroscopy
FACS	fluorescent-activated cell sorting
$FADH_2$	flavin adenine dinucleotide reduced
GFP	green fluorescent protein
GIP	general import pore
GTP	guanosine triphosphate
GTPase	guanosine triphosphatase
HA	hemagglutinin
HEK293	human embryonic kidney 293
HEXXH	histidine-glutamic acid-undetermined (2x)-histidine
HIF-1α	hypoxia-inducible factor 1α
HSP	heat shock protein
i-AAA	intermembrane space-AAA
IM	inner membrane

Imp1/2	intermembrane space protease 1/2
IMS	intermembrane space
kDa	kilodalton
KO	knockout
LRPPRC	leucine-rich pentatricopeptide repeat cassette
m-AAA	matrix-AAA
MDa	megadalton
MELAS	mitochondrial encephalopathy, lactic acidosis, and stroke like episodes
MERRF	myoclonic epilepsy and ragged-red fibers
Mia40	mitochondrial intermembrane space import and assembly 40
MPP	matrix processing peptidase
mRNA	messenger ribonucleic acid
mt	mitochondrial
MTS	mitochondrial targeting sequence
mt-tRNA	mitochondrial transfer ribonucleic acid
MW	molecular weight
NADH	nicotinamide adenine dinucleotide reduced
ND	NADH dehydrogenase
NO	nitric oxide
OM	outer membrane
OPA1	optic atrophy 1
ORF	open reading frame
OXA1	oxidase assembly 1
OXA1L	oxidase assembly 1-like
OXPHOS	oxidative phosphorylation system
Phb1	prohibitin 1
PNPase	polynucleotide phosphorylase
PPR	pentatricopeptide repeat
RNA	ribonucleic acid
ROS	reactive oxygen species
rRNA	ribosomal ribonucleic acid
SAM	sorting and assembly machinery
SCO	synthesis of cytochrome oxidase
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis

TACO1	translational activator of COX I
TCA	tricarboxylic acid
TIM	translocase of the inner membrane
TNFalpha	tumor necrosis factor alpha
TOB	topogenesis of mitochondrial outer membrane β-barrel proteins
TOM	translocase of the outer membrane
tRNA	transfer ribonucleic acid
UTR	untranslated region
VDAC	voltage-dependent anion-selective channel
YME1	yeast mitochondrial escape 1

INTRODUCTION

Mitochondria and OXPHOS

Mitochondria are highly dynamic semiautonomous organelles of endosymbiotic, α-proteobacterial descent found in virtually all eukaryotic cells. They are surrounded by two biological membranes that delimit two aqueous subcompartments, the intermembrane space and the mitochondrial matrix. They contain numerous copies of their own DNA (mtDNA) that codes for a limited number of protein products, almost exclusively subunits of the OXPHOS (Oxidative Phosphorylation System) machinery, and rRNA and tRNA components of a specific mitochondrial translation apparatus. In mammals, 13 protein products are synthesized within mitochondria, all of them being evolutionary conserved hydrophobic subunits of the OXPHOS system. The vast majority of the various ~1100 mammalian mitochondrial proteins is thus encoded in nucleus, synthesized on cytoplasmic ribosomes and subsequently sorted and imported into mitochondria by means of a specialized transport machinery (TOM/TIM translocases) (Lill et al., 1996; Voet and Voet, 1995).

Mitochondria are responsible for the conversion of energy and production of the bulk of cellular ATP via the TCA cycle and the OXPHOS machinery, serve as calcium (Ca^{2+}) stores and play a pivotal role in the mechanism of programmed cell death (apoptosis). They are the place of fatty acids oxidation, ketone body production, heme biosynthesis, cardiolipin metabolism, the biosynthesis of coenzyme Q, production of reactive oxygen species (ROS) and key steps of gluconeogenesis and the urea cycle (Voet and Voet, 1995).

The mammalian OXPHOS machinery is composed of five multi-subunit membrane-embedded enzyme complexes (the respiratory chain and the ATP synthase) build up of more than 90 protein subunits containing numerous prosthetic groups. According to nowadays widely accepted Mitchell's chemiosmotic hypothesis the electrons taken from the oxidation of NADH and succinate ($FADH_2$) undergo linear transfer through the four membrane embedded complexes of the respiratory chain, what is intimately coupled to vectorial proton pumping (translocation) across the inner membrane bilayer by three of these complexes (I, III, IV), so-called proton pumps (Mitchell, 1966; Mitchell, 1979; Voet and Voet, 1995). This results in the generation of electrochemical proton gradient (gradient of protons ΔpH and mitochondrial membrane potential ΔΨ) that might be subsequently used by the F_1F_O-ATP synthase to power

the synthesis of ATP (Voet and Voet, 1995). The individual OXPHOS complexes are further organized into higher order, supramolecular assemblies, so-called respiratory supercomplexes (Cruciat et al., 2000; Schagger and Pfeiffer, 2000). These appear to be the building blocks of a hypothetical macromolecular respiratory unit that was termed "respirasome" (Acin-Perez et al., 2008; Lenaz and Genova, 2007; Schagger and Pfeiffer, 2000).

The biogenesis of OXPHOS is complicated by the subcellular localization and intricate membrane topology of constituent complexes, the dual genetic origin of the individual subunits, the high hydrophobicity of most of them and by a plethora of prosthetic groups required for the activity and assembly/stability of the complexes. As a result, the biogenesis of OXPHOS requires coordinated expression of the nuclear and mitochondrial genome, a multilevel regulation of this process, the sorting and import of subunits and non-subunit (ancillary) proteins into the various subcompartments of the organelle, the biosynthesis and/or insertion of prosthetic groups, the membrane insertion/translocation and assembly of subunit proteins into assembly intermediates/subcomplexes and final oligomeric complexes, and the surveillance of protein quality control and proteolytic processing carried out by specific energy-dependent proteases (Fernandez-Vizarra et al., 2009; Kutik et al., 2009; Lill et al., 1996).

The quality (correct processing, folding, and topogenesis) and the abundance of proteins within the inner mitochondrial membrane are controlled by two contradictory processes: the assembly/membrane-integration and selective proteolytic degradation. The integration/assembly of proteins into the inner membrane is ensured by a number of more or less specific translocases, assembly factors and molecular chaperones that mediate the insertion of proteins or translocation of transmembrane segments from both sides of the membrane (Hildenbeutel et al., 2008; Kutik et al., 2009; Mokranjac and Neupert, 2009; Neupert and Herrmann, 2007). On the other hand, the proteolytic degradation of misfolded and/or non-assembled inner membrane proteins is conducted by two evolutionary conserved energy-dependent proteolytic machines, the m-AAA and i-AAA proteases. These ATP-dependent proteolytic complexes act at the opposite sides of the inner membrane. In addition to the metallopeptidase function, they also exert chaperone and translocase activities (Koppen and Langer, 2007; Leonhard et al., 1996; Tatsuta and Langer, 2008). Even though the processes of protein integration/assembly and degradation apparently act in a completely opposite manner, they share a common goal of maintaining the protein homeostasis within the inner mitochondrial membrane.

AIMS OF THE STUDY

- Characterize the impact of mutations in genes encoding CcO assembly factors Sco2 and Surf1 on the tissue pattern of CcO subcomplexes and the tissue levels of respective mutant proteins in order to gain new insights into the function of both factors and the process of human CcO assembly in various tissues

- Study the molecular role and biochemical properties of the human homologue, OXA1L, of yeast mitochondrial Oxa1 translocase involved in early posttranslational steps of CcO biogenesis

- Analyze the impact on CcO biogenesis and tissue copper levels of a novel mutation (G132S) in the juxtamembrane region of Sco1 metallochaperone and study the protein-protein interactions of wild-type Sco1 in order to characterize the molecular role of Sco1 metallochaperone in CcO biogenesis and cellular copper homeostasis maintenance

- Analyze the effects of mt-tRNA point mutations in various tissues from individuals affected by Leigh (8363G>A), MERRF (8344A>G), and MELAS (3243A>G) syndrome on the steady-state levels and activity of OXPHOS complexes

REVIEW OF THE LITERATURE AND DISCUSSION OF RESULTS

1. Import and membrane-integration of mitochondrial proteins

The vast majority of mitochondrial proteins, including ten of the thirteen cytochrome *c* oxidase (CcO) subunits, are encoded by nuclear genes, synthesized in cytoplasm as precursor proteins (preproteins) and further maintained in an unfolded, import-competent state by cytosolic chaperones (Hsp70, Hsp90). Subsequently, they are specifically recognized, imported and sorted into the various subcompartments of mitochondria by means of specialized translocation machinery. The mitochondrial targeting of about half of the mitochondrial proteins is ensured by a specific cleavable N-terminal amino acid presequence, so-called mitochondrial targeting sequence (MTS), often in the form of amphipathic α-helix (Neupert and Herrmann, 2007; Truscott et al., 2003). These signals are necessary and sufficient to target precursor proteins into the mitochondrial matrix, where the presequence is removed by the action of a specialized processing protease (MPP). The remaining half of mitochondrially destined proteins contains internal targeting signals as well as bipartite presequences (N-terminal MTS followed by internal hydrophobic loop signal) that can be present anywhere along the polypeptide sequence. Unlike the N-terminal MTS-containing precursors, the proteins containing internal targeting signals can be destined to all mitochondrial subcompartments (Neupert and Herrmann, 2007).

1.1. Transport across the outer membrane - the TOM complex

Depending on the protein's final destination, several multimeric membrane complexes are involved in its translocation across, and subsequent insertion into, the outer or inner mitochondrial membrane from either side.

The N-terminal targeting signals are recognized by the receptor subunits of the multimeric outer membrane TOM (Translocase of the Outer Membrane) complex that mediates the import of virtually all proteins of the mitochondria. In addition, the TOM complex is also responsible for the insertion of proteins into the outer mitochondrial

membrane. In conjunction with the TOB/SAM (Topogenesis of mitochondrial Outer membrane β-barrel proteins/Sorting and Assembly Machinery) complex, the TOM complex mediates insertion into the outer membrane of β-barrel proteins. TOM complex consists of a stable core, so-called general import pore complex (GIP complex) and loosely associated receptor proteins. The GIP complex consists of the central component Tom40 and three small associated subunits, Tom5, Tom6, and Tom7. Tom40 is a transmembrane protein that itself constitutes the import pore of the outer mitochondrial membrane. Tom40 is able to form pores in artificial membranes even in the absence of other TOM subunits. Similarly to other outer membrane channel protein porin (VDAC), Tom40 presumably forms a β-barrel membrane-spanning structure. However, it is still not clear whether the pores are formed by single or multiple Tom40 molecules. When purified using the mild detergent digitonin, the TOM complex has a molecular mass of roughly 490-600 kDa (Esaki et al., 2004; Neupert and Herrmann, 2007; van Wilpe et al., 1999; Yano et al., 2003).

1.2. Transport across the inner membrane - the TIM complexes

After crossing the outer membrane, preproteins destined to the inner membrane, IMS and matrix interact with one of the TIM (Translocase of the Inner Membrane) complexes. On the other hand, insertion into the outer membrane of β-barrel proteins, upon their release from TOM complex, is directly mediated by the TOB/SAM outer membrane-embedded complex (Neupert and Herrmann, 2007).

The mitochondrial inner membrane harbors a large variety of different membrane proteins that attain various membrane topologies, exposing active domains on one or both sides of the membrane. Although a limited number of these proteins is synthesized on mitochondrial ribosomes, the majority of protein constituents of the inner membrane is synthesized in cytosol and subsequently imported through the TOM complex. Three different import pathways were thus far described for the various inner membrane proteins. First, a specialized inner membrane transport complex TIM22 is involved in the membrane translocation of proteins (TIM subunits, metabolite carriers) with internal "hydrophobic loop" signals (TIM22 pathway). Second, some preproteins that use the inner membrane TIM23 complex are arrested at the level of the complex and then laterally inserted into the inner membrane (stop-transfer mechanism). Third, some, mostly evolutionary conserved, inner membrane destined preproteins are translocated into the matrix by the TIM23 complex and subsequently, upon cleavage of MTS, exported (inserted) into the inner membrane by

evolutionary conserved export machinery represented by the Oxa1 translocase complex (conservative sorting) (see 2.3) (Mokranjac et al., 2003; Mokranjac et al., 2005; Truscott et al., 2001).

Protein residents of the IMS, mostly of low molecular weight, exert essential functions in a variety of different processes, such as metabolic and bioenergetic reactions, transfer of metabolites, proteins and metal ions, and regulation of cell death. On the basis of their import characteristics, most IMS destined proteins can be attributed into one of these categories: (i) proteins with N-terminal MTS followed by a hydrophobic sorting sequence (bipartite presequence motifs) are imported via TOM and TIM23 complexes in a membrane potential, and in most cases, ATP-dependent manner. Upon import, such bipartite presequences are proteolytically removed, releasing the mature protein into IMS; (ii) the TOM complex-mediated import of small, mostly soluble IMS proteins is thought to be driven by the subsequent folding of proteins within IMS, triggered by the acquisition of cofactors or by the formation of intramolecular disulfide bridges. This so-called "folding trap hypothesis" presumes that the folded state hinders the back-translocation out of the mitochondria thereby conferring the unidirectional net import of these proteins. One example of such protein are cytochrome *c*, which is imported through the TOM complex in apo-form and subsequently locked in its folded state by cytochrome *c* heme lyase. Another example is the twin Cx_9C class of IMS proteins that are bound in precursor forms by inner membrane protein Mia40, which is able to convert its intramolecular disulfide bridges to intermolecular, covalently linking the precursors, thereby preventing their back-translocation. Lastly, (iii) the import reaction of some IMS proteins appears to be driven by affinities of these proteins to form complexes with other proteins of IMS. Examples are the import reactions of cytochrome *c* heme lyase, catalyzing the insertion of heme group to both cytochrome *c* and cytochrome c_1 in humans or creatine kinase (Brandner et al., 2005b; Herrmann and Kohl, 2007; Neupert and Herrmann, 2007).

1.3. Oxa1 translocase complex

During the evolution, most genes of α-proteobacterial descent were transferred to nucleus thus reducing the size of mitochondrial genome to the current state (Andersson and Kurland, 1999; Cavalier-Smith, 2002). However, mainly the hydrophobic nature of most mitochondrially-encoded proteins has hindered this process (Claros et al., 1995). Consequently, evolutionary conserved membrane-insertion machinery represented by the

Alb3/Oxa1/YidC protein family has evolved to ensure the cotranslational membrane insertion of hydrophobic proteins in mitochondria, chloroplasts and bacteria (Bonnefoy et al., 2009; Stuart, 2002). Hence, mitochondrial translation is thought to occur exclusively at the matrix face of the inner membrane bilayer (Liu and Spremulli, 2000). In contrast, the recognition and membrane-recruitment of translating ribosomes in the cytoplasm of eukaryotic and prokaryotic cells is mediated by signal recognition particles (Gilmore and Blobel, 1983) that appear to be absent from mitochondria (Glick and Von Heijne, 1996).

The insertion of mitochondrial translation products as well as of a subset of TIM23 conservatively sorted nuclear gene products into the inner membrane is ensured by a conserved integral inner-membrane protein Oxa1, the founding member of the Alb3/Oxa1/YidC protein family (Herrmann and Neupert, 2003; Hildenbeutel et al., 2008). Members of this family possess a hydrophobic core domain containing five transmembrane helices that facilitate the membrane export of their protein substrates (Herrmann et al., 1997; Kuhn et al., 2003). The best characterized member of this family, *Saccharomyces cerevisiae* Oxa1, is an intrinsic protein of the inner mitochondrial membrane. Unlike the bacterial homologue, YidC, mitochondrial Oxa1 proteins contain a C-terminal α-helical domain of roughly 100 residues that protrudes into the matrix (Jia et al., 2003; Preuss et al., 2005). This domain was shown in yeast to bind to the 60S ribosomal subunit protein L41, located near the polypeptide exit tunnel thereby physically recruiting the mitochondrial translation apparatus to the translocation complex (Jia et al., 2003) represented by approx. 200 kDa homooligomeric assembly of four Oxa1 subunits (Szyrach et al., 2003). In a crosslinking experiment, mitochondrially-encoded cytochrome c oxidase subunits Cox1, Cox2 and Cox3 were shown to transiently interact with Oxa1 as nascent chains (Hell et al., 2001). Although the yeast Oxa1 was shown to represent a rather general export machinery of the inner membrane, the co-translational membrane insertion of the mitochondrially encoded Cox2 precursor appears to exhibit the strictest dependency on its function. The other substrates of Oxa1, including Oxa1 itself, can be inserted independently of its function, albeit with significantly reduced efficiencies. This suggests the existence of an alternative insertion pathway(s). Recently, a novel post-translational role in the biogenesis of OXPHOS was demonstrated for yeast Oxa1. The protein was shown to stably interact in a posttranslational manner with the ATP synthase subunit c, mediating its assembly into the ATP synthase complex (Jia et al., 2007). The yeast Oxa1 null mutant is respiratory deficient, with no detectable CcO activity and markedly reduced levels of the cytochrome bc_1 complex and the F_1F_O-ATP synthase (Altamura et al., 1996; Bonnefoy et al., 1994). *Schizosaccharomyces*

pombe contains two distinct Oxa1 orthologues, both of which are able to complement the respiratory defect of yeast Oxa1-null cells. The double inactivation of these genes is lethal to this petite-negative yeast (Bonnefoy et al., 2000). Depletion of Oxa1 in *Neurospora crassa* results in a slow-growth phenotype accompanied by reduced subunit levels of CcO and NADH:ubiquinone oxidoreductase (complex I). The *N. crassa*, Oxa1 was shown to form a 170–180 kDa homo-oligomeric complex, most likely containing four Oxa1 monomers (Nargang et al., 2002).

Yeast Mba1, a protein associated with the matrix face of the inner membrane presumably functions as a ribosome receptor that cooperates with Oxa1 in the cotranslational insertion process (Ott et al., 2006). The lack of Mba1, together with the C-terminus of Oxa1, results in an association of mitochondrial translation products with mtHSP70 (Ott et al., 2006), a mitochondrial matrix chaperone known to specifically interact with unfolded polypeptides (Hartl and Hayer-Hartl, 2002). Yeast Mba1 exhibits sequence similarity with mitochondrial ribosomal L45 proteins from higher eukaryotes (Ott et al., 2006).

The human Oxa1 orthologue, referred to as Oxa1l, shares 33% sequence identity with the corresponding yeast polypeptide (Rotig et al., 1997). The human *OXA1L* cDNA was initially cloned by partial functional complementation of the respiratory growth defect of the yeast oxa1-79 mutant. It contains an open reading frame predicted to encode a protein of 435 amino acids (Bonnefoy et al., 1994). It was suggested that the ten exons of OXA1L might form an open reading frame (ORF) able to encode a precursor protein of 495 amino acids, and more recently the cDNA containing these additional 180 bp was cloned (Strausberg et al., 2002). However, this extended ORF version was shown to exhibit an even lower capacity to complement the respiratory growth defect of yeast *oxa1* cells than the original sequence. The human OXA1L mRNA was found to be enriched in mitochondria- bound polysomes from HeLa cells, and its 3′ untranslated region was shown to be functionally important when expressed in yeast cells (Sylvestre et al., 2003). We showed that human Oxa1l is a mitochondrial integral membrane protein that exists as part of a 600–700 kDa complex in mitochondria of human embryonic kidney 293 (HEK293) cells (Stiburek et al., 2007). We further demonstrated that the stable short hairpin RNA (shRNA)-mediated knockdown of human Oxa1l in HEK293 cells leads to markedly decreased protein levels and ATP hydrolytic activity of the F1Fo-ATP synthase and moderately reduced levels and activity of NADH:ubiquinone oxidoreductase (complex I), suggesting functional involvement of the protein in the assembly/stability of these two OXPHOS complexes (Stiburek et al., 2007). In sharp contrast to yeast *oxa1* mutant, the assembly/stability of cytochrome c oxidase (complex

IV) as well as of the cytochrome *c* reductase (complex III) was not negatively affected in human cells with downregulated expression of Oxa1l (Stiburek et al., 2007). These results indicate that human Oxa1l represents mitochondrial integral membrane protein required for the correct biogenesis of the F_1F_O-ATP synthase and to a lesser extent NADH:ubiquinone oxidoreductase.

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

2. Mitochondrial translational activators - Pet309, LRPPRC and TACO1

In addition to Oxa1 translocase, another class of inner membrane proteins, so-called translational activators exists in yeast mitochondria that mediate the membrane-recruitment of translating mitochondrial ribosomes (Naithani et al., 2003; Sanchirico et al., 1998). These proteins bind specifically to sequences in 5' untranslated regions (UTRs) of mitochondrial transcripts (Green-Willms et al., 2001; Manthey et al., 1998; McMullin and Fox, 1993). Similar mechanism was not expected to be conserved in mammals, since mammalian mitochondrial mRNAs lack significant 5' UTR sequences and the majority of genes involved in translation of mitochondrially encoded proteins lack mammalian homologues. The only exception was thought to be the human homologue, LRPPRC, of yeast translation activator Pet309 involved in translation and stabilization of *COX1* mRNA (Mootha et al., 2003; Xu et

al., 2004). Both Pet309 and LRPPRC contain several PPR motifs, consisting of degenerated 35-amino acid sequences proposed to form two antiparallel alpha helices (Delannoy et al., 2007). These repeats characterize a large protein family in plants with only a few examples found among fungi, animals, and protists. PPR proteins are known to participate mostly in different steps of sequence-specific RNA metabolism (Tavares-Carreon et al., 2008). Indeed, Pet309 is the only yeast translational activator that has been found to contain PPR motifs. LRPPRC was shown to be involved in the stabilization of both *COX1* and *COX3* mRNAs. Mutations in LRPPRC lead to French-Canadian form of cytochrome oxidase deficient Leigh syndrome (Mootha et al., 2003; Xu et al., 2004).

Very recently, a nuclear gene product named TACO1 (Translational Activator of COX I) was identified as first known specific mammalian mitochondrial translational activator (Weraarpachai et al., 2009). The mature polypeptide of roughly 30 kDa was shown to exist in mitochondrial matrix as an oligomer or as part of a complex of ~74 kDa. Loss of TACO1 results in late-onset Leigh syndrome with cytochrome oxidase deficiency, very likely due to specific defect in the synthesis of mitochondrially encoded subunit Cox1. The reduced amount of newly synthesized Cox1 in *TACO1* patient fibroblasts was shown to be accompanied by increase in some of the ND subunits, and *cyt b*, probably reflecting compensatory response. The substantially decreased levels of fully assembled CcO are not accompanied by appearance of any of the previously identified subcomplexes, confirming the role of Cox1 as an initial seed of CcO assembly. Interestingly, the inactivation of *S. cerevisiae* TACO1 ortholog, YGR021w that exhibits 29% sequence identity, resulted in almost normal CcO activity of cells grown on respiratory media (Weraarpachai et al., 2009).

3. Cytochrome c oxidase (CcO)

Eukaryotic cytochrome *c* oxidase (CcO) is the terminal multicomponent enzyme of the energy-transducing mitochondrial electron transport chain (Capaldi, 1990; Stiburek et al., 2006). It belongs to the superfamily of heme-copper containing terminal oxidases, characterized by the presence of histidine ligands to two heme groups and to a Cu_B copper ion (Michel et al., 1998). The mitochondrial enzyme, an aa_3-type terminal oxidase, catalyzes the sequential transfer of electrons from reduced cytochrome *c* to dioxygen, coupling this reaction with electrogenic proton pumping across the inner mitochondrial membrane. Eukaryotic CcO is a heterooligomeric complex composed of 7 (*Dictyostelium discoideum*), 11 (*Saccharomyces cerevisiae*) and 13 (mammals) protein subunits embedded in the protein-rich

highly convoluted inner mitochondrial membrane. The core of the enzyme is composed of three mitochondrially encoded subunits that exhibit high evolutionary conservation. Unlike prokaryotic enzymes, mitochondrial CcOs consist of additional small peripheral subunits, encoded by the nuclear genome and synthesized in cytoplasm (Ludwig et al., 2001; Taanman, 1997). The redox-active heme and copper cofactors, directly involved in electron transfer, are coordinated by the mitochondrially encoded subunits Cox1 and Cox2 (Tsukihara et al. 1995).

The biogenesis of eukaryotic CcO complex is complicated by its subcellular location, the dual genetic origin of constituent subunits, the hydrophobic nature of most of them, and mainly by a number of prosthetic groups required for function, including two heme *a* moieties, three copper ions, and zinc, magnesium and sodium ions (Carr and Winge, 2003; Stiburek et al., 2006). Consequently, a number of specific gene products have evolved to accommodate such complex requirements. Although some of these factors act in a general manner and participate also in the biogenesis of other respiratory chain complexes, studies on yeast have identified over thirty accessory factors essential exclusively for proper biogenesis of the eukaryotic enzyme, while a number of them were shown to have human homologues (Fontanesi et al., 2008b; Herrmann and Funes, 2005). Isolated CcO deficiency represents one of the most commonly recognized causes of respiratory chain defects in humans associated with a wide spectrum of clinical phenotypes (Bohm et al., 2006; Shoubridge, 2001a). Pedigree studies suggest that the majority of genetic defects associated with fatal infantile CcO deficiency are of nuclear origin and inherited as autosomal recessive traits. To date, autosomal recessive mutations in six nuclear-encoded factors (SURF1, SCO1, SCO2, COX10, COX15, LRPPRC) required for the assembly of functional CcO complex have been identified in humans (Shoubridge, 2001a; Shoubridge, 2001b; Stiburek et al., 2006). In addition, mutations in *FASTKD2* and *ETHE1*, involved in apoptosis and sulfide catabolism, respectively, were also reported to result in severe, tissue-specific CcO defect (Ghezzi et al., 2008; Tiranti et al., 2009). However, the effects on CcO biogenesis of mutations in both of the gene products are thought to be essentially secondary.

3.1. CcO structure and function

Mammalian CcO is a heterooligomeric complex with a combined molecular weight of 205 kDa composed of thirteen structural subunits encoded by both the mitochondrial and nuclear genes (Stiburek et al., 2006; Taanman, 1997). The enzyme is embedded in the inner mitochondrial membrane, with one part extending 37 Å into the intermembrane space (IMS)

and an opposite part protruding 32 Å into the mitochondrial matrix. The three mitochondrially encoded subunits, Cox1, Cox2 and Cox3 constitute the structural core of the enzyme that incorporates all redox-active cofactors (Tsukihara et al., 1996; Yoshikawa et al., 1998). Cox1, the largest and the most evolutionary conserved subunit of the enzyme, is a highly hydrophobic protein composed of twelve transmembrane helices connected by short extramembrane loops. This subunit coordinates the catalytic site of the enzyme, and constitutes the two proton translocation pathways (D- and K-pathway) (Wikstrom, 2000). Recently, it was reported that pro-inflammatory cytokine TNFalpha inhibits oxidative phosphorylation by mediating phosphorylation of tyrosine 304 of Cox1 (Samavati et al., 2008). Cox2 is the smallest and the least hydrophobic subunit of the enzyme core. It consists of a large polar C-terminal domain that protrudes into IMS, and a transmembrane α-helical hairpin that anchors the subunit within the inner membrane. The C-terminal domain of Cox2, composed of 10-stranded β barrel, coordinates the Cu_A center and constitutes the docking site for cytochrome *c*. Similarly to Cox1, subunit Cox3 is a highly hydrophobic protein spanning the inner membrane with seven transmembrane helices. This subunit does not bear any prosthetic groups and is not directly involved in proton translocation. However, studies of the *Rhodobacter sphaeroides* aa_3-type CcO indicate that the presence of Cox3 maintains the rapid proton uptake into the D-channel at physiological pH, which presumably reduces the half-life of reactive dioxygen reduction intermediates (Gilderson et al., 2003; Hosler, 2004). This is thought to prevent the turnover-induced inactivation of the enzyme (suicide inactivation), and the subsequent loss of Cu_B site (Bratton et al., 1999; Hosler, 2004). The remaining 10 evolutionary younger subunits that associate with the surface of the complex core are encoded by the nuclear genome and are imported into mitochondria upon synthesis on cytoplasmic polysomes (Margeot et al., 2005). They include small polypeptides required for the stability/assembly of the holoenzyme, with several of them believed to be involved in regulation of its catalytic activity (Huttemann et al., 2008; Ludwig et al., 2001). It was shown that the exchange of bound ADP by ATP at the matrix domain of subunit Cox4 leads to allosteric inhibition of the bovine enzyme at high intramitochondrial ATP/ADP ratios (Kadenbach et al., 2000). Very recently, the expression of subunit Cox4 in the axons of sympathetic neurons was shown to be regulated at the posttranscriptional level by brain-specific microRNA miR-338 (Aschrafi et al., 2008). Transfection of precursor miR-338 into the axons of primary sympathetic neurons decreased Cox4 transcript and protein levels and resulted in diminished ATP levels suggestive of decreased OXPHOS activity. Some of the nuclear encoded subunits were shown to be expressed in tissue- and developmentally-specific

isoforms (Huttemann et al., 2008; Linder et al., 1995). In mammals, subunit Cox4 exists as two tissue-specific isoforms, Cox4-1 and Cox4-2 expressed ubiquitously, and at high levels in the lungs and trachea, respectively. Their expression is further regulated by oxygen availability and confers different kinetic properties to the enzyme. This regulatory circuit was shown to involve hypoxia-inducible factor 1α (HIF-1α), which is either hydroxylated under aerobic conditions and subsequently degraded by proteasome, or left unmodified. The unmodified HIF-1α accumulates in cell, which leads to transcriptional induction of both COX4-2 gene and the gene encoding mitochondrial matrix LON protease, responsible for the degradation of Cox4-1 (Semenza, 2007).

Subunits Cox5a, Cox5b and Cox6b are hydrophilic extramembrane proteins, while the rest of the nuclear encoded subunits are hydrophobic polypeptides spanning the membrane once. Subunit Cox5a is unique in that it does not interact with any of the mitochondrially encoded core subunits being held by the matrix domain of Cox4 and an extramembrane segment of Cox6c (Tsukihara et al., 1996).

Several pathogenic mutations in each of the three mitochondrially encoded CcO subunits have been reported (Shoubridge, 2001a). To date, however, only a single pathogenic mutation was found in genes encoding nuclear-encoded CcO subunits (Massa et al., 2008). It was reported that the R19H amino acid substitution in COX6B1 results in diminished assembly capacity of otherwise stable polypeptide, which leads to impairment in the late stage of CcO assembly. Affected individuals presented with severe infantile encephalopathy associated with isolated CcO deficiency (Massa et al., 2008).

The CcO complex from *S. cerevisiae*, composed of three mitochondrially encoded and eight nuclear encoded subunits closely resembles the mammalian counterpart. Yeast null mutants for the homologues of mammalian nuclear encoded subunits Cox4, Cox5a, Cox5b, Cox6c or Cox7a are respiratory deficient, lacking CcO activity and the absorption bands representing heme aa_3 (Taanman and Williams, 2001). This suggests that potential loss-of-function mutations in at least some of the human nuclear encoded CcO subunits might lead to early developmental lethality, hindering their identification.

Crystalline preparations of bovine heart CcO were shown to contain 13 lipids per enzyme dimer. These included two cardiolipins, one phosphatidylcholine, three phosphatidylethanolamines, four phosphatidylglycerols and three triglycerides. The X-ray structure resolution showed that the four phospholipids stabilize the dimeric form. The

palmitates, together with vaccenate (*cis*-Δ^{11}-octadecenoat) of phosphatidylglycerol, probably play an important role in the O_2 transfer process (Shinzawa-Itoh et al., 2007).

CcO contains several metal centers involved in electron transfer and dioxygen reduction. Besides their function in catalysis, most of these prosthetic groups confer an important structural/assembly function within the complex, since the defects in the synthesis and/or insertion of these cofactors often result in markedly reduced levels of fully assembled complex accompanied by appearance of subcomplexes (Stiburek et al., 2006). In addition to catalytic cofactors, the matrix portion-associated peripheral subunit Cox5b contains bound Zn(II) ion, while single Mg(II) ion is found at the interface of Cox1 and Cox2 subunits. Moreover, Cox1 contains single Na(I) ion in a site that can also bind Ca(II) (Yoshikawa et al., 1998). However, the functional relevance of these additional cofactors as well as their import/insertion pathways are largely unknown. The zinc atom could play a role in structural stability of the complex (Coyne et al., 2007), whereas the magnesium/manganese site located in close proximity to the water exit channel is thought to facilitate the release of water produced during the dioxygen reduction (Schmidt et al., 2003). The low-spin heme *a* and the heterobimetallic heme a_3-Cu_B center are located relatively deep within the hydrophobic interior of Cox1. In contrast, the binuclear, mixed-valent Cu_A center extends 8 Å above the surface of the inner membrane, being held by the IMS-located, C-terminal domain of Cox2. The two copper ions (Cu(I) and Cu(II)) of the Cu_A center are coordinated by two bridging cysteines of the Cx_3C motif, two histidines, one methionine and a carbonyl oxygen of the peptide backbone of Cox2 (Tsukihara et al., 1995).

The Cu_A center serves as the primary acceptor of electrons channeled through the respiratory chain. The electrons donated by cytochrome *c* are rapidly distributed between the Cu_A center and heme *a*, further continuing to the catalytic site composed of high-spin heme a_3 and an electronically coupled Cu_B ion. This part of the catalytic cycle referred to as the "reductive phase", ensures the reduction of heme a_3-Cu_B center, a prerequisite for binding of dioxygen (and CO, but not NO) to this site, and subsequent water formation ("oxidative phase") (Brunori et al., 2005; Michel et al., 1998). Protons required for the reduction of dioxygen, together with those translocated through the complex to the IMS, are taken up from the matrix and transferred via two distinct pathways to the vicinity of the heme a_3-Cu_B catalytic site. The K-pathway, named after a conserved lysine residue, is responsible for one- or two-proton supply during the reductive phase. The D-pathway, named after a conserved aspartic residue, transfers the remaining two or three "substrate" protons required for water formation, as well as four protons that are pumped to the IMS (Wikstrom, 2000). Recently,

Belevich and colleagues revealed the fundamental mechanism of coupling of electron transfer with proton translocation in CcO (Belevich et al., 2006). They showed that the electron transfer from heme *a* to the catalytic site is kinetically linked to an internal vectorial proton transfer, initiating the proton pump mechanism of the enzyme. The free energy released during the electron-transfer reactions is thus transformed into the electrochemical transmembrane gradient of protons that might be eventually utilized by the F_1F_O-ATP synthase (complex V) to power ATP synthesis.

Under mild detergent conditions and even when crystallized the majority of CcO exists as a dimer of two 13-subunit assemblies with contacts between monomers mediated merely by subunits Cox6a and Cox6b (Musatov and Robinson, 2002; Tsukihara et al., 1996). The electron-transfer activity of monomeric and dimeric form is comparable. It was suggested that the dimeric form of CcO may be required for proton translocation since this activity can be observed only with dimer. However, when reconstituted into phospholipid vesicles CcO spontaneously dimerizes, hindering the performance of proton translocation assay with monomeric enzyme. Recently, Stanicova et al. reported that the dimeric, dodecyl maltoside-solubilized complex shows high resistance to elevated hydrostatic pressure without any marked perturbations of its quaternary structure or functional activity after release of the pressure. In contrast, 3 kbar of hydrostatic pressure triggered multiple structural and functional alterations within the monomeric form. These included sequential dissociation of subunits Cox6a, Cox6b followed by Cox7a and Cox3 as well as irreversible loss of catalytic activity that dropped by as much as 60% (Stanicova et al., 2007). Thus, it appears that the dimerization of CcO plays a key structural-functional role, conferring maximal structural stability for the complex.

3.2. Synthesis and incorporation of heme moieties

Heme *a* is a unique heme derivative found exclusively in all eukaryotic and certain prokaryotic CcO enzymes. In contrast, many bacterial terminal oxidases utilize heme *b* or heme *o*, instead of heme *a* (Michel et al., 1998). Heme *a* differs from protoheme (heme *b* or ferroprotoporphyrin IX) in that the C2 vinyl side chain is replaced by an isoprenoid substituent and a methyl group is oxidized into a formyl group (Caughey et al., 1975). Heme represents a potentially toxic, hydrophobic iron chelate, which may facilitate harmful cellular process through ROS formation, e.g. oxidative membrane damage (Ryter and Tyrrell, 2000). Hence, the synthesis, delivery, and final incorporation of heme *a* into CcO must be carefully

regulated. Despite this fact, almost nothing is known concerning the regulation of heme *a* homeostasis. Recently, the possible role for copper as a regulator of heme *a* biosynthesis was investigated. However, no functional correlations could have been found (Morrison et al., 2005). Most of the yeast CcO accessory factors mutants characterized by blocked holoenzyme assembly and rapid turnover of intermediates is unable to accumulate heme *a*. Initially, this phenomenon was ascribed to increased turnover of free heme *a*, as a consequence of reduced Cox1 levels. However, this appears inconsistent with the fact that some of the yeast mutants retain high heme *a* levels, even when Cox1 is almost undetectable (Barros and Tzagoloff, 2002). Instead, preliminary studies have suggested that the synthesis of heme *a* is subject to either positive or negative regulation by intermediate/subunit of CcO at the level of heme *a* synthase (Cox15) (Barros and Tzagoloff, 2002).

The biosynthesis of heme *a* involves sequential conversion of heme *b*. The first step in this reaction is catalyzed by an inner membrane-associated farnesyl transferase (Cox10). It involves the conversion of C2 vinyl group on pyrrole ring A into a 17-hydroxyethylfarnesyl moiety (Tzagoloff et al., 1993). This reaction yields heme O, found as a final cofactor in some prokaryotic terminal oxidases. In the next reaction, the C8 methyl substituent on pyrrole ring D of heme O is oxidized into an aldehyde, thus generating heme *a*. This oxidation proceeds via two successive monooxygenase steps catalyzed by Cox15, an inner membrane-anchored heme *a* synthase (Brown et al., 2002). The matrix localized ferredoxin (Yah1) and ferredoxin reductase (Arh1) are thought to provide reducing equivalents during this reaction (Barros et al., 2002). Interestingly, Cox15 is itself presumably a heme-containing enzyme, employing a heme *b* cofactor at the active site (Svensson et al., 1996). Human homologues of both yeast Cox10 and Cox15 were identified sharing 33 and 42% sequence identity, respectively, with the yeast counterparts (Glerum and Tzagoloff, 1994; Petruzzella et al., 1998)). Mutations in both human genes were reported to result in isolated CcO deficiency associated with severely reduced heme *a* levels (Antonicka et al., 2003a; Antonicka et al., 2003b). In *COX10*-deficient fibroblasts and *COX15*-deficient heart mitochondria, the CcO-specific assembly defect is not accompanied by any accumulation of subassemblies (Williams et al., 2004). Muscle-specific *Cox10* knockout mice start to develop slowly progressive myopathy at the age of 3 months. Despite the fact that the CcO activity in muscle is diminished to less than 5% of control already in 2.5 months, the affected muscle retains almost normal contractile activity without any additional signs of oxidative damage or apoptosis (Diaz et al., 2005).

Two heme *a* moieties are found within the eukaryotic CcO. The bis-histidine low-spin heme *a* is a six-coordinate heme responsible for electron transfer. The second heme *a*, present

in the complex, is a five-coordinate, high-spin heme a_3, that forms a heterobimetallic site with the Cu_B ion, a place where dioxygen, CO or NO binds (Brunori et al., 2005; Michel et al., 1998). Both heme planes are oriented perpendicular to the membrane with their iron centers being 14 Å apart (Yoshikawa et al., 1998). The insertion of heme *a* moieties into the Cox1 subunit has not been characterized so far. As both metal centers are enfolded within the hydrophobic interior of Cox1, buried 13 Å below the membrane surface, their incorporation is likely to occur either on nascent Cox1 or on an early CcO subassembly (Carr and Winge, 2003). The Cox1·Cox4·Cox5a subcomplex, as structurally present in the mature CcO, constitutes an open channel through which it would be possible for both heme moieties to be incorporated into Cox1 from the IMS side (Cobine et al., 2006c).

3.3. Surf1 assembly factor

Human Surf1 is an integral protein of the inner mitochondrial membrane required for the assembly of the CcO complex. The mature form with a molecular mass of ~30 kDa is composed of two transmembrane domains with a central loop region facing the IMS (Yao and Shoubridge, 1999). Both transmembrane domains and the central loop region are required for proper insertion, but the C-terminal tail of the protein is dispensable. In contrast to yeast homologue Shy1 (Mashkevich et al., 1997), separately expressed N- and C-terminal transmembrane domains of human Surf1 are not able to form a functional protein (Yao and Shoubridge, 1999). Although the precise molecular role of human Surf1 in CcO biogenesis remains unknown, several lines of evidence indicate that the protein plays a role in some of the early events of CcO assembly, ranging from the insertion of heme a/a_3 into Cox1 to promotion/stabilization of early subunits' assembly. Human cells lacking Surf1 accumulate early assembly intermediates composed of merely Cox1, Cox4 and Cox5a, suggesting that the assembly is stalled at an initial stage (Stiburek et al., 2009; Williams et al., 2004). Surf1 orthologues are found in terminal oxidase operons of several prokaryote species, in which the mature CcO consists of only three core subunits known to associate early in the assembly process in eukaryotes (Bundschuh et al., 2008; Fernandez-Vizarra et al., 2009). Bacterial Surf1 orthologues have repeatedly been implicated in the insertion and/or stabilization of heme a/a_3, thought to occur concurrent or immediately after membrane insertion of Cox1 (Bundschuh et al., 2009; Smith et al., 2005).

Since the yeast Surf1 homologue Shy1 was repeatedly observed in high-molecular weight CcO-containing complexes, the protein is likely to have an additional role in some of

the late CcO assembly events as well (Nijtmans et al., 2001). Recently it was demonstrated that Shy1 associates with different inner membrane protein modules, including CcO subcomplexes and supercomplex species composed of partially and fully assembled forms of CcO and the cytochrome bc_1 complex. The association with complex III was observed even in the absence of the core subunit Cox2 (Mick et al., 2007). Based on these findings it was proposed that Shy1, together with another yeast CcO assembly factor Cox14 accompany transient forms of CcO, thereby maintaining their competence for the incorporation of additional subunits. This appears consistent with the previous finding that Cox13, the yeast homologue of human Cox6a seems to be assembled subsequently to formation of supercomplexes (Brandner et al., 2005a).

Yeast Shy1 was shown to genetically and/or physically interact with several factors involved in translational and early post-translational steps of Cox1 biogenesis. In *S. cerevisiae*, Cox1 synthesis is controlled by translational activators Mss51 and Pet309. The high-copy expression of Mss51, which also has a posttranslational role in Cox1 biogenesis, is able to suppress the CcO deficient phenotype of *shy1*Δ cells. Mss51 and Cox1 form a transient complex that is stabilized by Cox14. The accumulation of this complex is postulated to downregulate the translation of Cox1 under conditions of impaired CcO assembly by sequestering Mss51. Shy1 is thought to facilitate the release of Mss51 from the ternary complex, making it available for Cox1 translation (Barrientos et al., 2009). Recently, Coa1 and Coa2 were identified as new CcO assembly factors in yeast acting in early steps of enzyme assembly (Mick et al., 2007; Pierrel et al., 2007; Pierrel et al., 2008). Coa1 is an inner membrane-associated protein that exists as part of the Mss51.Cox14.Cox1 ternary complex, as well as in a complex with Shy1 alone. Coa1 appears to be involved in the transition of newly synthesized Cox1 from the Mss51 complex to a downstream intermediate involving Shy1. The respiration in both *coa1Δ* and *shy1*Δ cells is enhanced by coexpression of Mss51 and Cox10, suggesting that Coa1 may link heme incorporation and cotranslational insertion of Cox1. Coa2, which may possess a chaperone function, appears to act downstream of Coa1, likely stabilizing Cox1 during the heme a_3 insertion step. This may be accomplished by promoting Cox5a/Cox6 (human Cox4/Cox5a) association with Cox1, suggested to increase the stability of the subunit by stabilizing the loops connecting the 12 transmembrane helices (Pierrel et al., 2008). This is consistent with the fact that the HAP4 induced upregulation of subunits Cox5 and Cox6 suppresses the respiratory deficient phenotype of *shy1* cells (Fontanesi et al., 2008a).

Several lines of evidence indicate that Surf1/Shy1 may be responsible for the insertion of heme a_3 into Cox1. The *Rhodobacter sphaeroides* Surf1 homologue is required for insertion/stabilization of heme a_3 within Cox1, as the majority of CcO lacks heme a_3 in *R. sphaeroides* Surf1 null mutant (Smith et al., 2005). When coexpressed in *E. coli*, together with enzymes for heme *a* synthesis, the *Paracoccus denitrificans* Surf1 homologues Surf1q and Surf1c bind heme *a in vivo*, probably providing a protein-bound heme *a* pool for the insertion into Cox1 (Bundschuh et al., 2009). *cox11*Δ cells are peroxide sensitive showing almost 2-fold increase in protein carbonylation, consistent with enhanced ROS production (Khalimonchuk et al., 2007). The increased peroxide sensitivity of these cells is thought to stem from accumulation of a transient pro-oxidant Cox1-heme a_3 assembly intermediate. Depletion of Shy1 in *cox11*Δ background abrogates the peroxide sensitivity of these cells. Furthermore, *shy1*Δ cells remain peroxide resistant even upon overexpression of Cox15, known to markedly increase heme *a* levels in CcO assembly mutants. The identity of the pro-oxidant heme is confirmed by the fact that only heme a_3 has an open coordination site, which, when solvent exposed could catalyze formation of the hydroxyl radical pro-oxidant (Khalimonchuk et al., 2007).

Mss51, Cox14, Coa1 and Coa2 are found exclusively in fungi, lacking homologues in both higher eukaryotes and bacteria (Barrientos et al., 2009). Furthermore, yeast Shy1 possess extra C-terminal segments not conserved in Surf1 proteins of other species. Thus, it is not clear whether similar mechanisms mediated by functional homologues of these proteins exist also in higher eukaryotes.

Ubiquitous posttranscriptional silencing of SURF1 in *Drosophila melanogaster* is associated with 100% egg-to-adult lethality, underdevelopment of CNS, particularly of the optic lobes, and impaired locomotor behavior in larvae. The altered motor patterns are likely not due to structural and/or functional abnormalities of muscle fibers or reduction in contractile efficiency, but rather results from defective energy provision due to CcO deficiency. In contrast, a panneuronal knockdown led to slight impairment in locomotor behavior and photoreactivity and strikingly increased longevity when compared to controls. However, histological analysis failed to reveal any marked neurodegenerative damage in brains of these flies (Zordan et al., 2006).

In vertebrates, SURF1 is part of the very tightly organized and highly conserved surfeit gene cluster containing six housekeeping genes (SURF1–6) that encode both structurally and functionally unrelated proteins (Duhig et al., 1998). The reason for the

conservation of this structure over 250 million years of divergent evolution between birds and mammals remains obscure. Posttranscriptional silencing of Surf1 in zebrafish (*Danio rerio*) resulted in 50% reduction in CcO activity, developmental defects in endodermal tissues, cardiac function and swimming behavior. The hindbrain and neuronal tube exhibited dramatically increased apoptosis and secondary motor neurons were absent or abnormal. In contrast, the cardiac dysfunction was likely due to impaired energy metabolism, since heart was devoid of apoptotic cells, exhibiting increasingly poor performance over time (Baden et al., 2007). The described phenotype of Surf1 deficient zebrafish was almost identical to that of Cox5 deficient animals, suggesting that it can be readily attributed to general CcO deficiency rather than a specific lack of Surf1 protein (Baden et al., 2007).

Two Surf1 knockout mouse models were generated so far by using either replacement of exons 5-7 by neomycin-resistance (NEO) cassette (Agostino et al., 2003) or insertion of a *loxP* sequence in exon 7 of the gene (Dell'agnello et al., 2007). The prominent characteristic of the *SURF1*^{NEO} KO mice was high embryonic lethality, subsequently attributed mainly to deleterious effects of the presence of the *NEO* cassette. In contrast, the later *SURF1*^{loxP} knockouts showed, in addition to mild CcO deficiency, altered neuronal Ca^{2+} homeostasis, moderate functional and morphological abnormalities in skeletal muscle and liver and substantially prolonged lifespan (Dell'agnello et al., 2007). In sharp contrast to severe CNS involvement observed in human patients, neither mouse *SURF1* KO model exhibited spontaneous neurodegeneration at any age. The positive effects of ablation of murine Surf1 on the lifespan of knockout animals is strikingly similar to that of *D. melanogaster* CNS-wide Surf1 knockout. On this account, it was suggested that the partial suppression of respiratory chain activity might, possibly mainly due to attenuated ROS production, positively affect lifespan of (-/-) animals (Fernandez-Vizarra et al., 2009). Consistent with these findings, CcO deficiency was associated with reduced oxidative stress in CNS of *COX10* knockout mice (Fukui et al., 2007).

Mutations in *SURF1*, which account for the majority of nuclear-encoded, isolated CcO deficiencies in humans, are characterized by the development of Leigh syndrome, a subacute necrotizing encephalomyopathy (Zhu et al., 1998). Most of the identified *SURF1* mutations are predicted to lead to loss of the protein. Human as well as yeast cells lacking Surf1/Shy1 retain approx. 10-20% of the CcO activity of wild-type cells, indicating that the function of the protein is partially dispensable in both organisms (Yao and Shoubridge, 1999). In contrast to human Surf1, the lack of Shy1 leads to partially pleiotropic effects including increased levels of cytochrome *c* and elevated NADH-cytochrome *c* reductase activity (Mashkevich et

al., 1997). Furthermore, the human and yeast proteins fail to complement each. However, the proteins exhibit striking sequence similarity in some conserved domains and the elevated cytochrome *c* concentration and complex III activity might well reflect a compensatory response (Nijtmans et al., 2001). Analogous to yeast, overexpression of NF-YA, a catalytic subunit of the human homologue of the yeast HAP complex, leads to increase in CcO activity of Surf1-deficient fibroblasts. Unlike the yeast HAP complex, the human NF-Y complex is not directly involved in regulation of mitochondrial biogenesis and the molecular mechanism responsible for the suppression of the CcO defect in *SURF1* fibroblasts is not known (Fontanesi et al., 2008a).

The severe CcO deficiency of *SURF1* fibroblasts is accompanied by barely detectable changes in cellular respiratory rates under normoxic conditions (Pecina et al., 2003). The measurement of CcO oxygen kinetics by the partial oxygen pressure at half-maximal respiration rate revealed markedly attenuated affinity for oxygen of the residual enzyme. This aspect could exacerbate the respiratory defect in tissues where high energy demand meets up with very low oxygen pressure, such as in the brain, a prominent pathology site of *SURF1* patients (Pecina et al., 2004).

The fatal neurological phenotype of CcO deficient Leigh syndrome is associated with remarkable tissue pattern of CcO assembly impairment, pointing to profound tissue-specific character of regulation of CcO biogenesis (Stiburek et al., 2005). Intriguingly, various tissue samples carrying mutations in *SURF1* exhibit, similar to *SCO1* and *SCO2* tissues, marked tissue-dependent copper deficiency. This suggests that Surf1 is required, in a tissue-specific manner, to maintain proper cellular copper homeostasis (Stiburek et al., 2009). Interestingly, yeast cells lacking Shy1 are deficient in mitochondrial copper, whereas the total cellular copper content remains normal (Pierrel et al., 2007). Supplementation of *shy1*Δ cultures with exogenous copper partially rescues the respiratory capacity of these cells (Fontanesi et al., 2008a).

3.4. Delivery and insertion of copper ions

Copper ions are required in mitochondria for the formation of Cu_A and Cu_B sites in CcO and for the incorporation into IMS-located fraction of Cu/Zn-superoxide dismutase (Cobine et al., 2006a). Due to its chemical reactivity that may lead to deleterious side effects, the amount of free cellular copper is maintained at extraordinary low levels under physiological conditions (Rae et al., 1999). As a result, the delivery and compartmentalization

of copper is mediated by a specific subset of proteins termed copper metallochaperones that are thought to transfer copper ions to their target proteins via transient protein-protein interactions (ligand-exchange reaction) (Huffman and O'Halloran, 2001). Despite the recent progress in detailed structure-function characterization of several members of the mitochondrial CcO-specific copper delivery pathway, the fundamental mechanism which ensures the copper uptake into mitochondria still remains to be elucidated (Cobine et al., 2006a; Cobine et al., 2006c). Recently, it was shown that yeast mitochondria contain a significant pool of copper bound neither to proteins nor mitochondrial DNA (Cobine et al., 2004). This pool is found in matrix as a soluble, anionic, low molecular weight complex, responding to changes in cytoplasmic copper content. Although the identity of the yeast matrix copper ligand was not revealed yet (Cobine et al., 2004), a compound with the same fluorescent and chromatographic properties was found to be conserved in mouse liver (Cobine et al., 2006c). This copper pool likely serves as a reserve for metallation of mitochondrial copper metalloenzymes, since the overexpression of heterologous copper-binding proteins in yeast matrix results in respiratory growth defect, suppressible by exogenous copper supplementation (Cobine et al., 2006a). A number of proteins engaged in mitochondrial, CcO-specific copper trafficking have been identified in eukaryotes, while mutations in two of them (Sco1 and Sco2) were reported to lead to fatal neonatal CcO deficiency in human (Shoubridge, 2001b; Stiburek et al., 2006).

The small hydrophilic protein Cox17 that localizes both to the cytoplasm and the mitochondrial IMS was the first to be implicated in copper ion provision to CcO. It delivers Cu(I) to two downstream CcO copper-chaperones Sco1 and Cox11. The interaction of Cox17 with Sco1 and Cox11 is thought to be of transient nature. Based on its dual localization, Cox17 was initially proposed to act as a copper shuttle between the cytoplasm and IMS (Beers et al., 1997). The tethering of Cox17 to the inner membrane by a heterologous transmembrane domain renders the protein fully functional, suggesting that migration between the cytoplasm and IMS is not essential for its function (Maxfield et al., 2004). Deletion of *COX17* does not affect mitochondrial copper levels (Cobine et al., 2004). However, *in vitro* studies with purified proteins and the yeast cytoplasm assay have demonstrated that Cox17 is able to deliver Cu(I) to both Sco1 and Cox11 (Horng et al., 2004). In addition to the Cx_9C motif, Cox17 possess another two conserved Cys residues upstream of the twin Cys motif. The protein is functional without either of the two disulfides in the twin Cx_9C motif. Two Cu(I) conformers of Cox17 were described so far. One is a mononuclear Cu(I)-Cox17 monomer in the form of helical hairpin stabilized by two disulfide bonds with

Cys residues in the twin Cx_9C motif. The second Cu(I) conformer is an oligomeric protein complex containing reduced thiolates that is capable of binding a polycopper-thiolate cluster. The purification of Cox17 from the IMS yields a protein devoid of bound copper (Khalimonchuk and Winge, 2008).

Knockout of *COX17* in mice leads to lethality of (-/-) individuals between embryonic days E8.5 and E10, confirming the essential character of Cox17 (Takahashi et al., 2002). Human Cox17 orthologue has been identified that shares 48% sequence identity with yeast counterpart (Amaravadi et al., 1997). Overexpression of the human Cox17 rescues the CcO activity defect of human *SCO2* but not *SCO1*-deficient cells (Leary et al., 2004). HeLa cells in which the expression of Cox17 was downregulated by the use of siRNA show diminished levels of CcO and CcO-containing supercomplexes. The accumulation of a Cox1-containing subcomplex of 158 kDa devoid of Cox2 in these cells points to the role of human Cox17 in maturation of Cox2, but not Cox1 (Oswald et al., 2009).

Cox19 and Cox23 are two additional soluble Cx_9C class proteins implicated in copper transfer to CcO. Yeast cells lacking either Cox19 or Cox23 are respiratory deficient and have reduced CcO activity. Cox19 exhibits dual localization in IMS and cytoplasm, albeit only upon overexpression (Nobrega et al., 2002). Cox23 localizes to both IMS and cytoplasm (Barros et al., 2004). The CcO-specific respiratory defect of Cox19 null strain is not associated with decreased mitochondrial copper. Moreover, the mutant phenotype can not be rescued by addition of exogenous copper salts. Cox19 exists as a stable dimer, and recombinant protein was reported to bind Cu(I). The tethering of Cox19 to the inner membrane via transmembrane domain of Sco2 does not abrogate its function in CcO assembly (Cobine et al., 2006a). Cox19 is likely present within the IMS mostly in the reduced state, since when purified it contains titrable thiolates (Khalimonchuk and Winge, 2008). There is a correlation in the ability of Cox19 to bind Cu(I) and its *in vivo* function in CcO assembly (Rigby et al., 2007). The CcO-specific respiratory defect of Cox23 null mutant is rescued by increased concentrations of copper, but only when *COX17* is overexpressed simultaneously (Barros et al., 2004). However, the deletion of *COX23* does not affect mitochondrial copper level (Cobine et al., 2006c). Human orthologue of COX19 was identified that shares 40% sequence identity with yeast protein. Subcellular localization studies with full-length, GFP-fused, human Cox19 showed predominant cytoplasmic localization in HEK 293 cells (Sacconi et al., 2005).

Pet191 is another member of the twin Cx_9C class of proteins found in the IMS. In contrast to Cox17, Cox19 and Cox23, Pet191 is tightly associated with the IM facing the IMS

and its import does not rely on Mia40. Yeast cells lacking Pet191 are respiratory deficient due to isolated CcO assembly defect (Khalimonchuk et al., 2008). The protein does not appear to have a prominent function in copper delivery to CcO, as *pet191*Δ cells have normal mitochondrial copper levels. Pet191 may be involved in the maintenance of twin Cx_9C proteins within IMS (Khalimonchuk and Winge, 2008).

Human Sco1 and Sco2 are closely related inner mitochondrial membrane copper-binding proteins encoded by paralogous genes. They have been demonstrated to exert nonoverlapping, cooperative roles in copper delivery to CcO (Leary et al., 2004). In addition, they have been shown to be involved in the maintenance of cellular copper homeostasis, presumably by controlling cellular copper export (Leary et al., 2007; Leary et al., 2009b). Very recently, human SCO proteins were reported to carry out distinct, stage-specific roles during Cox2 synthesis and Cu_A site maturation (Leary et al., 2009a). Interestingly, the tumor suppressor p53 was shown to directly regulate mitochondrial respiration through transactivation of human *SCO2* transcription (Matoba et al., 2006). Mutations in both *SCO1* and *SCO2* cause severe tissue-specific CcO assembly impairment accompanied by marked copper deficiency (Leary et al., 2007; Stiburek et al., 2009; Stiburek et al., 2005). However, both genes have been shown to be ubiquitously expressed, displaying a similar expression pattern across human tissues. Mutations of *SCO1* have originally been reported in only a single pedigree, where the two patients, presenting with fatal infantile encephalomyopathy and hepatopathy, were compound heterozygotes carrying a nonsense mutation on one allele and a P174L missense mutation on the second allele (Valnot et al., 2000). Recently, we have studied a novel *SCO1* missense mutation (G132S) found in a patient with CcO deficiency and early onset hypertrophic cardiomyopathy, hypotonia, encephalopathy, and hepatopathy (Stiburek et al., 2009). Thus, the lack of an apparent cardiac involvement in the previously published *SCO1* cases, which was in sharp contrast to *SCO2* mutations, very likely resulted either from the considerably reduced survival time of both siblings or the distinct nature of the missense allele expressed in these patients. Indeed, P174L mutant Sco1 exhibits markedly altered functional properties and almost normal polypeptide levels (Cobine et al., 2006b), whereas the G132S allele appears to lead to a simple, yet almost complete loss of protein and function (Stiburek et al., 2009). In contrast to *SCO1*, mutations of *SCO2* are more common, with all reported patients carrying at least one E140K missense allele (Vesela et al., 2004). Mutations in *SCO2* cause fatal infantile encephalomyopathy and hypertrophic cardiomyopathy. *SCO2* patients homozygous for the E140K substitution have a delayed onset

and slightly prolonged course of the disease compared with compound heterozygotes (Vesela et al., 2004).

SCO proteins are integral inner membrane components consisting of a globular copper-binding domain that protrudes into the IMS (Buchwald et al., 1991). This domain exhibits a thioredoxin fold composed of a central four-stranded β sheet surrounded by four α helices (Williams et al., 2005). A single Cu(I) binding site formed by cysteinyl residues of the Cx_3C motif and a histidyl residue is found within the globular domain. The structures of the metal-free human Sco1 conformer and Cu_1Sco1 complex are similar with only loop 8 showing significant rearrangements (Banci et al., 2006). Sco1 and Sco2 are tethered to the inner membrane by a single N-terminal transmembrane helix that was shown to be functionally important in Sco1 (Beers et al., 2002). The human Sco2 conformer resembles human Sco1 with the exception of greater conformational dynamics (Banci et al., 2007). SCO proteins are thought to act downstream of Cox17 in copper delivery pathway to Cu_A site in Cox2. Sco1 was shown to be copper-metallated by Cox17 *in vitro*. It is not known whether Sco1 delivers both Cu(I) and Cu(II) ions to build the binuclear, mixed valent Cu_A center in Cox2. Consistent with the composition of Cu_A center, SCO proteins can bind either Cu(I) or Cu(II) ions (Horng et al., 2005). It was suggested that SCO proteins might form a complex in order to deliver two copper ions to Cox2 simultaneously (Leary et al., 2004). Recently, however, a sequential delivery scenario is favored (Jaksch et al., 2000; Leary et al., 2009a). The involvement of human SCO proteins in copper delivery to CcO is further supported by the fact that the missense mutations in human *SCO1* (P174L) and *SCO2* (E140K and S240F) are located in the vicinity of the conserved Cx_3C copper-binding motif (Jaksch et al., 2000; Valnot et al., 2000). Additionally, it was shown that the CcO defect of both *SCO1* and *SCO2*-deficient fibroblasts and myoblasts is at least partially rescued by exogenous copper supplementation (Jaksch et al., 2001; Leary et al., 2004). Finally, the overexpression of the human SCO proteins with conserved cysteinyl and histidyl residues substituted by alanines, fail to rescue the CcO deficiency of either *SCO1* or *SCO2*-deficient fibroblasts (Horng et al., 2005).

Yeast cells lacking Sco1 are devoid of CcO activity and show markedly diminished protein levels of Cox2. Although yeast also encode a Sco2 protein, capable of binding copper ions (Cobine et al., 2006c), this has no apparent function in CcO assembly (Glerum et al., 1996). Both yeast Sco1 and Sco2 were shown to physically interact with Cox2, albeit only upon overexpression (Lode et al., 2000). Recently, we have reported that human Sco1 physically interacts with the fully assembled CcO complex in both skeletal muscle and

HEK293 cell mitochondria (Stiburek et al., 2009). Originally, based on sequence similarity of Sco1 with the peroxiredoxin protein family, the protein was proposed to be involved in the maintenance of Cu_A site cysteines in reduced state (Chinenov, 2000). Furthermore, on the basis of high-resolution structural data, human Sco1 has been implicated to function as a redox switch in the IMS (Williams et al., 2005).

We have demonstrated that human Sco2 acts in a highly tissue-specific manner at an early stage of CcO assembly, very likely during the maturation of Cox2 subunit (Stiburek et al., 2005). Furthermore, we showed that the E140K substitution leads to severely diminished Sco2 levels in all probed tissues. Since this substitution was shown to slightly perturb copper-binding of Sco2 it was speculated that the stability of Sco2 may depend on it being copper-loaded. The previously identified missense mutations in *SCO1* (P174L) and *SCO2* (E140K and S240F) are located in the vicinity of the conserved Cx_3C motif and the essential histidyl residue, suggesting that the loss-of-function may relate to perturbed copper-binding of the protein. However, both *SCO2* missense mutations are associated with severely impaired stability of the protein and the E140K mutant was shown to retain appreciable residual function in terms of Cu(I) binding (Foltopoulou et al., 2004). Furthermore, the overexpression of the E140K mutant Sco2 in the corresponding mutant background led to rescue of the CcO defect. In contrast, the P174L mutation does not affect the ability of Sco1 to bind and retain copper ions, however, its ability to be copper loaded by Cox17 is severely compromised. Based on our inability to detect the residual G132S mutant Sco1 in the dimeric form on blue-native gels and the fact that the mutation lies in a protein region shown to be required for dimerization, we proposed that the dimerization is required to stabilize Sco1. The G132S Sco1 skeletal muscle mitochondria accumulated two Cox2-containing subcomplexes, whereas corresponding Sco2-deficient samples are characterized by the complete absence of such species. This suggests that Sco1 is very likely responsible for a different posttranslational aspect of Cox2 biogenesis than Sco2 (Stiburek et al., 2009). This appears further supported by the fact that the steady-state level of Sco2 was virtually unaffected in Sco1-deficient background. Very recently, Leary and colleagues have reported that the synthesis of Cox2 is diminished in human *SCO2*, but not *SCO1* cells. On the other hand, the newly synthesized Cox2 exhibited increased stability in *SCO2* cells when compared to controls. It was concluded that Sco2 is required for the synthesis of Cox2, in a manner that depends on its ability to bind copper, acting upstream of Sco1 during the biogenesis of Cox2. It was further proposed that the association of Sco2 with Cox2 is required immediately following its synthesis, possibly to recruit Sco1 to the subunit. Otherwise the nascent Cox2 is rapidly degraded. Thus, the

maturation of Cox2 appears to require formation of a complex that contains both SCO proteins, each with a functional Cx_3C copper-coordinating motif (Leary et al., 2009a). Leary and colleagues further demonstrated that a fraction of total Sco2 acts as a thiol-disulfide oxidoreductase, oxidizing the copper-coordinating cysteines in Sco1 during Cox2 maturation. Under physiological conditions the cysteines in Cx_3C motif of Sco1 exists as a mixed population of oxidized disulfides and reduced thiols. In contrast, Sco1 molecules from either SCO background exhibited altered ratio of oxidized to reduced cysteines. This ratio was shown to be shifted towards disulfides upon overexpression of wild-type Sco2, and towards thiols upon knockdown of mutant Sco2, in *SCO2* background (Leary et al., 2009a).

Tissues and/or fibroblasts cultures harboring mutations in *SCO1*, *SCO2*, *COX10* or *COX15* were found to exhibit marked copper-deficient phenotype, consistent with involvement of the corresponding gene products in regulation of cellular copper homeostasis. Importantly, the copper deficiency phenotypes of *SCO1*, *SCO2* and *COX15* mutant fibroblasts were shown to be fully dissociable from the respective CcO defects. Kinetic labeling studies using ^{64}Cu indicated that the copper defect of *SCO1* and *SCO2* patient fibroblasts is caused by a defect in cellular copper retention, rather than copper uptake. Although overexpression of *SCO2* was shown to suppress the copper defect, this rescue was only partial in *SCO1* background. This was attributed to aberrant signaling of the P174L Sco1 variant (Leary et al., 2007). Based on these findings, it was postulated that Sco1 and Sco2 are bifunctional proteins that interplay in order to generate a signal that modulates the rate of copper efflux from the cell. Since it was not possible to alter the copper content of control fibroblasts by changing the levels or relative ratio of SCO proteins, it is unlikely that similar mechanism could be involved under physiological conditions. On the other hand, it was observed that the cysteinyl residues of Sco1, but not those of other CcO assembly factors, shows altered oxidized-to-reduced ratio in copper-deficient mutant *COX10*, *COX15* and *SCO2* backgrounds relative to controls. And based on the observation that the cysteinyl residues of the P174L Sco1 variant are completely oxidized in the *SCO1* background, it was proposed that the mutant protein elicits a signal equivalent to that of the wild-type copper-loaded conformer, thereby signaling a state of cellular copper overload. Thus, it was concluded that the copper-related signaling of SCO proteins is modulated through changes in the redox state of Sco1's cysteine thiols (Leary et al., 2009a). Recently, we have shown that also affected tissues harboring mutations in *SURF1* exhibit marked tissue-dependent copper deficiency, further expanding the list of CcO assembly factors with possible role in cellular copper homeostasis maintenance. The observed association of human Sco1 with the fully assembled CcO prompted us to hypothesize that the

regulation of cellular copper homeostasis may involve cytochrome oxidase, as an important cellular copper recipient (Stiburek et al., 2009).

The inner-membrane protein Cox11 represents another mitochondrial CcO-specific copper metallochaperone. Similarly to SCO proteins, Cox11 is thought to function downstream of Cox17 in copper delivery to CcO, presumably inserting Cu(I) into Cox1 to build the Cu_B site. Yeast Cox11 null mutant have diminished CcO activity and reduced levels of Cox1 (Tzagoloff et al., 1990). The role for Cox11 in Cu_B site formation was implicated by the observation that CcO isolated from *R. sphaeroides* Cox11 null mutant lacked Cu_B site, along with diminished magnesium content and altered heme a_3 environment, but contained both heme moieties (Hiser et al., 2000). Like Sco1 and Sco2, yeast Cox11 is tethered to the inner membrane by a single N-terminal transmembrane helix, while the soluble C-terminal domain harboring three copper-binding cysteinyl residues protrudes into the IMS (Carr et al., 2002). These cysteinyl residues are responsible for both Cu(I)-binding and *in vivo* activity. Truncation of the transmembrane domain leads to soluble protein that dimerizes upon Cu(I) binding (Carr et al., 2002). Cox11 was shown to be copper-metallated by Cox17 (Horng et al., 2004). Two *COX11* homologues have been identified in human genome, however only one represents active gene predicted to encode protein with 55% sequence identity with yeast polypeptide (Petruzzella et al., 1998).

3.5. Assembly of the mammalian CcO complex

The spatiotemporal assembly of mammalian CcO within the inner mitochondrial membrane is a sequential, tissue-specific and relatively slow process (Nijtmans et al., 1998; Wielburski and Nelson, 1983). The half-life of the holoenzyme is thought to be about three days (Leary et al., 2002). Little is known about the sequential order in which prosthetic groups are delivered/synthesized and inserted, and constituent subunits are assembled to form the mature membrane-embedded complex. The fact that CcO subcomplexes are allowed to accumulate in human mitochondria, have permitted the *bona fide* definition of several key stages of this intricate process (Stiburek et al., 2006). In contrast, yeast CcO subcomplexes are difficult to detect as they likely undergo rapid proteolytic degradation (Horan et al., 2005). The nuclear encoded CcO subunits are imported into mitochondria upon synthesis on free cytoplasmic polysomes (Margeot et al., 2005). It is not known whether all of these subunits undergo conservative sorting or whether a subset of them is inserted from the IMS side. In contrast, most of the CcO accessory proteins are translated on outer membrane-attached

polysomes, and might be imported through the TOM machinery in a cotranslational manner (Margeot et al., 2005). The intramitochondrial steady-state levels of various unassembled CcO subunits differ considerably. Significant pools of free Cox1 and Cox5a appear to exist in mitochondria of various human tissues, whereas the levels of unassembled Cox4, and in particular of Cox2 are substantially lower (Stiburek et al., 2005). Cox1 appears to stably interact with several nonsubunit proteins before it associates with Cox4 and Cox5a, since it is readily detected as part of three 60-100 kDa complexes that apparently lack other CcO subunits. Subsequently, upon membrane insertion, Cox1 associates with the Cox4·Cox5a heterodimer, forming Cox1·Cox4·Cox5a subassembly (Stiburek et al., 2005). This subcomplex readily accumulates under conditions of blocked/retarded assembly, pointing to its high intrinsic stability (Nijtmans et al., 1998; Stiburek et al., 2005; Williams et al., 2004).

Two lines of evidence suggest that the insertion of heme *a* occurs either on unassembled Cox1 or during the formation of Cox1·Cox4·Cox5a subassembly. First, both heme moieties are buried deep within the transmembrane interior of Cox1, making the incorporation at the later stages unlikely (Tsukihara et al., 1996). Second, human cells deficient in heme *a* synthesis do not accumulate Cox1·Cox4·Cox5a subassembly (Antonicka et al., 2003b; Williams et al., 2004). The later finding also suggests that the presence of heme *a* within Cox1 might stabilize the binding of Cox4·Cox5a heterodimer to this subunit. In contrast, heme *a* is not required for the assembly of the core subunits in *R. sphaeroides* CcO (Hiser and Hosler, 2001). The insertion of active site heme might require the inner-membrane protein Surf1, since a significant fraction of CcO isolated from *R. sphaeroides* Surf1 null mutant was devoid of heme a_3 (Smith et al., 2005). Owing to the location of Cu_B site, its formation is likely to occur more or less concurrently with the insertion of heme groups. However, the presence of Cu_B ion within Cox1 does not seem to be essential for stable incorporation of heme a_3 (Hiser et al., 2000). The intrinsic inner-membrane protein Cox11 might be responsible for the formation of Cu_B site (Hiser et al., 2000). Upon assembly of heme moieties and formation of the Cu_B center, the Cu_A-containing Cox2 is believed to join the Cox1·Cox4·Cox5a subcomplex. Since the diminished formation of Cu_A site apparently leads to an accelerated turnover of Cox2 (Stiburek et al., 2005; Williams et al., 2004), formation of the Cu_A site in Cox2 appears to constitute a prerequisite for an efficient association of this subunit with Cox1·Cox4·Cox5a subcomplex. The increased proteolytic degradation of such Cox2 might result either from the lowered intrinsic stability of the protein or its reduced binding to Cox1. Conversely, the proper assembly of Cox2 appears indispensable for subsequent association of Cox3, and hence for the stable binding of most of

the remaining nuclear-encoded subunits. Indeed, a transmitochonrial cell line (cybrid) with 100% mutant load of a large C-terminal truncation in Cox3 was shown to lack the holoenzyme complex and accumulate subcomplex composed of Cox1, Cox2, Cox4 and Cox5a (Tiranti et al., 2000). Cox2 might be required to secure the incorporation of heme a_3, or whole active site, via capping the proposed heme-insertion channel formed in Cox1·Cox4·Cox5a subassembly (Cobine et al., 2006c). Upon assembly of Cox2 and Cox3 the remaining nuclear encoded subunits, with the exception of Cox6a and Cox7a or Cox7b, are thought to join the complex (Nijtmans et al., 1998). The resulting assembly intermediate S3 represents a ubiquitous, although minor form of CcO in lauryl maltoside preparations. Subsequent association of the rest of the subunits completes the assembly of the holoenzyme complex. In the next, maturation step a covalent bond is formed on assembled Cox1 bridging His^{240}, one of the three histidine ligands of Cu_B, with conserved Tyr^{244} located at the end of the proton translocation K-channel (Yoshikawa et al., 1998). This posttranslational modification is thought to secure the Cu_B ion in a certain configuration and distance from heme a_3, thus preventing the coordination of Cu_B via histidine ligands of the active site heme (Pinakoulaki et al., 2002). Finally, the mature holoenzyme complex associates with complex I and dimeric complex III, to form the 1.7-MDa respiratory supercomplex (Schafer et al., 2006; Schagger and Pfeiffer, 2000). The role of cardiolipin in final maturation of CcO and the function of cytochrome *c* during CcO assembly remains elusive.

4. Respiratory supercomplexes

Growing body of experimental evidence suggests that the interactions among the complexes of the mitochondrial oxidative phosphorylation system, and in particular of the respiratory chain, are not limited to their functional interplay during electron transfer reactions, mediated merely by the electron carriers coenzyme Q (CoQ) and cytochrome *c*, but extend well beyond that point to their stable and organized physical association within the membrane and their interdependence during assembly/biogenesis. Nowadays, it became widely accepted that the respiratory complexes are found within the inner membrane in the form of stable high order, supramolecular assemblies, so-called respiratory supercomplexes, the building blocks of functional association that was termed respirasome (Lenaz and Genova, 2007; Schagger and Pfeiffer, 2000). It was suggested that the functional relevance of supramolecular associations of respiratory complexes resides in facilitating the electron flux

between the complexes by reducing the distance of diffusion of mobile carriers, and by substrate channeling (Lenaz and Genova, 2007; Schagger and Pfeiffer, 2000).

Although stable assemblies of complexes III and IV were isolated in past from several prokaryote species including *P. denitrificans* and *Sulfolobus sp*., they were recognized as being characteristic of these bacteria (Berry and Trumpower, 1985; Iwasaki et al., 1995). More recently, mainly the development of BN-PAGE enabled detailed quantitative analysis of this phenomenon in both yeast and mammals (Schagger and Pfeiffer, 2000). In *S. cerevisiae*, which lacks complex I, the major supramolecular assemblies of respiratory complexes were found to be two species with apparent masses of 750 and 1,000 kDa containing merely subunits of complexes III and IV (Cruciat et al., 2000; Schagger and Pfeiffer, 2000). The smaller supercomplex was shown to consist of a complex III dimer and a complex IV monomer (III_2IV_1), whereas the larger supercomplex (III_2IV_2) contained a complex III dimer associated with two complex IV monomers. In contrast, supercomplex composed of complex I monomer and complex III dimer (I_1III_2) appears to be the most stable assembly of respiratory complexes found in mammalian mitochondria. On the other hand, supercomplex consisting of complex I monomer, complex III dimer and of one to two copies of complex IV monomer ($I_1III_2IV_{1-2}$) appears as the most physiologically relevant. However, only <10% of total mitochondrial complex IV was found to exist in supercomplex form in mammalian mitochondria (Schagger and Pfeiffer, 2000). The molecular architecture of both I_1III_2 and $I_1III_2IV_1$ supercomplexes was characterized using electron microscopy single particle image analysis (Schafer et al., 2006). Digitonin-solubilized mammalian mitochondria contain various additional minor stoichiometric variants of these two major species. Very recently, Acín-Pérez and colleagues demonstrated that some of the previously identified mammalian supercomplexes contain also complex II, and complex V, as well as electron carriers CoQ and cytochrome *c* (Acin-Perez et al., 2008). Importantly, the authors were also able to demonstrate that the supercomplexes containing complexes I, II, III, and IV, and the mobile carriers are capable of transferring electrons from NADH or succinate to oxygen, exhibiting properties of true respirasome.

In addition to the functional relevance with respect to electron transfer activity, supramolecular organization of respiratory complexes appears to play a role in the assembly/stability of the individual complexes, particularly of complex I. It has been repeatedly demonstrated in various systems that in the absence of appreciable amounts of either complex III or complex IV, the amount of fully assembled complex I is severely diminished. Human and mouse cells with complex III deficiency due to mutations in

mitochondrial gene encoding cytochrome *b* showed dramatic loss of complex I (Acin-Perez et al., 2004). Attempts to isolate complex I from *P. denitrificans* mutant strains lacking complexes III or IV led to complete dissociation of complex I under the conditions of BN-PAGE (Stroh et al., 2004). Mouse cells with severely reduced complex IV due to either downregulated expression of nuclear encoded subunit Cox4 or chromosomal deletion of *COX10* showed markedly diminished levels of complex I (Li et al., 2007). Finally, it was reported that the knockdown of Cox4 and Cox5a in *Caenorhabditis elegans* leads to enzymatic defect of complex I with otherwise normal amount of the assembled complex (Suthammarak et al., 2009). While the dependence of complex I biogenesis on the presence of complex III is relatively easy to reconcile due to ubiquitous character of I_1III_2 supercomplex, the contingence of complex I biogenesis upon complex IV remains obscure.

In yeast it was shown that complex IV associates with complex III already in the form of incomplete subcomplexes, with some of the late assembled subunits being likely added directly to the III/IV supercomplex (Mick et al., 2007). An example of such subunit was shown to be Cox13, the yeast homologue of human Cox6a (Brandner et al., 2005a). However, since complex IV assembly does not depend on complex III in yeast (Mick et al., 2007), the physiological relevance of this observation remains to be elucidated.

5. Degradation of proteins within the inner mitochondrial membrane

In contrast to most of the constituents of mammalian cellular proteome that were shown to exhibit half-lives of about 0.5 to 2 hours (Yen et al., 2008), most mitochondrial proteins are quite stable with half-lives of several days. A number of more or less specific proteases, including processing peptidases, ATP-dependent proteases, and oligopeptidases are found within the various subcompartments of mitochondria that mediate the selective proteolysis/degradation of mitochondrial proteins. However, if mitochondria are severely damaged or become superfluous due to altered physiological demands, they can be removed by a non-selective process, the autophagy (Mijaljica et al., 2007; Yorimitsu and Klionsky, 2005). Two ATP-dependent proteolytic machines, the *m*- and *i*-AAA metalloproteases that expose their catalytic domains to the opposite sides of the inner membrane are known to mediate the surveillance of protein quality control and eventually the selective degradation of non-assembled and damaged proteins in the inner mitochondrial membrane (Koppen and Langer, 2007).

5.1. i-AAA protease - Yme1

Yme1l1 is the human homologue (42% sequence identity, MW of processed protein ~69 kDa) of the yeast ATP-dependent i-AAA metalloprotease subunit Yme1 (*Y*east *m*itochondrial *e*scape 1) (Aldridge et al., 2007; Coppola et al., 2000; Shah et al., 2000). The yeast i-AAA protease is an inner membrane-anchored homooligomeric complex of approx. 1MDa with the active domain protruding into the IMS. It was shown to conduct protease, chaperone and translocase functions on the IMS side of the inner membrane (Leonhard et al., 1996; Leonhard et al., 1999). The yeast i-AAA protease complex belongs, along with the bacterial FtsH and mitochondrial m-AAA protease, to the group of so-called AAA (*A*TPases *A*ssociated with a variety of cellular *A*ctivities) proteases characterized by the presence of (i) a highly conserve Walker type ATPase module, (ii) a conserved HEXXH metal-binding module and (iii) membrane localization. Subunits of these complexes share a similar domain structure: they are anchored to the membrane by one or two membrane spanning segments at their N-terminal end, which are followed by one AAA domain and a metallopeptidase domain (Koppen and Langer, 2007). Inactivation of yeast Yme1 leads to temperature-sensitive respiratory growth defect, an increased frequency of DNA escape from mitochondria to nucleus, a petite-negative (ρ^-) phenotype and accumulation of mitochondria with aberrant morphology (Thorsness et al., 1993; Weber et al., 1996). Thus far, the yeast Yme1 was, either directly or indirectly, connected with the following protein substrates: Cox2, Phb1, Nde1, Atp4, Atp6, Atp17, Yme2, Inh1 and Yta10. It was shown that the ectopic overexpression of human Yme1l1 partially complements the termosensitive respiratory growth defect of the yeast *yme1* mutant, suggesting at least partial functional conservation of the human orthologue (Shah et al., 2000). Using a conserved *S. cerevisiae* import system, it was demonstrated that yeast Yme1 is required for mitochondrial translocation of ectopically expressed human polynucleotide phosphorylase (PNPase) (Rainey et al., 2006). On the other hand, the human Yme1l1 was so far indirectly connected only with the constitutive proteolysis of several splice variants of the intermembrane space dynamin GTPase Opa1 involved in mitochondrial fusion and cristae remodeling during apoptosis (Griparic et al., 2007; Song et al., 2007).

5.2. m-AAA protease - paraplegin (Spg7) and/or Afg3l2

In addition to the *i*-AAA complex, inner mitochondrial membrane harbors another homologous protease complex of the AAA type, the m-AAA protease. This oligomeric proteolytic machine with ATP-dependent metallopeptidase activity is active on the matrix side of the inner membrane. It exists in mammalian mitochondria in two forms: as a heterooligomer of paraplegin (Spg7)/Afg3l2 subunits and as a homooligomeric complex composed of solely Afg3l2 subunits (Koppen et al., 2007). In contrast to human i-AAA protease Yme1l1, much experimental attention was given to the human m-AAA complex in the past decade. This was mainly due to the fact that mutations in SPG7, which encodes the subunit paraplegin, are responsible for one form of Hereditary Spastic Paraplegia, a genetically heterogeneous neurological disorder characterized by a selective degeneration of long axons of the corticospinal tract and fasciculus gracilis (Atorino et al., 2003; Nolden et al., 2005; Rugarli and Langer, 2006). In yeast, the use of proteolytically inactive m-AAA protease as a substrate trap has led to the identification of mitochondrial ribosomal protein MrpL32 as a substrate of the m-AAA protease (Nolden et al., 2005). Unexpectedly, the protease does not affect the overall stability of the protein but cleaves off its N-terminal targeting sequence upon import of the precursor into mitochondria. The processing of MrpL32 is a prerequisite for the assembly of the protein with the mitochondrial ribosome, substantiating the mitochondrial translation defect and respiratory incompetence of yeast m-AAA protease deficient cells (Nolden et al., 2005). The yeast ROS-scavenger protein Ccp1 is another protein whose processing was shown to depend on m-AAA protease. Interestingly, it was demonstrated that m-AAA protease is not directly responsible for the cleavage of the bipartite presequence of Ccp1, but that it is required for ATP-dependent vectorial dislocation of Ccp1 precursor from the membrane bilayer. This activity mediated by the AAA domain of m-AAA protease is thought to ensure correct positioning of Ccp1 relative to membrane, most likely allowing intramembrane cleavage of the presequence by the rhomboid protease (Tatsuta et al., 2007).

CONCLUSIONS

- The loss-of-function of human Sco2 and Surf1 leads to highly tissue-specific patterns of CcO assembly impairment in terms of both diminished holoenzyme levels and accumulation of incomplete CcO subcomplexes. Together with the lack of pronounced tissue-specific differences in the expression of Sco2 and Surf1, it suggests tissue-dependent functional differences of both proteins that likely evolved to accommodate the profound tissue-specific requirements of metazoan mitochondrial function and biogenesis

- The dissection of subunit composition of CcO subcomplexes from Sco2 deficient mitochondria argues for the role of Sco2 in a distinct posttranslational step of Cox2 biogenesis, most probably in Cu_A site maturation. The accumulation of early CcO assembly intermediates in mitochondria with loss of Surf1 point to the role of the protein in some of the early stages of the process. The interdependence of association of Cox4 and Cox5a subunits with Cox1 during the assembly process brings new important addition to the current knowledge of human CcO assembly pathway

- The biochemical characterization of human OXA1L gene product showed that the processed form is a 42-kDa integral mitochondrial membrane protein that exists as part of a 600-700 kDa complex in mitochondria of HEK293 cells. The RNAi knockdown of OXA1L in HEK293 cells showed that the protein plays an important role in the biogenesis of F_1F_O-ATP synthase and respiratory complex I. In sharp contrast to the yeast orthologue, the loss of human Oxa1l does not lead to any impairments of assembly of CcO or the cytochrome bc_1 complex, suggesting functional divergence during evolution.

- Analysis of protein-protein interactions of wild-type Sco1 demonstrated that a fraction of the protein associates with the fully assembled CcO complex in both human muscle and HEK293 cell mitochondria. The immunoblot analysis of skeletal muscle expressing mutated (G132S) version of Sco1 showed that the mutation, which lies in a region required for protein dimerization, compromises stability of the protein, presumably by hindering its dimerization, leading to impairment of CcO assembly. The dissection of CcO

subcomplex pattern in Sco1-deficient muscle mitochondria suggests that the protein functions in a different posttranslational stage of Cox2 biogenesis than its paralogue Sco2.

- The severe copper defect of tissues harboring loss-of-function mutations in Sco1, Sco2 and Surf1 indicates either that each of these proteins plays an important role in cellular copper homeostasis maintenance or that the marked secondary CcO deficiency *per se* leads to highly tissue-specific pattern of cellular copper impairment in human. The former suggestion is supported by the association of a fraction of Sco1 with the CcO holoenzyme complex.

- Mutations that affect mt-tRNA^{Lys} (8363G>A, 8344A>G) lead to combined deficiency of complexes I and IV, compared to an isolated defect of complex I in the 3243A>G sample with impaired mt-tRNA$^{Leu(UUR)}$. The patterns of OXPHOS deficiencies in frontal cortex mitochondria of 8363G>A and 3243A>G patients differed substantially from those of other tissues. Particularly, in the frontal cortex mitochondria of the 3243A>G patient the assembly of complex IV appeared to be hindered by some factor other than the sole availability of mtDNA-encoded subunits.

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LIST OF ORIGINAL ARTICLES

Stiburek, L., Vesela, K., Hansikova, H., Pecina, P., Tesarova, M., Cerna, L., Houstek, J., Zeman, J. (2005) Tissue-specific cytochrome *c* oxidase assembly defects due to mutations in SCO2 and SURF1. *Biochem J,* 392, 625-32.

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Stiburek, L., Vesela, K., Hansikova, H., Pecina, P., Tesarova, M., Cerna, L., Houstek, J. and Zeman, J.

Tissue-specific cytochrome c oxidase assembly defects due to mutations in SCO2 and SURF1

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In this paper we addressed the roles of human Sco2 and Surf1 in the assembly of cytochrome *c* oxidase as well as the sequence of early events in the CcO assembly pathway. We analyzed the assembly state of CcO and the levels of Sco2 protein in various tissues of six patients carrying previously identified mutations in *SCO2* and *SURF1*.

We used fibroblast cell culture, spectrophotometric enzyme activity assays, isolation of crude mitochondrial fraction by cellular fractionation and differential centrifugation, blue-native (BN), two-dimensional (2D) BN/SDS and SDS-PAGE with downstream immunoblotting using battery of monoclonal and polyclonal antibodies against various mitochondrial proteins.

The biogenesis of eukaryotic CcO requires several accessory proteins in addition to structural subunits and prosthetic groups. SCO2 is a copper-binding protein presumably involved in formation of the CuA centre of the COX2 subunit. The function of SURF1 is unknown. Immunoblot analysis of native gels demonstrated that CcO holoenzyme is reduced to 10–20% in skeletal muscle and brain of *SCO2* and *SURF1* patients and to 10–30% in heart of *SCO2* patients, whereas liver of *SCO2* patients' contained normal holoenzyme levels. The steady-state levels of mutant SCO2 protein ranged from 0 to 20% in different *SCO2* patient tissues. In addition, eight distinct CcO subcomplexes and unassembled subunits were found, some of them identical with known assembly intermediates of the human enzyme. Heart, brain and skeletal muscle of *SCO2* patients contained accumulated levels of the COX1·COX4 ·COX5A subcomplex, three COX1-containing subcomplexes, a COX4·COX5A subcomplex and two subcomplexes composed of only COX4 or COX5A. The accumulation of COX1·COX4·COX5A subcomplex, along with the virtual absence of free COX2, suggests that the lack of the CuA centre may result in decreased stability of COX2. The appearance of COX4·COX5A subcomplex indicates that association of these nucleus-encoded subunits

probably precedes their addition to COX1 during the assembly process. Finally, the consequences of SCO2 and SURF1 mutations suggest the existence of tissue-specific functional differences of these proteins that may serve different tissue-specific requirements for the regulation of CcO biogenesis.

I contributed to this study by designing the research, performing part of the mitochondrial isolations, carrying out the vast majority of electrophoretic and immunoblot analyses, and by writing the manuscript. The work on this project was done in part in collaboration with Department of Bioenergetics, Institute of Physiology, ASCR.

Stiburek, L., Fornuskova, D., Wenchich, L., Pejznochova, M., Hansikova, H. and Zeman, J.

Knockdown of human Oxa1l impairs the biogenesis of F1Fo-ATP synthase and NADH:ubiquinone oxidoreductase.

Journal of Molecular Biology *2007*; *374*: 506-16

In this paper we addressed the molecular role and biochemical properties of human OXA1L gene product in the biogenesis of oxidative phosphorylation system.

We used human HEK293 cell culture, stable shRNA-mediated RNA interference approach, expression cloning, eukaryotic transfections and bacterial transformations, immunocytochemistry, confocal microscopy, subcellular and submitochondrial fractionation and localization, co-immunoprecipitation, spectrophotometric enzyme activity assays, oxygen consumption analysis, FACS analysis, isolation of crude mitochondrial fraction by differential centrifugation, rabbit OXA1L antibody design and preparation, BN, 2D BN/SDS and SDS-PAGE with downstream immunoblotting using battery of monoclonal and polyclonal antibodies against various mitochondrial proteins.

The Oxa1 protein is a founding member of the evolutionarily conserved Oxa1/Alb3/YidC protein family, which is involved in the biogenesis of membrane proteins in mitochondria, chloroplasts and bacteria. The predicted human homologue, Oxa1l, was originally identified by partial functional complementation of the respiratory growth defect of the yeast oxa1 mutant. We demonstrate that both the endogenous human Oxa1l, with an apparent molecular mass of 42 kDa, and the Oxa1l-FLAG chimeric protein localize exclusively to mitochondria in HEK293 cells. Furthermore, human Oxa1l was found to be an integral membrane protein, and, using two-dimensional blue native/denaturing PAGE, the majority of the protein was identified as part of a 600–700 kDa complex. The stable short hairpin (sh) RNA-mediated knockdown of Oxa1l in HEK293 cells resulted in markedly decreased steady-state levels and ATP hydrolytic activity of the F1Fo-ATP synthase and moderately reduced levels and activity of NADH:ubiquinone oxidoreductase (complex I). However, no significant accumulation of corresponding sub-complexes could be detected on

blue native immunoblots. Intriguingly, the achieved depletion of Oxa1l protein did not adversely affect the assembly or activity of cytochrome *c* oxidase or the cytochrome bc1 complex. Taken together, our results indicate that human Oxa1l represents a mitochondrial integral membrane protein required for the correct biogenesis of F1Fo-ATP synthase and NADH:ubiquinone oxidoreductase.

I contributed to this study by designing the research, maintaining the HEK293 cell culture, carrying out expression cloning, transfections and transformations, subcellular and submitochondrial fractionation and localization studies, electrophoretic and immunoblot analyses, immunocytochemical staining, co-immunoprecipitation assays, and by writing the manuscript.

Stiburek, L., Hansikova, H., Tesarova, M., Cerna, L. and Zeman, J.

Biogenesis of eukaryotic cytochrome c oxidase

Physiological Research *2006*; *55*, Suppl 2, S27-41.

In this paper we reviewed recent advancements in the understanding of the biogenesis of cytochrome *c* oxidase, with a focus on mammalian enzyme, and presented several unpublished results on the same subject.

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

I contributed to this study by writing the manuscript and performing all the mentioned studies including 2D BN/SDS-PAGE immunoblotting of Surf1 in Sco2-deficient heart mitochondria as well as expression cloning and immunocytochemistry of OXA1L-FLAG fusion protein in HEK293 cells.

Stiburek, L., Vesela, K., Hansikova, H., Hulkova, H. and Zeman, J.

Loss of function of Sco1 and its interaction with cytochrome c oxidase

American Journal of Physiology - Cell Physiology *2008*; *296*(5):C1218-26.

In this paper we assessed the impact on CcO assembly and tissue copper levels of a G132S mutation in the juxtamembrane region of SCO1 metallochaperone associated with early onset hypertrophic cardiomyopathy, encephalopathy, hypotonia, and hepatopathy, assessed the total copper content of various *SURF1* and *SCO2*-deficient tissues, and investigated the possible physical association between CcO and Sco1.

We used spectrophotometric enzyme activity assays, HEK293 cell culture, isolation of crude mitochondrial fraction by cellular fractionation and differential centrifugation, BN, 2D BN/SDS and SDS-PAGE with downstream immunoblotting using battery of monoclonal and polyclonal antibodies against various mitochondrial proteins, co-immunoprecipitation and copper content analysis using flame atomic absorption spectroscopy (FAAS).

Sco1 and Sco2 are mitochondrial copper-binding proteins involved in the biogenesis of the CuA site in the cytochrome *c* oxidase (CcO) subunit Cox2 and in the maintenance of cellular copper homeostasis. Human Surf1 is a CcO assembly factor with an important but poorly characterized role in CcO biogenesis. The steady-state level of mutant Sco1 was severely decreased in the muscle mitochondria of the *SCO1* patient, indicating compromised stability and thus loss of function of the protein. Unlike the wild-type variant, residual mutant Sco1 appeared to migrate exclusively in the monomeric form on blue native gels. Both the activity and content of CcO were reduced in the patient's muscle to ~10-20% of control values. *SCO1*-deficient mitochondria showed accumulation of two Cox2 subcomplexes, suggesting that Sco1 is very likely responsible for a different posttranslational aspect of Cox2 maturation than Sco2. Intriguingly, the various *SURF1*- deficient samples analyzed showed a tissue-specific copper deficiency similar to that of *SCO*-deficient samples, suggesting a role for Surf1 in copper homeostasis regulation. Finally, both blue native immunoblot analysis and coimmunoprecipitation revealed that a fraction of Sco1 physically associates with the CcO

complex in human muscle mitochondria, suggesting a possible direct relationship between CcO and the regulation of cellular copper homeostasis.

I contribute to this study by designing the research, performing part of the mitochondrial isolations, all electrophoretic and immunoblot analyses, human cell culture, and by writing the manuscript.

Fornuskova, D., Brantova, O., Tesarova, M., Stiburek, L., Honzik, T., Wenchich, L., Tietzeova, E., Hansikova, H., Zeman, J.

The impact of mitochondrial tRNA mutations on the amount of ATP synthase differs in the brain compared to other tissues

Biochimica et Biophysica Acta-Molecular Basis of Disease *2008*; *1782*: 317-25

In this paper we compared deficiency patterns of the individual OXPHOS complexes of various tissue samples of patients with Leigh (8363G>A), MERRF (8344A>G), and MELAS (3243A>G) syndromes due to mutations in mt-tRNA genes.

We used spectrophotometric enzyme activity assays, isolation of crude mitochondrial fraction by cellular fractionation and differential centrifugation, blue-native and two-dimensional blue-native/SDS-PAGE immunoblotting with battery of monoclonal and polyclonal antibodies against various mitochondrial proteins and oxygen consumption analysis.

The impact of point mutations in mitochondrial tRNA genes on the amount and stability of respiratory chain complexes and ATP synthase (OXPHOS) has been broadly characterized in cultured skin fibroblasts, skeletal muscle samples, and mitochondrial cybrids. However, less is known about how these mutations affect other tissues, especially the brain. Both mutations that affect mt-tRNA^{Lys} (8363G>A, 8344A>G) resulted in severe combined deficiency of complexes I and IV, compared to an isolated severe defect of complex I in the 3243A>G sample (mt-tRNA$^{Leu(UUR)}$). Furthermore, we compared obtained patterns with those found in the heart, frontal cortex, and liver of 8363G>A and 3243A>G patients. In the frontal cortex mitochondria of both patients, the patterns of OXPHOS deficiencies differed substantially from those observed in other tissues, and this difference was particularly striking for ATP synthase. Surprisingly, in the frontal cortex of the 3243A>G patient, whose ATP synthase level was below the detection limit, the assembly of complex IV, as inferred from 2D-PAGE immunoblotting, appeared to be hindered by some factor other than the availability of mtDNA-encoded subunits.

I contributed to this study by assisting in research design, performing part of the electrophoretic and immunoblot analyses, and by helping to write the final manuscript.

Tissue-specific cytochrome *c* oxidase assembly defects due to mutations in *SCO2* and *SURF1*

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The biogenesis of eukaryotic COX (cytochrome *c* oxidase) requires several accessory proteins in addition to structural subunits and prosthetic groups. We have analysed the assembly state of COX and SCO2 protein levels in various tissues of six patients with mutations in *SCO2* and *SURF1*. SCO2 is a copper-binding protein presumably involved in formation of the Cu_A centre of the COX2 subunit. The function of SURF1 is unknown. Immunoblot analysis of native gels demonstrated that COX holoenzyme is reduced to 10–20 % in skeletal muscle and brain of *SCO2* and *SURF1* patients and to 10–30 % in heart of *SCO2* patients, whereas liver of *SCO2* patients' contained normal holoenzyme levels. The steady-state levels of mutant SCO2 protein ranged from 0 to 20 % in different *SCO2* patient tissues. In addition, eight distinct COX subcomplexes and unassembled subunits were found, some of them identical with known assembly intermediates of the human enzyme. Heart, brain and skeletal muscle of *SCO2* patients contained accumulated levels of the COX1 · COX4 · COX5A subcomplex, three COX1-containing subcomplexes, a COX4 · COX5A subcomplex and two subcomplexes composed of only COX4 or COX5A. The accumulation of COX1 · COX4 · COX5A subcomplex, along with the virtual absence of free COX2, suggests that the lack of the Cu_A centre may result in decreased stability of COX2. The appearance of COX4 · COX5A subcomplex indicates that association of these nucleus-encoded subunits probably precedes their addition to COX1 during the assembly process. Finally, the consequences of SCO2 and SURF1 mutations suggest the existence of tissue-specific functional differences of these proteins that may serve different tissue-specific requirements for the regulation of COX biogenesis.

Key words: assembly pathway, Cu_A centre, cytochrome *c* oxidase, mitochondria, SCO2, SURF1.

INTRODUCTION

Eukaryotic COX (cytochrome *c* oxidase), the terminal enzyme of the mitochondrial respiratory chain, is embedded in the inner mitochondrial membrane where it catalyses the transfer of electrons from reduced cytochrome *c* to molecular oxygen and further couples this reaction with proton translocation across the inner membrane. Mammalian COX is a multisubunit complex of approx. 200 kDa composed of 13 subunits encoded by both the mitochondrial and nuclear genes. The mitochondrially encoded subunits COX1, COX2 and COX3 are evolutionarily conserved and form the catalytic and structural core of the enzyme [1]. The remaining ten evolutionarily younger subunits are encoded by the nuclear genome and are associated with the surface of the complex core. These small polypeptides are required for the stability and assembly of the holoenzyme and are also involved in modulation of its activity in response to various cellular stimuli [2]. Tissue-specific isoforms of subunits COX4, COX6A, COX6B and COX7A were identified in humans [3,4]. In addition to the constituent protein subunits, COX contains several redox-active prosthetic groups directly involved in electron transfer. These are two haem A moieties (*a* and a_3) and two copper centres (Cu_A and Cu_B). The Cu_B centre and both haem A moieties are located within the hydrophobic interior of COX1 subunit composed of 12 transmembrane α-helices. The binuclear Cu_A centre is located within the ten-stranded β-barrel that forms the polar C-terminal domain of COX2, which protrudes into the intermembrane space. This subunit is anchored to the membrane by two N-terminal transmembrane α-helices that make extensive contacts with COX1 [5]. The assembly pathway of mammalian COX in the inner mitochondrial membrane is a sequential and relatively slow process that is still not fully understood [6,7]. Studies on yeast have identified more than 30 accessory proteins essential for proper biosynthesis or assembly of the enzyme. To date, mutations in six nucleus-encoded factors (SURF1, SCO1, SCO2, COX10, COX15 and LRPPRC) required for the assembly of the COX complex have been identified in humans [6–8].

SCO2 is an inner mitochondrial membrane copper-binding protein presumably involved in copper transfer to the Cu_A centre of COX2. The molecular mass of its fully processed form is approx. 25 kDa. Mutations in human *SCO2* cause fatal infantile COX deficiency with the predominant symptoms being encephalopathy and hypertrophic cardiomyopathy. To date, all patients identified were either compound heterozygotes for 1541G > A (where 1541G > A denotes the guanine > adenine nucleotide transition at the position 1541 of the DNA) mutation, with the other allele carrying either a nonsense or missense mutation, or homozygotes for this common 1541G > A transition, predicting a E140K amino acid substitution near the highly conserved CXXXC putative copper-binding motif [9–11]. The most severe cases (early onset) are compound heterozygotes, while patients homozygous for E140K substitution have a comparatively milder phenotype (delayed onset, less progressive).

Human SURF1 is a 30 kDa transmembrane protein localized in the inner mitochondrial membrane [12,13]. The precise function of this protein is still unknown, but recently it was suggested that

Abbreviations used: BN-PAGE, Blue Native PAGE; COX, cytochrome *c* oxidase; CS, citrate synthase; DDM, n-dodecyl-β-D-maltoside; SDH, succinate: ubiquinone oxidoreductase; VDAC, voltage-dependent anion channel.

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human SURF1 promotes the association of COX2 with the COX assembly intermediate composed of COX1, COX4 and COX5A [14], which was originally described as COX1- and COX4-containing assembly intermediate S2 [15]. Mutations in human *SURF1* cause Leigh syndrome, a fatal neurological disorder associated with severe isolated COX deficiency and characterized mainly by bilaterally symmetrical necrotic lesions in the basal ganglia and brainstem [16,17]. Nearly all reported *SURF1* patients carried loss-of-function mutations that predict either truncated and unstable protein product or unstable mRNA [18].

Histochemical studies and enzyme activity measurements show that *SCO2* mutations result in a tissue-specific decrease of COX activity, with heart and skeletal muscle being most severely affected. In contrast, cultured fibroblasts and liver were shown to retain high residual activity [9–11], indicating tissue-specific differences in COX biogenesis or maintenance. The COX activity was reduced to approx. 10 % of control values in *SURF1* patient fibroblasts [14], and the skeletal muscle of *SURF1* patients was repeatedly shown to retain approx. 20 % of residual COX activity [19]. In *SURF1* fibroblasts, the reduction of COX activity was shown to be accompanied by a similar decrease in holoenzyme levels and also by a marked accumulation of COX subcomplexes, suggesting that the residual enzyme is fully active and that the enzyme deficiency stems from impaired assembly or maintenance of the protein complex [14].

The aim of the present study was to examine and compare the consequences of *SCO2* and *SURF1* mutations in various human tissues. We have investigated the steady-state levels of COX holoenzyme and the presence and composition of COX subcomplexes in tissues and primary fibroblast cultures from three patients harbouring *SCO2* mutations and from three patients carrying mutations in *SURF1*. We directly demonstrate that mutations in both genes result in tissue-specific decrease of COX holoenzyme levels with a parallel reduction in the COX activity, accompanied by accumulation of specific COX subcomplexes and unassembled subunits. We further show that all investigated *SCO2* mutations result in severely decreased levels of mutant SCO2 protein in heart, brain, liver and fibroblasts. The subunit composition of COX subcomplexes identified demonstrates the involvement of human SCO2 in biogenesis or maintenance of COX2 and suggests an addition to the current model of the human COX assembly pathway.

EXPERIMENTAL

Ethics

The present study was carried out in accordance with the Declaration of Helsinki of the World Medical Association, and was approved by the Committees of Medical Ethics at all collaborating institutions. Informed parental consent, in accordance with guidelines of the participating institutions, was obtained for all biopsies and autopsies.

Patients

Patients P1 and P2 were homozygous for 1541G > A transition in *SCO2* leading to E140K amino acid substitution. Patient P3 was a compound heterozygote for the 1280C > T transition in *SCO2*, leading to the formation of a premature termination codon, and a common missense 1541G > A mutation [20]. Patient P4 was a compound heterozygote for the combined frameshift deletion-insertion mutation in *SURF1* 312_321del 311_312insAT, resulting in the formation of a premature termination codon, and a 821del18 deletion in *SURF1* leading to highly unstable mRNA [21]. Patient P5 was homozygous for the frameshift deletion 845_846delCT leading to the formation of a premature termination codon, and patient P6 was homozygous for a 688C > T nonsense substitution in *SURF1* leading to the formation of a premature termination codon truncating the protein at Arg^{230} [22]. Patients P1 and P2 presented with progressive encephalopathy since the third month of age, and they died at < 1 year of age [20]. Patient P3 presented with hypertrophic cardiomyopathy since birth and he died at 7 weeks of age [20]. Patients P4–P6 presented with failure to thrive, progressive hypotonia and hypertrichosis at the end of the first year of life, followed by a total arrest of psychomotor development, and all died around the third year of age [21,22].

Cell cultures and tissues

All studied tissues and primary fibroblast cultures were obtained from three patients (P1–P3) harbouring two different combinations of *SCO2* mutations, three patients (P4–P6) carrying mutations in *SURF1* and age-related controls. Primary skin fibroblast cultures were established from forearm skin biopsies. Open muscle biopsies were obtained from the tibialis anterior muscle and were frozen at − 80 °C. Post-mortem heart, liver, brain (basal ganglia) and kidney tissue specimens were removed and frozen less than 2 h after death.

Isolation of mitoplasts and mitochondria

Skeletal muscle, brain and kidney mitochondria were isolated according to standard differential centrifugation procedures [23] in a buffer comprising 150 mM KCl, 10 mM Tris/HCl, 2 mM EDTA and 2 μg/ml aprotinin (pH 7.4) at 4 °C. Heart and liver mitochondria were isolated in a buffer comprising 250 mM sucrose, 20 mM Tris/HCl, 2 mM EDTA and 2 μg/ml aprotinin (pH 7.4) at 4 °C. Mitoplast-enriched fractions were prepared from cultured fibroblasts using digitonin (Sigma–Aldrich) as described in [24], with a final digitonin/protein ratio of 0.6 mg/mg. Protein concentrations were determined with the Bio-Rad Protein Assay kit (Bio-Rad Laboratories). All samples were stored at − 80 °C.

Enzyme activity assays

Activities of COX and CS (citrate synthase) were measured spectrophotometrically in fibroblasts and isolated tissue mitochondria essentially as described in [25].

Electrophoresis

BN-PAGE (Blue Native PAGE) [26] was used for separation of mitochondrial membrane protein complexes on polyacrylamide 8–15, 8–16 and 10–18 % (w/v) gradient gels using a Mini Protean® 3 System (Bio-Rad Laboratories). Mitoplasts or mitochondria were solubilized with DDM (n-dodecyl β-D-maltoside; Sigma–Aldrich) with a final DDM/protein ratio of 1.0 mg/mg in a buffer containing 1.5 M aminocaproic acid, 2 mM EDTA and 50 mM Bis-Tris (pH 7.0) at 4 °C. Serva Blue G (Serva) was added to solubilized protein at a concentration of 0.1 mg/mg of detergent, and 5–50 μg of protein was loaded for each lane. The electrophoresis was performed at 40 V, 4 °C for 1 h and then at 100 V, 4 °C. Tricine SDS/PAGE was carried out under standard conditions with 12 % polyacrylamide, 0.1 % (w/v) SDS and 5.5 M urea gels. Mitochondrial fractions were dissociated in 50 mM Tris/HCl (pH 6.8), 12 % (v/v) glycerol, 4 % SDS, 2 % (v/v) 2-mercaptoethanol and 0.01 % (w/v) Bromophenol Blue for 30 min at 37 °C, and approx. 10 μg of protein was loaded for each lane. For two-dimensional BN/SDS/PAGE [26], strips of the

Table 1 Comparison of relative protein content of COX holoenzyme with relative COX activity in various tissues of *SCO2* patients P1–P3 and *SURF1* patients P4–P6

Protein content of COX holoenzyme normalized to protein content of SDH is expressed as a percentage of control values (COX/SDH content). Values of COX activity normalized to CS activity are expressed as a percentage of the mean reference range (COX/CS activity). n.d., not determined.

	P1		P2		P3		P4		P5		P6	
Tissue	COX/SDH content (%)	COX/CS activity (%)	COX/SDH content (%)	COX/CS activity (%)	COX/SDH content (%)	COX/CS activity (%)	COX/SDH content (%)	COX/CS activity (%)	COX/SDH content (%)	COX/CS activity (%)	COX/SDH content (%)	COX/CS activity (%)
Heart	25	8	30	34	10	30	40	n.d.	n.d.	n.d.	n.d.	n.d.
Muscle	10	19	20	28	n.d.	21	n.d.	11	15	22	10	9
Brain	20	16	20	18	20	13	15	20	n.d.	n.d.	n.d.	n.d.
Liver	100	76	100	64	100	100	80	n.d.	n.d.	n.d.	n.d.	n.d.
Fibroblasts	70	62	70	82	60	100	10	12	15	6	15	14

first-dimension gels were incubated for 40 min in 1 % 2-mercaptoethanol and 1 % SDS and then for 10 min in 1 % SDS, and denatured proteins were then resolved in the second dimension on 13 % polyacrylamide, 0.1 % SDS and 5.5 M urea gels [14,26].

Preparation of a polyclonal antibody raised against human SCO2

An SCO2-specific antibody was generated by injecting rabbits with a synthetic peptide specific for the C-terminal part of human SCO2 (CGRSRSAEQISDSVRRHMAAF). Testing of the specificity of the SCO2 antiserum revealed that affinity purification was not required, and crude serum was used in all subsequent experiments.

Immunoblot analysis

Proteins were electroblotted from the gels on to Immobilon™-P PVDF membranes (Millipore) using semi-dry transfer for 2 h at a constant current of 0.8 mA/cm^2. Membranes were air-dried overnight, rinsed twice with 100 % (v/v) methanol and blocked in PBS and 10 % (w/v) non-fat dried milk for 1 h. Primary detection was performed with mouse monoclonal antibodies raised against COX subunits COX1 (A-6403; 1 μg/ml), COX2 (A-6404; 1 μg/ml), COX4 (A-21348; 2 μg/ml), COX5A (A-21363; 2 μg/ml) and COX6B (A-21366; 1 μg/ml) (Molecular Probes), with rabbit polyclonal antiserum raised against human SCO2 (1:1000) and with monoclonal antibodies raised against the flavoprotein subunit of SDH (succinate:ubiquinone oxidoreductase) (A-11142; 0.1 μg/ml) (Molecular Probes) and the VDAC (voltage-dependent anion channel) (31HL Ab-1; 1.4 μg/ml) (Calbiochem) at indicated dilutions. Blots were incubated with primary antibodies in PBS, 0.3 % (v/v) Tween 20 and 1 % non-fat dried milk for 2 h. Secondary detection was carried out with goat anti-mouse IgG–horseradish peroxidase conjugate (A8924; 1:1000) (Sigma–Aldrich) or with goat anti-rabbit IgG–horseradish peroxidase conjugate (A0545; 1:2000) (Sigma–Aldrich) in PBS, 0.1 % Tween 20 and 1 % non-fat dried milk for 1 h. The blots were developed with West Pico Chemiluminescent substrate (Pierce) and exposed to Kodak BioMax Light films (Kodak). The films were subsequently scanned and digital images were analysed using the Quantity One application (Bio-Rad Laboratories).

RESULTS

Activities of COX in *SCO2* and *SURF1* patient tissues

Previous respiratory chain enzyme activity measurements of tissues and cell cultures from our patients with *SCO2* and *SURF1* mutations showed an isolated tissue-specific COX deficiency in all patients [20–22]. To determine the residual COX activity of the mitochondrial preparations used in the present study, we expressed COX activity relative to the activity of the mitochondrial marker enzyme, CS. Severe isolated defects of COX activity were found in the *SCO2* patient heart, skeletal muscle and brain, whereas in fibroblasts and liver the activity was only moderately affected (Table 1). In contrast, severe reduction of COX activity was found in all of our *SURF1* fibroblast cultures (Table 1).

Steady-state levels of COX holoenzyme in *SCO2* and *SURF1* patient tissues

All mitochondrial preparations used in the present study were balanced on the basis of the immunoblot signal of the mitochondrial inner membrane protein complex SDH. To determine the residual steady-state levels of COX holoenzyme in tissues of patients as a percentage of control values, dilutions of control mitochondria were loaded on the same gels. Mitochondrial samples were resolved using BN-PAGE and subsequently probed with an anti-COX1 antibody. In heart mitochondria from patients P1, P2, P3 and P4, the steady-state levels of COX holoenzyme were found to be approx. 25, 30, 10 and 40 % of control values respectively (Figures 1A and 1B). Mitochondria from basal ganglia of patients P1–P3 and P4 contained approx. 20 and 15 % of residual holoenzyme respectively (Figure 1C). In skeletal muscle from patients P1, P2, P5 and P6, the holoenzyme levels were approx. 10, 20, 15 and 10 % of control values respectively (Figure 1D). In primary fibroblasts, the steady-state levels of COX holoenzyme were found to be approx. 70 % of control values in the case of patients P1 and P2, approx. 60 % in the case of patient P3 (Figure 1E) and approx. 15 % in the case of patients P5 and P6 (Table 1). The liver samples of *SCO2* patients (P1–P3) were the least affected and contained similar steady-state levels of COX holoenzyme to control samples, whereas in *SURF1* patient liver (P4) the holoenzyme was found to be approx. 80 % of control values (Figure 1F) (Table 1).

Subcomplexes of COX in *SCO2* and *SURF1* patient tissues

Mitochondrial preparations from various *SCO2* and *SURF1* patient tissues, primary fibroblast cultures and control samples were resolved using either BN-PAGE or two-dimensional BN/SDS/PAGE and subsequently probed with anti-COX subunit-specific monoclonal antibodies in order to detect the presence and possible accumulation of COX subcomplexes and to uncover their subunit composition. In addition to holoenzyme complex (Figures 1A and 3A, complex a), heart samples of *SCO2* patients contained

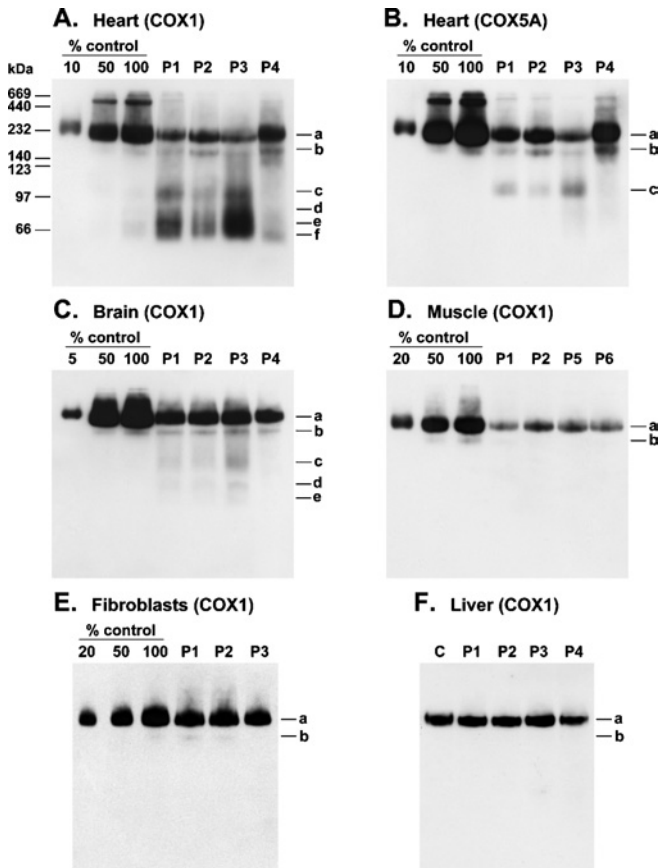


Figure 1 Steady-state levels of COX holoenzyme and subcomplexes in tissues and fibroblast cultures of *SCO2* and *SURF1* patients

Mitochondrial fractions from *SCO2* (P1–P3) and *SURF1* (P4–P6) patient samples were resolved by BN-PAGE (8–15 % polyacrylamide), electroblotted on to PVDF membranes and probed with monoclonal antibodies specific for subunits COX1 (**A**, **C**–**F**) or COX5A (**B**). The amount of protein loaded per lane (~5 μg) was normalized to SDH. Three aliquots of control mitochondria corresponding to indicated dilutions of control samples were loaded on the same gels. Immunoreactive material was visualized by chemiluminescence. The positions of COX holoenzyme (a), COX subcomplexes (b–f) and molecular-mass standards (kDa) are indicated.

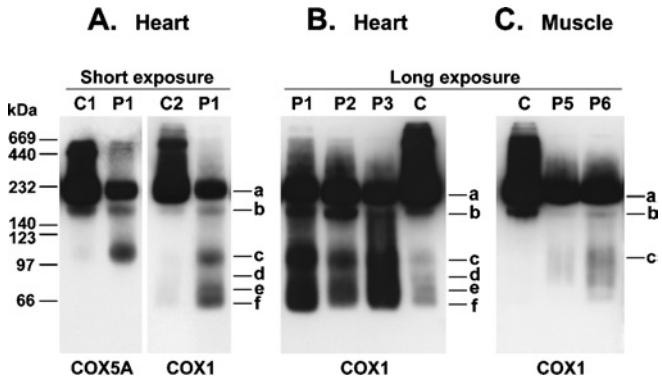


Figure 2 COX subcomplexes in control heart, heart of *SCO2* patients and skeletal muscle of *SURF1* patients

Mitochondrial fractions from *SCO2* patient heart (P1–P3) (**A**, **B**), *SURF1* patient skeletal muscle (P5 and P6) (**C**) and control samples were resolved by BN-PAGE (8–15 % polyacrylamide), electroblotted on to PVDF membranes and probed with monoclonal antibodies specific for subunits COX1 or COX5A. The amount of protein loaded per lane (~5 μg) was normalized to SDH. Immunoreactive material was visualized by chemiluminescence. The positions of COX holoenzyme (a) and COX subcomplexes (b–f) and the molecular-mass standards (kDa) are indicated.

eight distinct COX subcomplexes (b–i). Prolonged exposure of the blots revealed the presence of six of them (b–f and i) also in control heart samples (Figures 2B and 3A). Subcomplexes c–i were found in *SCO2* heart samples at highly accumulated levels, whereas only subcomplexes b and f were found slightly increased in the heart of the *SURF1* patient (Figure 1A). Mitochondria from *SCO2* skeletal muscle contained increased levels of seven distinct subcomplexes (Figure 3C, c–i), apparently identical with that found in *SCO2* patient heart. Their steady-state levels were, however, found to be substantially lower in this tissue (Figure 1D), and their full detection thus required higher protein loads (~50 μg) and longer exposure times (Figure 3C). In line with this, control heart samples contained substantially higher steady-state levels of COX subcomplexes than skeletal muscle controls (Figures 2B and 2C). Also, *SCO2* brain samples revealed accumulated COX subcomplexes of approx. 10–120 kDa, detectable mainly with anti-COX1 and anti-COX5A antibodies, but their profile differed from that found in *SCO2* heart and skeletal muscle mitochondria (Figures 3A–3C). In particular, the COX4 signal in the brain was very weak in this region. In contrast with *SCO2* patient samples, we did not detect any accumulated subcomplexes in *SURF1* patient brain (Figure 1C). In skeletal muscle of *SURF1* patients, we found substantially decreased levels of subcomplex b and faint accumulation of subcomplexes with similar electrophoretic mobility to subcomplexes c–f from *SCO2* samples (Figure 2C). Mitochondria from *SCO2* patient kidney contained moderately increased subcomplexes with migration similar to subcomplexes c–f and i from *SCO2* patient heart (Figure 3D). We did not detect any conclusive accumulation of COX subcomplexes in *SCO2* patient fibroblasts (Figures 1E and 3E).

Alignment of parallel-run immunoblots probed with different antibodies indicated, together with immunoblots of two-dimensional native/denaturing gels, that subcomplex b consists of at least COX1, COX2, COX4, COX5A and COX6B. Subcomplex c comprised at least subunits COX1, COX4 and COX5A, whereas subcomplexes d–f were recognized solely with an anti-COX1 antibody (Figures 1–3). Subcomplex g was detectable with both anti-COX4 and anti-COX5A antibodies, whereas subcomplexes h and i were recognized only with single anti-COX4 and anti-COX5A antibodies respectively (Figures 3A–3C). Low-molecular-mass subcomplexes g–i were not detectable on immunoblots of one-dimensional native gels, since the polyacrylamide gradient used (8–15 %) was optimal for fractionation of subcomplexes b–f, while lower-molecular-mass polypeptides were allowed to migrate out of the gel.

Steady-state levels of SCO2 protein in various tissues of *SCO2* patients

Mitochondrial fractions and fibroblast lysates were resolved using SDS/PAGE and subsequently probed with polyclonal antiserum raised against human SCO2. Equal loading was verified with an antibody raised against the mitochondrial outer membrane protein VDAC. The SCO2 protein was undetectable in all *SCO2* brain samples and in heart of patients P2 and P3 (Figure 4). In heart of patient P1 and liver of patients P1 and P3, the levels of mutant SCO2 were approx. 5 % of control values. In fibroblasts of patients P1–P3, the residual SCO2 was approx. 20 % of control values, while in liver of patient P2 it was approx. 10 % of control values (Figure 4).

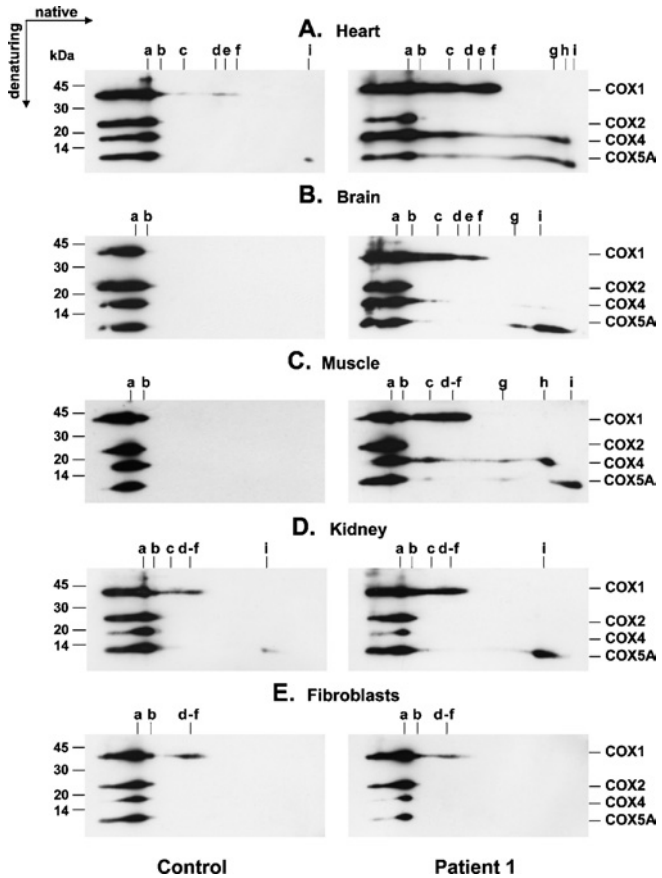


Figure 3 Subunit composition of COX subcomplexes in various tissues of the *SCO2* patient P1

Mitochondrial fractions (10–50 μg) from various tissues of *SCO2* patient P1 and control samples were resolved using two-dimensional BN/SDS/PAGE, electroblotted on to PVDF membranes and probed simultaneously with monoclonal antibodies specific for subunits COX1, COX2, COX4 and COX5A. Sample loads and exposures of films to the blots were chosen such that the signals corresponding to holoenzyme complex a were of similar intensities within both control and patient immunoblots. Immunoreactive material was visualized by chemiluminescence. The polyacrylamide gradient used in the first dimension (BN) was 8–16 % for (**A**, **B**) and 10–18 % for (**C**–**E**). The positions of COX holoenzyme (a) and COX subcomplexes (b–i) and the migration of molecular-mass standards (kDa) are indicated.

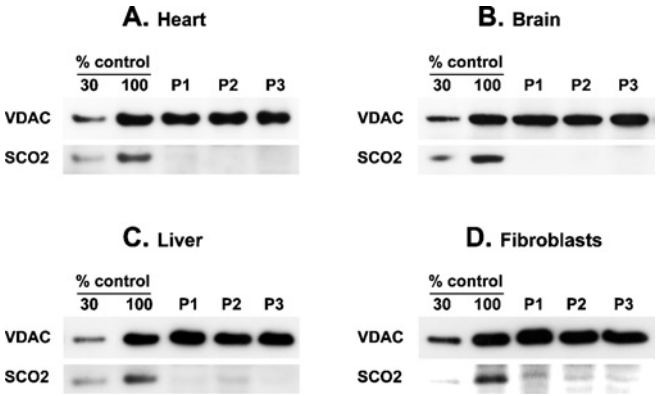


Figure 4 Steady-state levels of SCO2 protein in heart, brain, liver and fibroblasts of *SCO2* patients

Mitochondrial fractions from heart (**A**), brain (**B**) and liver (**C**) samples (~10 μg) of *SCO2* patients (P1–P3) and whole cell lysates (~10 μg) of *SCO2* patient fibroblasts (**D**) were resolved using SDS/PAGE (12 % polyacrylamide), electroblotted on to PVDF membranes and probed with polyclonal antiserum raised against human SCO2 or with monoclonal antibody raised against the mitochondrial outer membrane protein VDAC. Two aliquots of control mitochondria corresponding to indicated dilutions of control samples were loaded on the same gels. Immunoreactive material was visualized by chemiluminescence.

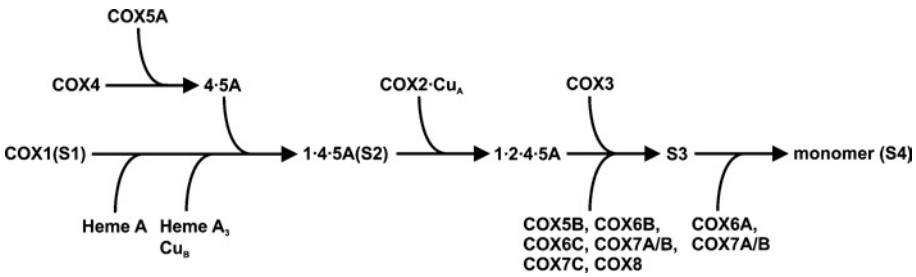
DISCUSSION

The present study represents the first investigation of the assembly state of COX in various clinically affected tissues from patients with *SCO2* and *SURF1* mutations. Although both SCO2 and SURF1 proteins are thought to act at a similar stage of COX assembly, patients carrying mutations in respective genes present with distinct clinical phenotypes [9–11,16–19]. Quantitative immunoblot analysis of native gels revealed tissue-specific COX assembly defects in all patients studied that corresponded to the enzyme activity measurements. The steady-state levels of mutant SCO2 protein were found severely reduced in all the probed *SCO2* patient tissues. The subunit composition of COX subcomplexes identified demonstrates the involvement of human SCO2 protein in biogenesis or maintenance of COX2 and suggests an addition to the current model of the COX assembly pathway.

In our previous work, we have determined the COX activity in several tissues from six patients with mutations in *SCO2* [20] that revealed the COX defect to be most pronounced in heart, brain and muscle, moderate or low in liver, and very low or undetectable in fibroblasts. In this study, we present a detailed proteomic analysis of three of these cases in order to reveal how the *SCO2* genetic defect manifests at the level of COX biosynthesis and assembly in different tissues.

The COX holoenzyme was repeatedly shown to be reduced to approx. 15 % in *SURF1* patient fibroblasts, including our patients P4–P6 [12,14,22,27]. Although skeletal-muscle samples of our *SURF1* patients revealed a similar decrease in COX holoenzyme to cultured fibroblasts, SURF1-deficient heart and liver contained substantially higher levels of residual holoenzyme. In contrast, the tissue-specific consequences of *SCO2* mutations, mainly the profound difference between the residual COX activity in skeletal muscle and fibroblasts, have previously been reported [9,20]. Recently, it was shown that COX holoenzyme is only moderately decreased in immortalized *SCO2* patient fibroblasts [28]. In the present study, we show that despite very low levels of mutant protein, the livers of *SCO2* patients display practically no reduction of fully assembled COX, corresponding to high residual activity. In our previous study, we have found more pronounced decrease in COX activity (39 % of COX/CS) in liver homogenate of an additional *SCO2* E140K homozygote patient [20], but due to the lack of material we could not examine the COX assembly state and enzyme content in this case. In contrast, liver and fibroblasts with mutations in *SCO1*, the paralogous gene of *SCO2*, were reported with severe reduction of COX activity and holoenzyme content respectively [14,29]. The recombinant human E140K SCO2 protein was shown to have a diminished copper-binding capacity and an altered conformational state [30]. Consistent with the latter finding, our results demonstrate that E140K mutation leads to lowered stability of SCO2 protein, as its levels are drastically decreased in mitochondria of patients. The *SCO2* E140K homozygotes present with delayed onset of hypertrophic cardiomyopathy when compared with compound heterozygotes [20,31]. In line with this, heart samples from our E140K homozygotes revealed a substantially milder assembly impairment than that from the E140K/Q53X compound heterozygote. However, we did not detect any substantial difference in the amount of residual SCO2 between the heterozygote and both homozygotes.

In addition to reduced holoenzyme, patient tissues contained varying levels of COX subcomplexes and unassembled subunits.



Scheme 1 Proposed model of the assembly pathway of human COX

The Arabic numerals denote subunits of the enzyme. S1–S4 indicate previously identified assembly intermediates. Prosthetic groups are also indicated. Dimerization of the 13-subunit holoenzyme (S4) completes the assembly of the COX complex.

Fibroblasts with mutations in *SURF1* were repeatedly shown with increased levels of COX assembly intermediates S1 and S2 [12,14,27], and *SCO1* fibroblasts were shown to accumulate subcomplexes comprising COX1 and COX4 [14]. We have identified eight distinct COX subcomplexes (b–i) in various tissues of our *SCO2* and *SURF1* patients. In addition to monomeric holoenzyme complex (a), all samples contained subcomplex b of approx. 180 kDa that corresponds to the previously identified assembly intermediate S3, which, except for COX6A and either COX7A or COX7B, contains all COX subunits [15]. Heart, skeletal muscle and brain of *SCO2* patients contained highly increased levels of a 110 kDa subcomplex c composed of COX1, COX4 and COX5A, very likely identical with the assembly intermediate S2 [15] and with COX1 · COX4 · COX5A subassembly from *SURF1* fibroblasts [14]. In addition, *SCO2* heart, skeletal muscle and brain contained increased levels of subcomplexes d–f of 60–90 kDa. They involved solely COX1 subunit and might be identical with subassemblies d–f from *SURF1* patient fibroblasts [14]. We assume that subcomplex f represents unassembled COX1 subunit and might thus correspond to assembly intermediate S1 [15]. In *SCO2* brain mitochondria, subcomplex c was detectable almost exclusively with anti-COX1 antibody, and the ratio of subcomplexes d–f to subcomplex c was substantially lower here when compared with *SCO2* heart and skeletal muscle. This might reflect more efficient clearance of partially assembled COX subunits in brain mitochondria. This is further supported by the fact that, in contrast with heart and skeletal muscle, control brain mitochondria did not reveal, even after very sensitive detection, any COX subcomplexes, except for very low levels of ubiquitous subcomplex b (results not shown). Interestingly, only subcomplexes b and f were found slightly accumulated in the heart of our *SURF1* patient, although fibroblasts from this patient contained highly increased levels of four subcomplexes comprising COX1, COX4 and COX5A [14]. Furthermore, despite severe reduction of holoenzyme, the brain sample from the identical patient did not contain any accumulated COX subcomplexes, and also *SURF1* skeletal-muscle samples showed rather faint accumulation of subcomplexes, pointing to another tissue-specific aspect of SURF1 deficiency.

In the absence of one or more mitochondrially encoded subunits, the levels of COX4 and COX5A are the least affected [32–39]. Both subunits were also shown to be closely positioned within the X-ray structure of the bovine enzyme [5]. Therefore it was suggested that they might already be associated before assembly with COX1 [40]. Apparently, the COX4 · COX5A subcomplex of approx. 40 kDa (g) from *SCO2* patient samples represents the proposed heterodimer. According to the current model of the COX assembly pathway, the association of subunits starts with the interaction of COX1 with either COX4 or COX5A [14,15,40]. Instead, our results demonstrate that the mutual association of these nucleus-encoded subunits probably precedes their addition to COX1 during the assembly process (Scheme 1).

It was previously shown that pools of unassembled COX subunits exist [41]. In fact, subcomplexes h and i with a molecular mass of approx. 10–20 kDa are composed of single COX4 and COX5A subunits respectively and very likely represent unassembled subunits. This indicates that high residual levels of these subunits reported from *SCO2* patient tissues [9,11,42] and from cells devoid of one or more mitochondrially encoded subunits [33–40] are attributable to (i) high intrinsic stability of both subunits and (ii) their association with each other and COX1. The lower levels of unassembled COX4, compared with COX5A, observed in most of the probed tissues, very likely reflect the lower intrinsic stability of the COX4 polypeptide. This is further supported by the appearance of detectable levels of free COX5A subunit, but not COX4, under physiological conditions (Figures 3A and 3D).

Most of the subcomplexes that we have identified in probed tissues apparently correspond to known assembly intermediates [15] and/or to subassemblies identified in cultured human cells [14]. Therefore it is unlikely that they represent irrelevant aggregates or parts of labile enzyme with disrupted tertiary interactions. To rule out possible proteolytic breakdown, the correct size of detected subunits was confirmed on two-dimensional native/denaturing immunoblots. Taken together, we assume that the subcomplexes identified in the present study correspond to protected rate-limiting steps relevant to the normal assembly pathway (assembly intermediates) that accumulate in mitochondria of patients due to the impaired biogenesis or maintenance of COX.

The *SCO2* mutations carried by our patients are thought to reduce the efficiency with which the Cu_A centre of COX2 is formed [11,28]. In the present study, we show that, in SCO2-deficient mitochondria, the assembly process is stalled before COX2 associates with the COX1 · COX4 · COX5A subcomplex. Indeed, all investigated SCO2-deficient tissues with severely reduced holoenzyme contained increased levels of COX1-, COX4- and COX5A-comprising subcomplexes. The fact that the immunoblots did not reveal accumulation of free COX2 or COX2-containing subcomplex(es) suggests that the lack of Cu_A centre within COX2 results either in (i) decreased stability of the subunit or (ii) diminished efficiency of its interaction with the COX1 · COX4 · COX5A subcomplex. However, the latter would require that the stability of Cu_A lacking free COX2 is low enough, albeit not directly responsible for assembly impairment, to prevent its accumulation in mitochondria of patients. Interestingly, the profile of COX assembly defects observed in *SCO2* patient tissues closely resembles the situation in cells with inhibited mitochondrial protein synthesis due to doxycycline treatment [15].

The precise molecular basis of tissue-specific consequences of *SCO2* and *SURF1* mutations remains unresolved. In addition to different levels of COX holoenzyme, variable levels of subcomplexes were found among different tissues, although some of them displayed the same residual level of the holoenzyme (e.g. heart and muscle). This is likely to be attributable to different rates of clearance of partially assembled or unassembled subunits. The tissue-specific pattern of assembly defects only partially overlaps with the expression of particular tissue-specific isoforms of COX subunits, suggesting the involvement of a rather different mechanism. In our patients, the mutant SCO2 protein was almost undetectable in brain and heart with profound COX deficiency, whereas the liver, and particularly fibroblasts, contained small but significant amounts of residual SCO2. However, we find it unlikely that this minor difference could account for the distinct biochemical and clinical involvement of these tissues, unless there is a pronounced difference in 'spare capacity' of SCO2 for copper delivery to the Cu_A centre in these tissues. Therefore we speculate that tissue-specific consequences of *SCO2* and *SURF1* mutations, in terms of both holoenzyme and subcomplex levels, suggest the existence of tissue-specific functional differences of these proteins that may have evolved to meet different requirements for the regulation of COX biogenesis.

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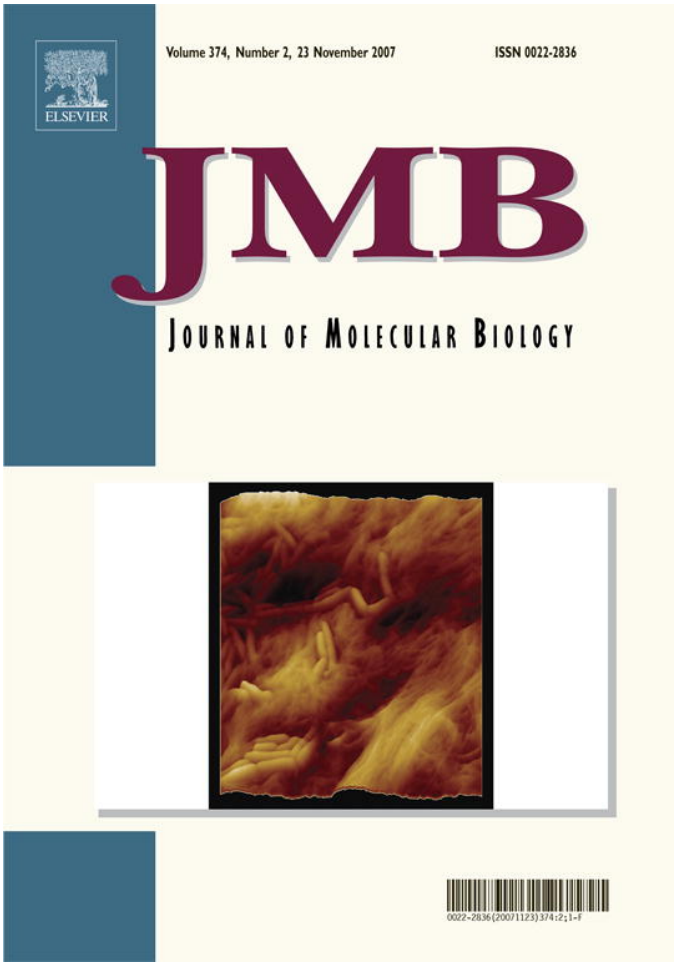

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Knockdown of Human Oxa1l Impairs the Biogenesis of F_1F_o-ATP Synthase and NADH:Ubiquinone Oxidoreductase

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The Oxa1 protein is a founding member of the evolutionarily conserved Oxa1/Alb3/YidC protein family, which is involved in the biogenesis of membrane proteins in mitochondria, chloroplasts and bacteria. The predicted human homologue, Oxa1l, was originally identified by partial functional complementation of the respiratory growth defect of the yeast *oxa1* mutant. Here we demonstrate that both the endogenous human Oxa1l, with an apparent molecular mass of 42 kDa, and the Oxa1l-FLAG chimeric protein localize exclusively to mitochondria in HEK293 cells. Furthermore, human Oxa1l was found to be an integral membrane protein, and, using two-dimensional blue native/denaturing PAGE, the majority of the protein was identified as part of a 600–700 kDa complex. The stable short hairpin (sh) RNA-mediated knockdown of Oxa1l in HEK293 cells resulted in markedly decreased steady-state levels and ATP hydrolytic activity of the F_1F_o-ATP synthase and moderately reduced levels and activity of NADH:ubiquinone oxidoreductase (complex I). However, no significant accumulation of corresponding sub-complexes could be detected on blue native immunoblots. Intriguingly, the achieved depletion of Oxa1l protein did not adversely affect the assembly or activity of cytochrome *c* oxidase or the cytochrome bc_1 complex. Taken together, our results indicate that human Oxa1l represents a mitochondrial integral membrane protein required for the correct biogenesis of F_1F_o-ATP synthase and NADH:ubiquinone oxidoreductase.

Keywords: mitochondria; Oxa1l; ATP synthase; NADH:ubiquinone oxidoreductase; biogenesis

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Introduction

The mitochondrial oxidative phosphorylation system (OXPHOS) is responsible for the vast majority of ATP produced in aerobic cells. It is composed of four respiratory chain complexes and the F_1F_o-ATP synthase (complex V) embedded within the inner membrane of the organelle. The biogenesis of OXPHOS is complicated by its sub-cellular location, dual genetic origin, the large number of constituent subunits and prosthetic groups, and the high hydrophobicity of some of the membrane subunits. Consequently, a number of specific gene products have evolved to accommodate such complex requirements. One of them is the Oxa1 protein, a founding member of the evolutionarily conserved Oxa1/Alb3/YidC protein family, which is involved in the biogenesis of membrane proteins in mitochondria, chloroplasts and bacteria.[1,2] The best characterized member of this family, *Saccharomyces cerevisiae* Oxa1, is an intrinsic protein of the inner mitochondrial membrane that mediates the insertion of mitochondrial translation products as well as of conservatively sorted nuclear gene products into the inner membrane from the mitochondrial matrix.[1,3,4] Although the yeast Oxa1 was shown to represent a rather

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Abbreviations used: OXPHOS, oxidative phosphorylation system; CcO, cytochrome *c* oxidase; SDH, succinate:ubiquinone oxidoreductase; CS, citrate synthase; SDHA, 70 kDa flavoprotein subunit of SDH; PDH, pyruvate dehydrogenase; HEK293, human embryonic kidney 293; RNAi, RNA interference; shRNA, short hairpin RNA; shRNAmir, miR-30–based shRNA; FCCP, carbonyl cyanide *p*-(trifluoromethoxy) phenylhydrazone; GFP, green fluorescent protein; TMPD, *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine dihydrochloride.

general export machinery of the inner membrane, the co-translational membrane insertion of the mitochondrially encoded Cox2 precursor appears to exhibit the strictest dependency on its function.[5–7] The other substrates of Oxa1, including Oxa1 itself, can be inserted independently of its function, albeit with significantly reduced efficiencies.[8,9] Very recently, a novel post-translational role in the biogenesis of OXPHOS was demonstrated for yeast Oxa1. The protein was shown to stably interact in a post-translational manner with the ATP synthase subunit c, mediating its assembly into the ATP synthase complex.[10] Yeast *oxa1* cells are respiratory-deficient, with undetectable cytochrome *c* oxidase (CcO or complex IV) activity and markedly reduced levels of the F_1F_o-ATP synthase and cytochrome bc_1 complex (complex III).[3,4] *Schizosaccharomyces pombe* contains two distinct Oxa1 orthologues, both of which are able to complement the respiratory defect of yeast Oxa1-null cells. The double inactivation of these genes is lethal to this petite-negative yeast.[11] The depletion of Oxa1 in *Neurospora crassa* results in a slow-growth phenotype accompanied by reduced subunit levels of CcO and NADH:ubiquinone oxidoreductase (complex I). The *N. crassa*, Oxa1 was shown to form a 170–180 kDa homo-oligomeric complex, most likely containing four Oxa1 monomers.[12] Mitochondrial Oxa1 homologues possess a hydrophobic core domain composed of five transmembrane helices, and a C-terminal matrix domain that was shown in yeast to bind mitochondrial ribosome, thereby mediating co-translational membrane recruitment of nascent mitochondrial translation products.[13,14]

The predicted human Oxa1 orthologue, referred to as Oxa1l, shares 33% sequence identity with the corresponding yeast polypeptide. The human *OXA1L* cDNA was originally cloned by partial functional complementation of the respiratory growth defect of the yeast *oxa1-79* mutant.[15] It contains an open reading frame predicted to encode a protein of 435 amino acids. It was suggested that the ten exons of *OXA1L* might form an open reading frame able to encode a precursor protein of 495 amino acids,[16] and more recently the cDNA containing those additional 180 bp was cloned.[17] However, this extended version was shown to exhibit an even lower capacity to complement the respiratory growth defect of yeast *oxa1* cells than the original sequence.[18] The human *OXA1L* mRNA was found to be enriched in mitochondria-bound polysomes isolated from HeLa cells, and its 3′ untranslated region was shown to be functionally important when expressed in yeast cells.[18]

Here we address the role of human Oxa1l in the biogenesis of OXPHOS. We demonstrate here that human Oxa1l is a mitochondrial integral membrane protein that exists as part of a 600–700 kDa complex in mitochondria of human embryonic kidney 293 (HEK293) cells. We further show that the stable short hairpin RNA (shRNA)-mediated knockdown of human Oxa1l in HEK293 cells leads to markedly decreased protein levels and ATP hydrolytic activity of the F_1F_o-ATP synthase and moderately reduced levels and activity of NADH:ubiquinone oxidoreductase. Intriguingly, the activity and content of cytochrome *c* oxidase and the cytochrome bc_1 complex were unaffected or even increased in Oxa1l knockdown cells. Hence, these results indicate that human Oxa1l represents a mitochondrial integral membrane protein which is required for the correct biogenesis of the F_1F_o-ATP synthase and NADH:ubiquinone oxidoreductase.

Results

Human Oxa1l localizes to mitochondria in HEK293 cells

The predicted human Oxa1l precursor (Q15070) has a calculated molecular mass of 48.5 kDa and possesses a characteristic N-terminal mitochondrial targeting sequence. Both Mitopred and MitoProt II predict a significant score for location of the protein within mitochondria. To demonstrate the mitochondrial targeting of Oxa1l, a chimeric construct con-

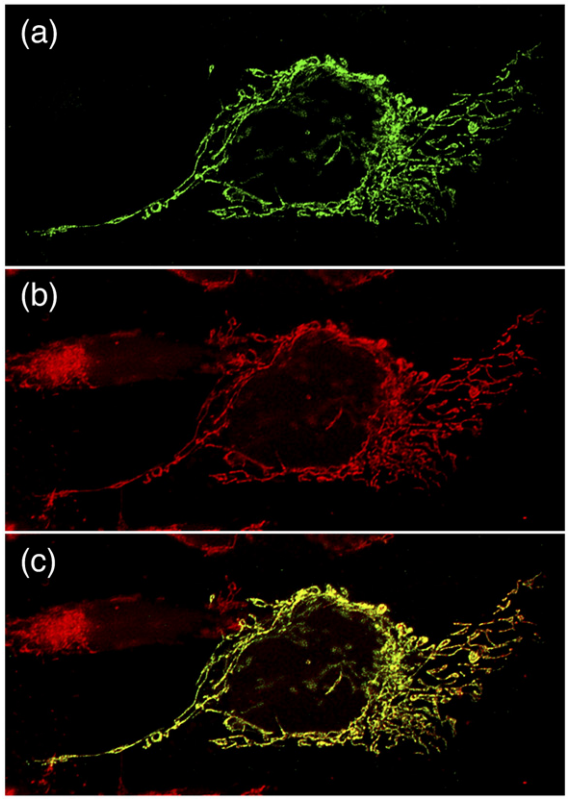

Figure 1. Overexpressed human Oxa1l–FLAG chimeric protein is targeted exclusively to mitochondria in HEK293 cells. (b) HEK293 cells were transiently transfected with the OXA1L–FLAG expression construct, stained with MitoTracker Red, and (a) subsequently labeled with a monoclonal M2 anti-FLAG antibody and with an anti-mouse Alexa Fluor 488 antibody. The fluorescence signal was recorded with a Nikon Eclipse TE2000 microscope, and deconvolved using the Huygens Professional Software (SVI). (c) Superimposition of (a) and (b) shows complete overlap of the two staining patterns.

taining the OXA1L coding sequence tagged with a C-terminal FLAG epitope was transiently expressed in HEK293 cells. As shown in Figure 1, labeling of the cells with an anti-FLAG monoclonal antibody and with the mitochondria-selective dye Mito-Tracker Red followed by confocal microscopy revealed a characteristic mitochondrial staining pattern, as well as a complete overlap of the two signals. To analyze the sub-cellular location of the endogenous human Oxa1l, we prepared samples of whole cell lysates, isolated mitochondria and cytoplasmic fractions from HEK293 cells. Immunoblot analysis using an antibody to human Oxa1l showed that the protein, with an apparent molecular mass of approximately 42 kDa, is clearly more enriched in isolated mitochondria than in whole cell lysates, and is absent from the cytoplasmic fraction (Figure 2(a)). Antibodies to Cox1 (mitochondria), eIF2α (cytoplasm) and mtHSP70 (mitochondria and cytoplasm) were used as compartment markers. These results suggest that human Oxa1l localizes exclusively to mitochondria in HEK293 cells.

Human Oxa1l is an integral membrane protein

Human Oxa1l is predicted to be a transmembrane protein. We have determined its solubility by fractionation of sonically disrupted mitochondria into soluble matrix proteins and membrane vesicles. Immunoblot analysis of the two fractions revealed that Oxa1l is found exclusively in the membrane fraction (Figure 2(b)). To find out whether Oxa1l is a peripheral or integral membrane protein, its susceptibility to alkaline carbonate extraction was assessed. Mitochondria were extracted with sodium carbonate (pH 11.5), and the supernatant and pellet fractions were subjected to immunoblot analysis. Oxa1l was found exclusively in the pellet fraction, indicating that it is an integral membrane protein (Figure 2(b)).

Human Oxa1l exists as part of a 600–700 kDa complex in the mitochondria of HEK293 cells

The fact that both *S. cerevisiae* and *N. crassa* Oxa1 proteins were reported to be part of an oligomeric assembly prompted us to assess this property in the human protein. Mitochondria isolated from HEK293 cells were solubilized with 1.3% (w/v) dodecyl maltoside and fractionated using two-dimensional blue native/denaturing PAGE, on a 6%–16% (w/v) polyacrylamide gradient in the first dimension. Probing of the resulting immunoblots with the antibody to Oxa1l and with the corresponding pre-immune serum revealed that Oxa1l is specifically present in one major, high molecular mass complex, and in several minor, faster migrating assemblies. As shown in Figure 2(c), simultaneous detection with the antibody to Oxa1l and with an antibody to ATPase F1-α revealed that the major Oxa1l-containing complex has an estimated molecular mass of approximately 600–700 kDa. Since the yeast Oxa1 was recently shown to physically interact with components of ATP synthase holoenzyme, we have asked whether the human Oxa1l complex also contains some of the ATP synthase subunits.

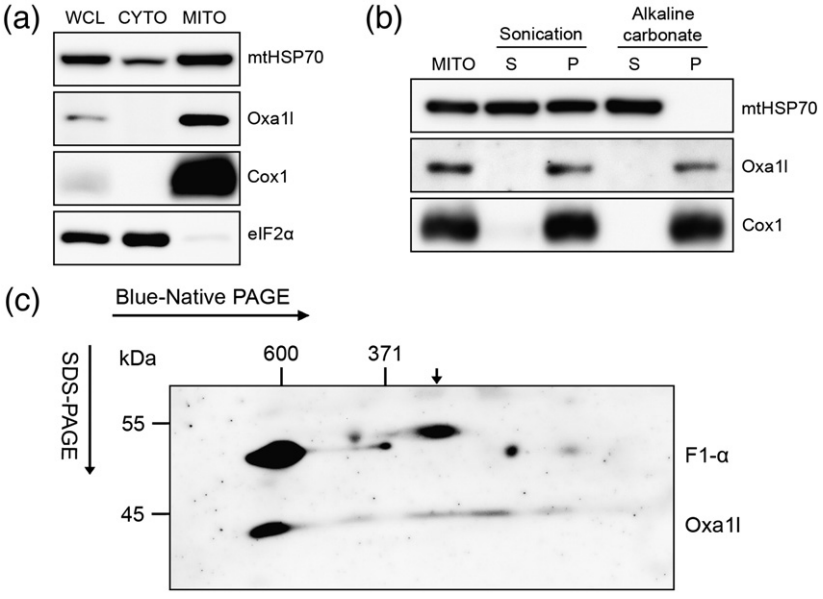

Figure 2. Human Oxa1l is a mitochondrial integral membrane protein that exists as part of a 600–700 kDa complex. (a) Equal amounts of protein (20 μg) from isolated mitochondria, cytoplasmic fraction and whole cell lysate from the HEK293 cells were fractionated using SDS–10% PAGE, and the resulting immunoblots were decorated with an antibody to Oxa1l. Antibodies to Cox1 (mitochondria), eIF2α (cytoplasm) and mtHSP70 (mitochondria and cytoplasm) were used as compartment markers. (b) Mitochondria isolated from HEK293 cells were either sonically disrupted or extracted with sodium carbonate (pH 11.5) and centrifuged at 100,000*g* for 30 min, and the resulting fractions were processed for SDS–PAGE. The aliquots of resulting samples (5 μg of protein) were, together with the whole mitochondria lysate, used to prepare denaturing immunoblots that were subsequently decorated with antibodies to Oxa1l, mtHSP70 (peripheral protein) and Cox1 (integral protein). s, supernatant; p, pellet. (c) Mitochondria isolated from HEK293 cells were solubilized with 1.3% dodecyl maltoside and fractionated (50 μg of protein) using two-dimensional blue native/denaturing PAGE, on a 6%–16% polyacrylamide gradient in the first dimension. The resulting immunoblot was decorated with antibodies to Oxa1l and ATPase F_1-α. Under the conditions employed, an antibody to F_1-α recognized the ATP synthase holoenzyme of approximately 600 kDa and the F_1-ATPase sub-complex of 371 kDa. The small arrow indicates a non-specific binding of Oxa1l antibody that was also achieved using the Oxa1l preimmune serum alone.

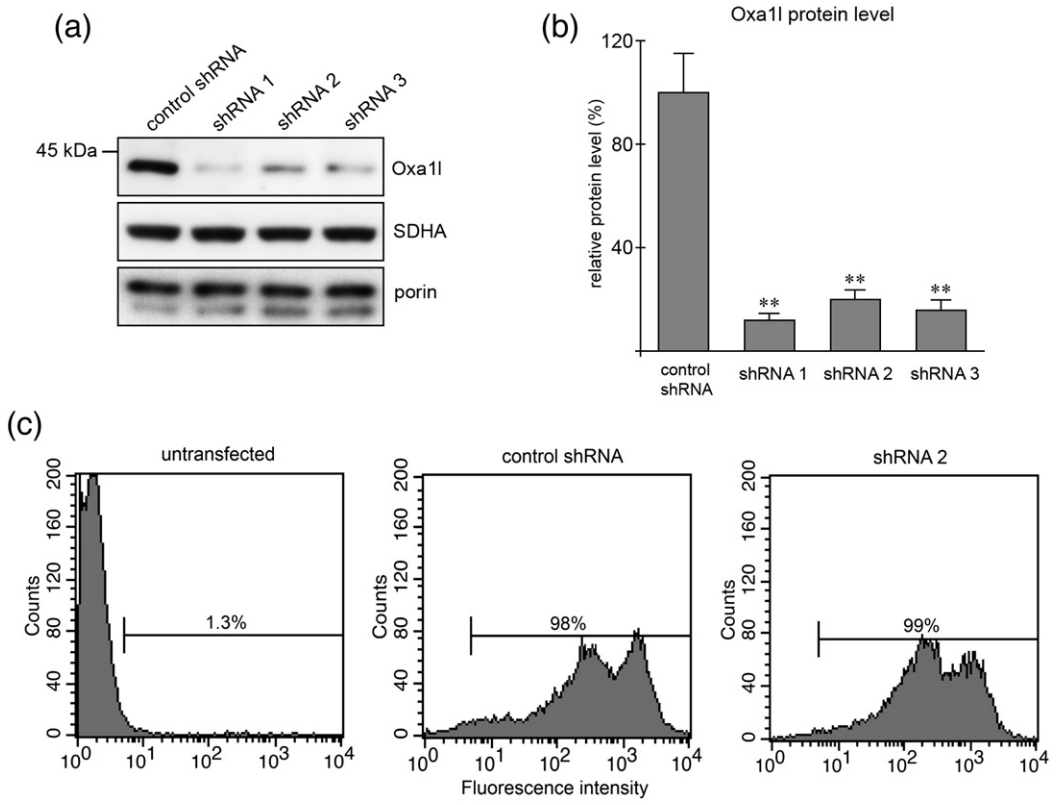

Figure 3. Oxa1l is substantially depleted in HEK293 cells by the stable expression of shRNAmir. (a) Equal amounts of protein (10 μg) from mitochondria isolated from G418-resistant cells stably transfected with one of the OXA1L-targeted shRNA constructs (shRNA 1, 2 and 3) and from cells expressing the negative control shRNA were fractionated using SDS–10% PAGE. The resulting immunoblot was decorated with antibodies to Oxa1l, SDHA and porin. (b) The results of densitometric quantification (Quantity One; Bio-Rad) of three independent experiments from (a) are presented in the histogram. **, $p<0.01$ (c) Flow cytometric analysis of GFP expression in HEK293 cells stably transfected with the OXA1L-targeted shRNA construct (shRNA 2) or with the negative control shRNA construct. The analysis of untransfected parental HEK293 cells is included as a negative control.

HEK293 cells were transiently transfected with the OXA1L–FLAG expression construct and solubilized with 1% (v/v) Triton X-100 and the fusion protein was then immunoprecipitated from the lysate using an ANTI-FLAG® M2 affinity agarose resin. The immunoprecipitate was fractionated using SDS–PAGE and the resulting immunoblots were probed with available antibodies against the ATP synthase subunits and against various respiratory chain subunits. However, despite high efficiency of Oxa1l–FLAG protein precipitation we could not detect any OXPHOS subunits in the immunoprecipitate using available antibodies (data not shown). These results suggest that a substantial fraction of human Oxa1l exists in the mitochondria of HEK293 cells as part of an approximately 600–700 kDa assembly that do not appear to contain any of the mitochondrial OXPHOS complexes.

Stable knockdown of Oxa1l in HEK293 cells

To uncover the functional involvement of human Oxa1l in the biogenesis of OXPHOS, we down-regulated its expression in HEK293 cells using RNA interference (RNAi). Given the considerable half-life of the OXPHOS complexes, a stable vector-based approach was utilized that incorporates the RNA polymerase II-driven expression of short hairpin RNAs embedded within the context of a mammalian miR-30 microRNA primary transcript. This method was shown to provide high-level knockdown even in polyclonal cell populations of single-copy integrants.[19] Sequences of eight different miR-30–based shRNAs (shRNAmirs) targeted to the coding sequence of human OXA1L were downloaded from the publicly accessible RNAi Codex database†, synthesized, cloned into the lentiviral pCMV-GIN-Zeo vector, and functionally validated by their transient co-expression with the OXA1L-FLAG construct (data not shown). The three shRNA constructs that proved to be effective were selected (shRNA 1, mp_id 433861; shRNA 2, mp_id 19616 and shRNA 3, mp_id 19615), and together with the negative control (non-silencing) shRNA construct and the empty vector were used to establish G418-resistant stably expressing cells. The fact that the expression of shRNAmir in the pCMV-GIN-Zeo vector is transcriptionally coupled with that of green fluorescent protein (GFP) permitted an estimation of

† http://codex.cshl.org

shRNA expression levels in stable cells. Flow cytometric analysis revealed that practically all the cells were GFP positive, without marked differences in expression levels (Figure 3(c)). The extent of endogenous Oxa1l protein depletion was then determined by immunoblot analysis of mitochondrial fractions isolated from pooled colonies of stable cells (Figure 3(a)). The cells carrying the OXA1L-targeted constructs expressing shRNA 1, shRNA 2 and shRNA 3 showed substantial reductions in Oxa1l protein levels to approximately 12%, 20% and 16% of control values, respectively (Figure 3(b)). No significant differences in the Oxa1l protein levels were observed between cells carrying the negative control shRNA construct and cells carrying the empty vector (data not shown). Given the substantial reduction in Oxa1l protein levels, pooled colonies were used for subsequent analysis, obviating the need for additional sub-cloning.

Knockdown of human Oxa1l leads to markedly diminished protein content and ATP hydrolytic activity of the F_1F_o-ATP synthase and moderately reduced content and activity of NADH:ubiquinone oxidoreductase (complex I)

We initially determined the steady-state levels of several mitochondrial proteins, mainly OXPHOS subunits, in our cellular Oxa1l knockdown model. Since we did not detect significant alterations in the content of mitochondria (porin, 70 kDa flavoprotein subunit of succinate:ubiquinone oxidoreductase (SDHA)), the whole-cell lysates, normalized to the immunoblot signal of α-tubulin, were used for denaturing immunoblots. The steady-state levels of SDHA, core protein 2, porin, mtHSP70 and pyruvate dehydrogenase (PDH)-E2 were found to be similar to those in negative controls, whereas the levels of Cox1, Cox2 and Cox5a were either unaffected or slightly increased to approximately 120%–130% of control values. In contrast, the steady-state levels of ATP synthase subunits F1-α, F1-β, OSCP and subunit d were reduced to approximately 25%–50%. In addition, the levels of complex I subunits NDUFB6 and NDUFA9 were decreased to approximately 50%–60% of control values in Oxa1l-depleted cells (Figure 4(a), or data not shown).

To determine the holoenzyme content and assembly state of each of the OXPHOS complexes, blue native immunoblots of dodecyl maltoside-solubilized mitochondria were prepared. Consistent with denaturing analysis, native immunoblots showed unaffected levels of respiratory complexes II and III, whereas the content of cytochrome *c* oxidase holoenzyme was increased to approximately 130% of control values (Figure 4(b)). On the contrary, the levels of F_1F_o-ATP synthase holoenzyme were reduced to approximately 25%–50%, whereas the holoenzyme content of complex I was decreased to approximately 50%–60% of control values in the mitochondria of Oxa1l-depleted cells (Figure 4(b)). However, no significant accumulation of either the

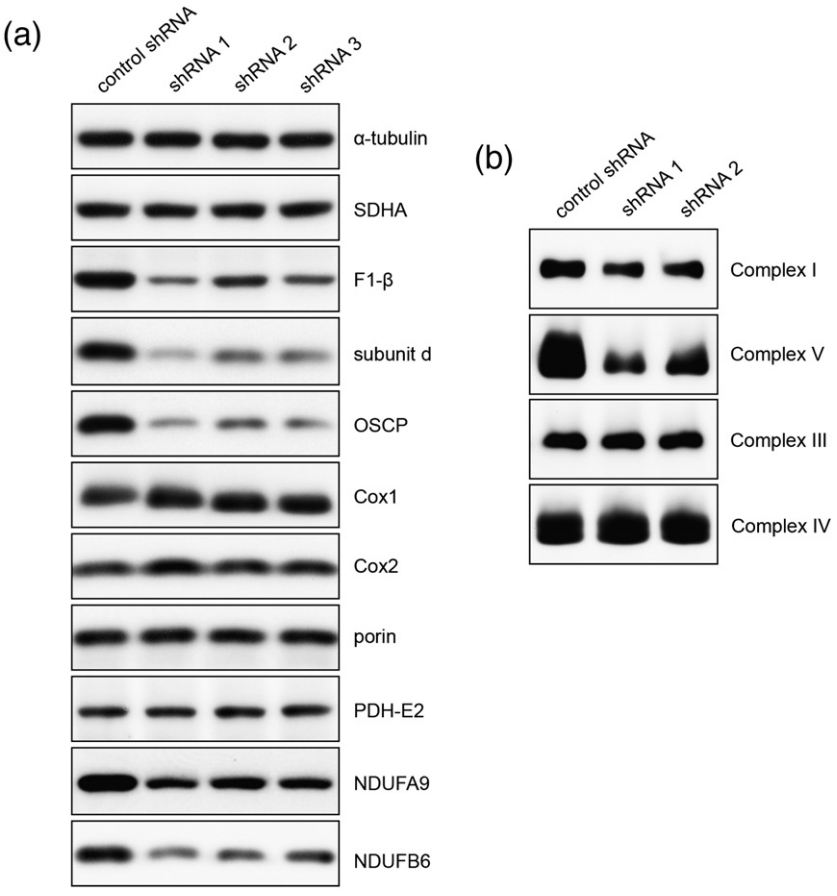

Figure 4. Depletion of Oxa1l in HEK293 cells results in decreased steady-state levels of the F_1F_o-ATP synthase and NADH:ubiquinone oxidoreductase holoenzyme and subunits. (a) Equal amounts of protein (10 μg) from whole cell lysates prepared from G418-resistant cells stably transfected with one of the OXA1L-targeted shRNA constructs (shRNA 1, 2 and 3) and from cells carrying the negative control shRNA construct were fractionated using SDS–PAGE. The resulting immunoblots were decorated with antibodies to α-tubulin, SDHA, F_1-β, subunit d, OSCP, Cox1, Cox2, porin, PDH-E2, NDUFA9 and NDUFB6. (b) Isolated mitochondria were solubilized with 1.3% dodecyl maltoside and fractionated using blue native PAGE on 6%–15% polyacrylamide gradient. The amount of protein loaded per lane (~5 μg) was normalized to the immunoblot signals of porin and SDHA. The resulting immunoblots were decorated with antibodies to NDUFA9 (complex I), subunit d (complex V), Cox1 (complex IV) and core protein 2 (complex III). The immunoblots shown are representative of at least three independent experiments.

ATP synthase or complex I sub-complexes could be detected on blue native immunoblots using available antibodies (data not shown).

To confirm and extend the above results, enzymatic activities of respiratory chain complexes, as well as the aurovertin-sensitive ATP hydrolytic activity, were determined. The activities of succinate:ubiquinone oxidoreductase (SDH), cytochrome *c* reductase and citrate synthase (CS) were found to be at control levels (data not shown), whereas the activity of CcO, normalized to that of CS, was either unaffected or insignificantly increased to approximately 110%–120% of control values. In contrast, the aurovertin-sensitive ATP hydrolytic activity was substantially decreased to approximately 30%–60% of control values in the mitochondria of Oxa1l-depleted cells (Figure 5(a)). In line with the moderately reduced protein content, the activity of complex I, normalized to that of CS, was diminished to approximately 70%–80% of control values. The values of aurovertin-sensitive ATPase activity correlated with the relative residual content of ATP synthase holoenzyme, consistent with the fact that no significant accumulation of F_1 sub-complex(es) could be detected on blue native immunoblots.

To further confirm the results of CcO activity and content determination, high-resolution polarographic measurements of ascorbate/*N,N,N′,N′*-tetramethyl-*p*-phenylenediamine dihydrochloride (TMPD)-fuelled, sodium azide-sensitive oxygen consumption were carried out with digitonin-permeabilized cells. The measurements showed significantly increased ascorbate/TMPD-fuelled respiration in Oxa1l-depleted cells to approximately 150%–160% of control values, indicating increased activity and/or content of the membrane-embedded CcO complex in these cells (Figure 5(b)).

Discussion

This study represents the first characterization of a mammalian member of the evolutionarily conserved Oxa1/Alb3/YidC family of proteins. The evidence presented herein indicates that human Oxa1l is a mitochondrial integral membrane protein that exists as part of a 600–700 kDa complex in the mitochondria of HEK293 cells. We further demonstrate here that the shRNA-mediated depletion of human Oxa1l leads to compromised biogenesis of the F_1F_o-ATP synthase and, to a lesser extent, NADH:ubiquinone oxidoreductase.

The mitochondrial membrane localization of human Oxa1l is supported by several lines of evidence. First, the protein possesses a characteristic N-terminal mitochondrial targeting sequence and a hydrophobic core region composed of five predicted transmembrane helices. Second, the sub-cellular fractionation of HEK293 cells together with subsequent immunoblotting using an antibody to Oxa1l revealed that the protein, with an apparent molecular mass of approximately 42 kDa, is highly enriched in isolated mitochondria and absent from the cytoplasmic fraction. Third, immunofluorescence labeling of HEK293 cells transfected with the OXA1l–FLAG expression construct, followed by confocal microscopy, showed an exclusive mitochondrial targeting of the overexpressed Oxa1l-FLAG chimeric protein. Finally, the human Oxa1l was found exclusively in the pellet fraction of sonically disrupted mitochondria, and could not be extracted from the mitochondrial membrane by alkaline carbonate treatment. Similarly, the yeast Oxa1 was shown to be a mitochondrial integral membrane protein, spanning the inner membrane with five transmembrane helices.[20,21]

Both *S. cerevisiae* and *N. crassa* Oxa1 were reported to be part of an oligomeric assembly.[8,12,22] Here we demonstrate that the majority of human Oxa1l exists in the mitochondria of HEK293 cells as part of a 600–700 kDa complex of unknown composition. In contrast to yeast Oxa1,[10] no ATP synthase subunits to which we have antibodies were found to co-purify with the human Oxa1l–FLAG protein expressed in HEK293 cells. Similarly, we could not detect any respiratory chain subunits in the Oxa1l–

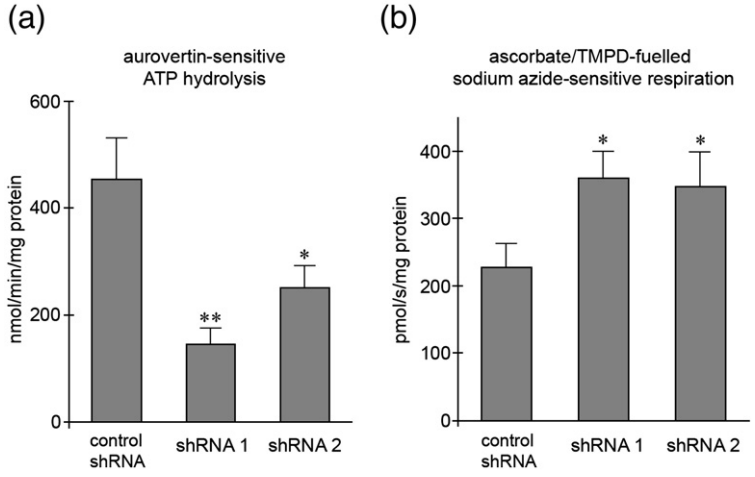

Figure 5. Depletion of Oxa1l in HEK293 cells leads to diminished aurovertin-sensitive ATP hydrolytic activity and increased ascorbate/TMPD-fuelled, sodium azide-sensitive oxygen consumption. (a) Aurovertin-sensitive ATP hydrolytic activity was measured using an ATP-regenerating system in mitochondria isolated from G418-resistant cells stably transfected with one of the OXA1L-targeted shRNA constructs (shRNA 1 and 2) and from cells carrying the negative control shRNA construct. (b) Ascorbate/TMPD-fuelled, sodium azide-sensitive oxygen consumption was measured in digitonin-permeabilized cells in the presence of FCCP using an OROBOROS oxygraph. Values are expressed as mean±S.D. *, $p<0.05$; **, $p<0.01$.

FLAG immunoprecipitate. Since the Oxa1 complex from *N. crassa* was shown to be composed exclusively of Oxa1 monomers,[12] it is possible that also the human complex might be similarly organized. However, given the higher estimated molecular mass of the human complex, it seems likely that some other protein(s) might be involved.

To investigate the functional involvement of human Oxa1l in the biogenesis of OXPHOS, we down-regulated its expression in HEK293 cells by the stable expression of shRNAmir. The depletion of Oxa1l achieved was specific, since a qualitatively identical loss-of-function phenotype was induced using three different shRNA species. The Oxa1l-depleted cells showed largely normal mitochondrial content, and both functionally and quantitatively intact respiratory chain complexes II and III. Intriguingly, the cytochrome oxidase activity and content of CcO, as well as the ascorbate/TMPD-fuelled, sodium azide-sensitive oxygen uptake, were either unaffected or even increased when compared to negative controls. On the contrary, markedly reduced protein content and ATPase activity of the F_1F_o-ATP synthase and moderately reduced content and activity of NADH:ubiquinone oxidoreductase were found in Oxa1l-depleted cells, indicating selectively compromised assembly or stability of these two OXPHOS complexes. However, no significant accumulation of sub-complexes containing either the ATP synthase or complex I subunits could be detected in the mitochondria of Oxa1l-depleted cells.

The observed defect of ATP synthase and complex I is consistent with the mitochondrial localization and integral membrane nature of Oxa1l and, to a certain extent, with the functional involvement of fungal Oxa1 homologues. However, the inactivation of *OXA1* in yeast was shown to cause more pleiotropic OXPHOS defects, characterized mainly by the complete absence of CcO activity,[4] pointing to the essential requirement of Oxa1 in the biogenesis of CcO.[23] In our opinion, there are at least four reasons that might account for the observed difference between the human cell line and fungi. First, the human protein might have functionally diverged from its fungal counterparts, most likely in terms of an adapted substrate preference. Since the yeast Oxa1 is strictly required for the N-terminal export of Cox2 precursor, it is possible that the complete absence of the N-terminal leader peptide in nascent human Cox2 might be responsible for the observed Oxa1-independent assembly of human CcO. Second, another protein that provides a partially overlapping function (e.g. Cox18) might be able to compensate, to a certain extent, for the lack of Oxa1l in human mitochondria. Third, Oxa1l may exhibit a tissue-specific loss-of-function phenotype, similar to several other factors involved in human OXPHOS biogenesis.[24] Finally, the depletion of Oxa1l achieved might not reach the threshold necessary to induce a detectable defect of cytochrome *c* oxidase and cytochrome bc_1 complex, though the increased respiratory activity and content of CcO argues against this alternative.

The fact that human Oxa1l is able to partially restore the respiratory growth of yeast *oxa1* cells suggests that both proteins share at least basic functional features. However, given the wide exchangeability among the members of the Oxa1/Alb3/YidC family,[25] this cannot be extrapolated to a precise endogenous role. In this context, it was suggested that the potential homo-oligomerization of Oxa1 proteins might be responsible for their functional conservation, as interaction with other components may be dispensable for basic (translocase) function.[12]

The slightly increased activity and content of CcO in Oxa1l-depleted cells may likely represent a part of an adaptive response to OXPHOS impairment. This explanation appears conceivable, since CcO was repeatedly shown to exhibit a low reserve capacity and a large control coefficient over the respiratory chain.[26–28] Furthermore, CcO was shown to be required for the assembly/stability of complex I in mouse fibroblasts.[29] Indeed, a similar increase in the protein content of CcO was observed in human fibroblasts with a selectively reduced content of complex I holoenzyme.[30] The observed discrepancy between the cytochrome oxidase activity and ascorbate/TMPD-driven respiration was reported[31] and probably stems from different preparations of the enzyme complex. Unlike the low-concentration digitonin treatment (respirometry), which preserves the inner membrane intact, the lauryl maltoside solubilization (spectrofotometric CcO assay) removes the enzyme complex from the inner membrane, eventually leading to altered enzyme function (e.g. due to abrogated allosteric interactions).

Since we did not detect any significant accumulation of ATP synthase or complex I sub-complexes in Oxa1l-depleted mitochondria, it seems unlikely that the holoenzyme reduction observed could stem from a compromised stability of the complex. The lack of significant accumulation of F_1-ATPase also suggests that the assembly defect of ATP synthase cannot be attributed solely to selectively affected accumulation of mitochondrially encoded subunits a and A6L, since human ρ^0 cells were shown to contain high levels of F_1-ATPase.[32] The F_1-ATPase was, however, repeatedly found to be more or less stably associated with subunit c (oligomer) in ρ^0 cells.[33,34] In contrast to yeast and *S. pombe*, the biogenesis of mammalian (and *N. crassa*) ATP synthase is likely to be further complicated by the fact that the highly hydrophobic subunit c is encoded in the nucleus and has to be imported from the cytoplasm prior to its membrane insertion and assembly. Nargang *et al.*[12] have studied the *N. crassa oxa1* mutant but the authors did not address the effects of Oxa1 depletion on the ATP synthase in their paper. Recently, it was reported that the ATP synthase defect of yeast *oxa1* cells probably stems directly from the compromised assembly of subunit c into the holoenzyme.[10] Indeed, the expression level of

subunit c was suggested to determine the content of ATP synthase in mammalian tissues.[35] It was shown that in mouse and rat thermogenic brown fat, the low expression level of subunit c mRNA correlates with the low relative content of ATP synthase in this tissue, irrespective of high mRNA levels of the remaining ATP synthase subunits.[36–38] Furthermore, no accumulated ATP synthase sub-complexes could be detected in brown adipose tissue.[39] Therefore, we speculate that the ATP synthase defect of human Oxa1l-depleted cells could stem from the impaired assembly and/or membrane integration of the nuclear-encoded ATP synthase subunit c.

In conclusion, this study has revealed a possible role for the human member of the Oxa1/Alb3/YidC protein family in the biogenesis of OXPHOS that differs to a certain extent from that demonstrated for fungal Oxa1 homologues. The specific RNAi phenotype suggests that Oxa1l is involved in the biogenesis of ATP synthase and complex I in human mitochondria, consistent with its mitochondrial localization and integral membrane properties. Future work will involve investigation of the exact composition of the Oxa1l-containing high molecular mass complex, as well as knockdown of Oxa1l expression in another (primary) human cell type.

Materials and Methods

Cell culture, transfections and flow cytometry

Human embryonic kidney cells (HEK293, CRL-1573) were obtained from ATCC (Rockville, MD) and grown at 37 °C in a 5% (v/v) CO_2 atmosphere in high-glucose Dulbecco's modified Eagle's medium (PAA, Austria) supplemented with 10% (v/v) fetal calf serum (PAA). Cell transfections were carried out either with a Nucleofector™ device (Amaxa, Cologne, Germany) using the HEK293 cell-specific transfection kit (Amaxa) or with the Lipofectamine 2000 reagent (Invitrogen, Paisley, UK). The GFP fluorescence of stable G418-resistant HEK293 cells was measured with a FACSCalibur flow cytometer and analyzed using the Cell Quest 3.3 application (Becton Dickinson, San Jose, CA).

Immunofluorescence

HEK293 cells (1.5×10^5) grown on 70 mm^2 glass chamber slides (BD Falcon, Palo Alto, CA) were transfected with an OXA1L–FLAG expression construct using the Lipofectamine 2000 reagent. At 24 h after the transfection, the cells were incubated with 200 nM MitoTracker Red CMX Ros (Molecular Probes, Eugene, OR) for 15 min, and then fixed and permeabilized with phosphate-buffered saline, 4% (v/v) paraformaldehyde and 0.1% (v/v) Triton X-100 solutions, respectively. Subsequently, the cells were incubated in phosphate-buffered saline, 10% (w/v) bovine serum albumin solution for 1 h at 37 °C to block non-specific binding. Immunocytochemical detection was then performed with a monoclonal M2 anti-FLAG antibody (1:1000) and with an anti-mouse IgG_1 Alexa Fluor® 488 antibody (1:500).

Laser scanning confocal microscopy

xyz images sampled according to the Nyquist criterion were acquired using a Nikon Eclipse TE2000 microscope equipped with a C1*si* confocal scanning head and an Apo TIRF 60× (N.A. 1.49) objective. The 488 nm and 543 nm laser lines and appropriate 515(±15) and 590(±15) nm band pass filter sets were used for excitation and fluorescence detection, respectively. Individual channel images were acquired separately. Images were restored using the measured point spread function (PSF) and the classic maximum likelihood deconvolution algorithm in Huygens Professional Software (SVI, Hilversum, Netherlands).

Plasmid construction

The nucleotide sequences of eight different miR-30–based shRNAs (shRNAmirs) targeted to the coding sequence of human OXA1L were downloaded from the publicly accessible RNAi Codex database.[40] The corresponding 97-mer oligonucleotides were synthesized (Invitrogen) and used as template sequences for PCR amplification to produce clonable double-stranded products. The corresponding XhoI/EcoRI restriction fragments were inserted downstream of the CMV-GFP-IRES-Neo expression cassette of the lentiviral pCMV-GIN-Zeo vector (Open Biosystems, Huntsville, AL). The negative control (non-silencing) shRNAmir pCMV-GIN-Zeo derivative was obtained from Open Biosystems. The full-length human OXA1L coding sequence was amplified from the IMAGE (integrated molecular analysis of genomes and their expression) clone 40017377 and inserted into the C-FLAG fusion mammalian expression vector pCMV-Tag4 (Stratagene, La Jolla, CA). The fidelity of all constructs was confirmed by automated DNA sequencing.

Validation of shRNAs and generation of stable transfectants

To assess the efficiency of the shRNAmir constructs, HEK293 cells were co-transfected using a Nucleofector™ device with either one of the eight OXA1L-targeted shRNA constructs or the negative control (non-silencing) shRNA construct and with the OXA1L–FLAG expression construct. At 48 h after the transfection, the cells were lysed and the expression level of the Oxa1l-FLAG fusion protein was examined by immunoblot analysis. The three OXA1L-targeted constructs that contained shRNA 1 (mp_d 433861, 5′-CAAGTTAGCAGGAGACCAT-3′), shRNA 2 (mp_id 19616, 5′-CCTACAACCTGGAAAG GAT-3′) and shRNA 3 (mp_id 19615, 5′-GAGACCATATTGAGTATTA-3′) (RNAi Codex) showed the highest potential to interfere with the expression of Oxa1l–FLAG protein in the transient assay. For the generation of stable transfectants, HEK293 cells (10^6) were transfected using a Nucleofector™ device with the three functionally validated OXA1L-targeted shRNA constructs, the negative control (non-silencing) shRNA construct and with the empty vector. At 48 h after the transfection the cells were split into culture medium containing 720 μg/ml of G418 sulfate (Clontech, Mountain View, CA) and antibiotic-resistant colonies were selected over a period of three weeks. The cells were further maintained in the presence of 720 μg/ml G418.

Preparation of a polyclonal antibody to human Oxa1l

An Oxa1l-specific antiserum was generated by immunizing chicken with a synthetic peptide (KLH-coupled) corresponding to the C-terminal part of human Oxa1l (CKPKSKYPWHDT). The polyclonal antibody to human Oxa1l was affinity-purified from the total IgY with the respective peptide-packed column. The specificity of the produced antibody was tested by immunodetection of the Oxa1l-FLAG fusion protein.

Immunoblot analysis

Protein sample preparations, electrophoresis and immunoblot analysis were performed essentially as described.[24] Monoclonal antibodies against the mitochondrial outer membrane protein porin, CcO subunits Cox1, Cox2 and Cox5a, the 70 kDa flavoprotein subunit of SDH (SDHA), complex III subunit core protein 2, complex I subunits NDUFA9 and NDUFB6, complex V subunits F1-α, F1-β, subunit d and OSCP, and pyruvate dehydrogenase (PDH) subunit E2 were obtained from Mitosciences (Eugene, OR). The mouse monoclonal antibody to mtHSP70 was from Alexis Biochemicals (San Diego, CA). Polyclonal antibodies to α-tubulin and eIF2α were from Cell Signaling Technology (Beverly, MA), and the monoclonal anti-FLAG antibody was from Sigma (Prague, Czech Republic). Signal acquisition was performed using either a VersaDoc 4000 imaging system (Bio-Rad Laboratories, Hercules, CA) or Kodak BioMax Light films (Eastman Kodak Co., Rochester, NY). Digital images were analyzed using the Quantity One application (Bio-Rad Laboratories).

Immunoprecipitation of Oxa1l–FLAG protein

HEK293 cells ($\sim 10^6$) were transiently (48 h) transfected with the Oxa1l–FLAG expression construct, lysed with a buffer containing 1% Triton X-100, 150 mM NaCl, 1 mM EDTA and 50 mM Tris–HCl (pH 7.4), and the lysate was incubated for 6 h at 4 °C with previously washed 50 μl of an ANTI-FLAG® M2 affinity agarose resin (flagipt-1; Sigma, Prague, Czech Republic). Subsequently, the resin was washed five times with a buffer containing 150 mM NaCl, 50 mM Tris–HCl (pH 7.4), and the bound protein was eluted by competition with 3× FLAG peptide. Finally, the eluted immunoprecipitate was combined with SDS sample buffer and resolved using SDS–PAGE.

Enzyme activity assays

The activities of enzyme complexes NADH:ubiquinone oxidoreductase (complex I), succinate:ubiquinone oxidoreductase (SDH or complex II), cytochrome bc_1 complex (complex III), cytochrome *c* oxidase (complex IV) and citrate synthase (CS) were measured spectrophotometrically in whole cell extracts and isolated mitochondria using standard methods.[41] Aurovertin-sensitive ATP hydrolytic activity was measured in isolated mitochondria using an ATP-regenerating system as described.[41,42]

Polarographic measurements

The ascorbate/TMPD-fuelled sodium azide-sensitive oxygen consumption of digitonin-permeabilized HEK293 cells was measured at 30 °C using an OROBOROS Oxygraph (Anton Paar, Innsbruck, Austria) in a medium containing 0.5 mM EGTA, 3 mM $MgCl_2$, 60 mM potassium lactobionate, 20 mM taurine, 10 mM KH_2PO_4, 110 mM sucrose, 1 g/l BSA, 20 mM Hepes (pH 7.1). The measurements were carried out in the presence of 30 μg/ml of digitonin, 2.5 μM antimycin A, 2 mM ascorbate, 500 μM TMPD and 1.5 μM carbonyl cyanide *p*-(trifluoromethoxy) phenylhydrazone (FCCP). Respiration was inhibited by the addition of sodium azide to a final concentration of 10 mM.

Sub-cellular and submitochondrial fractionation

HEK293 cells (10^7) were harvested, washed twice with phosphate-buffered saline, resuspended in an isotonic buffer containing 250 mM sucrose, 10 mM Tris–HCl (pH 7.4), 1 mM EDTA, 1% (w/v)Protease inhibitor cocktail (Sigma), and disrupted on ice using a Dounce homogenizer. Unbroken cells and nuclei were removed from the homogenate by centrifugation at 600*g* for 15 min. The post-nuclear supernatant was centrifuged at 10,000*g* for 25 min to pellet the mitochondria. The resulting supernatant corresponding to the cytoplasmic fraction was collected, and the mitochondrial pellet was washed once with the isotonic buffer. For sonical disruption, isolated mitochondria were adjusted to a protein concentration of 2.5 mg/ml, sonicated and centrifuged at 100,000*g* for 30 min. Alkaline sodium carbonate extraction of mitochondrial membranes was performed essentially as described.[43]

Statistical analysis

A Student's *t* test was performed using Microsoft Excel. Results are expressed as mean±S.D. A *p* value of less than 0.05 was considered as statistically significant, and asterisks are used to denote significance as follows: *, $p<0.05$; **, $p<0.01$.

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Biogenesis of Eukaryotic Cytochrome c Oxidase

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Summary

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

Key words

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

Introduction

Eukaryotic cytochrome *c* oxidase (CcO) is the terminal multicomponent enzyme of the energy-transducing mitochondrial electron transport chain (Capaldi 1990). It belongs to the superfamily of heme-copper containing terminal oxidases, characterized by the presence of histidine ligands to two heme groups and to a Cu_B copper ion (Michel *et al.* 1998). The mitochondrial enzyme, an aa_3-type terminal oxidase, catalyzes the sequential transfer of electrons from reduced cytochrome *c* to dioxygen, coupling this reaction with electrogenic proton pumping across the inner mitochondrial membrane. Eukaryotic CcO is a heterooligomeric complex composed of 7 (*Dictyostelium discoideum*), 11 (*Saccharomyces cerevisiae*) and 13 (mammals) protein subunits embedded in the protein-rich, highly convoluted inner mitochondrial membrane. The core of the enzyme is composed of three mitochondrially encoded subunits that exhibit high evolutionary conservation. Unlike prokaryotic enzymes, mitochondrial CcO consists of additional small peripheral subunits, encoded by the nuclear genome and synthesized in cytoplasm (Taanman 1997, Ludwig *et al.* 2001). The redox-active heme and copper cofactors, directly involved in electron transfer, are coordinated by the mitochondrially encoded subunits Cox1 and Cox2 (Tsukihara *et al.* 1995).

The biogenesis of eukaryotic CcO complex is complicated by its subcellular location, dual genetic origin of constituent subunits, the hydrophobic nature of most of them, and mainly by a number of prosthetic groups required for function, including two heme *a* moieties, three copper ions, and zinc, magnesium and sodium ions (Carr and Winge 2003). Consequently, a

number of specific gene products have evolved to accommodate such complex requirements. Although some of these factors act in a general manner and participate also in the biogenesis of other respiratory chain complexes, studies on yeast have identified over thirty accessory factors essential exclusively for proper biogenesis of the eukaryotic enzyme, while a number of them were shown to have human homologues (Khalimonchuk and Rödel 2005, Herrmann and Funes 2005). Isolated CcO deficiency represents one of the most commonly recognized causes of respiratory chain defects in humans associated with a wide spectrum of clinical phenotypes (Shoubridge 2001a, Böhm *et al.* 2005). Pedigree studies suggest that the majority of genetic defects associated with fatal infantile CcO deficiency are of nuclear origin and inherited as autosomal recessive traits. To date, autosomal recessive mutations in six nuclear encoded factors (SURF1, SCO1, SCO2, COX10, COX15, LRPPRC) required for the assembly of functional CcO complex have been identified in humans (Shoubridge 2001a, 2001b, Barrientos *et al.* 2002, Pecina *et al.* 2004). In this review we summarize current knowledge pertinent to the eukaryotic CcO biogenesis, with a special focus on mammalian enzyme whenever possible.

1. CcO structure and function

Mammalian CcO is a heterooligomeric complex of approximately 200 kDa composed of thirteen structural subunits encoded by both the mitochondrial and nuclear genes (Capaldi 1990, Taanman 1997). The enzyme is embedded in the inner mitochondrial membrane, with one part extending 37 Å into the intermembrane space (IMS) and an opposite part protruding 32 Å into the mitochondrial matrix (Tsukihara *et al.* 1996). The three mitochondrially encoded subunits, Cox1, Cox2 and Cox3 constitute the catalytic and structural core of the enzyme that incorporates all redox-active cofactors (Taanman 1997). Cox1, the largest and the most conserved subunit of the enzyme, is a highly hydrophobic protein composed of twelve transmembrane helices connected by short extramembrane loops. This subunit coordinates the catalytic site of the enzyme, and constitutes the two proton translocation pathways (D- and K-pathway) (Wikström *et al.* 2000). Cox2 is the smallest and the least hydrophobic subunit of the enzyme core. It consists of a large polar C-terminal domain that protrudes into IMS, and a transmembrane α-helical hairpin that anchors the subunit within the inner membrane. The C-terminal domain of Cox2, composed of ten stranded β barrel, coordinates the Cu_A center and constitutes the docking site for cytochrome *c*. Similarly to Cox1, subunit Cox3 is a highly hydrophobic protein spanning the inner membrane with seven transmembrane helices. It does not bear any prosthetic groups (Tsukihara *et al.* 1996) and is not directly involved in proton translocation. However, studies of the *Rhodobacter sphaeroides* aa_3-type CcO indicate that the presence of Cox3 maintains the rapid proton uptake into the D-channel at physiological pH, which presumably reduces the half-life of reactive dioxygen reduction intermediates (Gilderson *et al.* 2003, Hosler 2004). This is thought to prevent the turnover-induced inactivation of the enzyme (suicide inactivation), and the subsequent loss of Cu_B site (Bratton *et al.* 1999, Hosler 2004). The remaining 10 evolutionary younger subunits that associate with the surface of the complex core are encoded by the nuclear genome, and imported into mitochondria upon synthesis on cytoplasmic polysomes (Taanman 1997, Margeot *et al.* 2005). They include small polypeptides required for the stability/assembly of the holoenzyme, with several of them believed to be involved in regulation of catalytic activity (Ludwig *et al.* 2001). It was shown that the exchange of bound ADP by ATP at the matrix domain of subunit Cox4 leads to allosteric inhibition of the bovine enzyme at high intramitochondrial ATP/ADP ratios (Kadenbach *et al.* 2000). In addition, some of the nuclear encoded subunits were shown to be expressed in tissue- and developmentally-specific isoforms (Kadenbach *et al.* 1990, Linder *et al.* 1995). Subunits Cox5a, Cox5b and Cox6b are hydrophilic extramembrane proteins, while the rest of the nuclear encoded subunits are hydrophobic polypeptides, spanning the membrane once. Subunit Cox5a is unique in that it does not interact with any of the core subunits being held by the matrix domain of Cox4 and an extramembrane segment of Cox6c (Tsukihara *et al.* 1996). Although mutations in the three mitochondrially encoded subunits have been reported in several cases, mutations in the nuclear encoded subunits have not been found yet (Shoubridge 2001a, Barrientos *et al.* 2002). The CcO complex from *S. cerevisiae*, composed of three mitochondrially encoded and eight nuclear encoded subunits closely resembles the mammalian counterpart (Taanman 1997). Yeast null mutants for homologues of mammalian nuclear encoded subunits Cox4, Cox5a, Cox5b, Cox6c or Cox7a are respiratory deficient, lacking CcO activity and the

absorption bands representing heme aa_3 (Taanman and Williams 2001). This suggests that loss-of-function mutations in at least some of the human nuclear encoded CcO subunits might confer lethality during the early stages of intrauterine development.

In addition to the constituent protein subunits CcO contains several metal centers involved in electron transfer and dioxygen reduction (Taanman 1997). Besides their function in catalysis, most of these prosthetic groups confer an important structural/assembly function within the complex, since the defects in the synthesis and/or insertion of these cofactors often result in markedly reduced levels of fully assembled complex. In addition to catalytic cofactors, the matrix portion-associated peripheral subunit Cox5b contains a bound Zn(II) ion, while a Mg(II) ion is found at the interface of Cox1 and Cox2 subunits. Moreover, Cox1 contains single Na(I) ion in a site that can also bind Ca(II) (Tsukihara *et al.* 1996). However, the functional relevance of these three cofactors, as well as their import/insertion pathways, remains to be clarified. The low-spin heme *a* and the heterobimetallic heme a_3-Cu_B center are located relatively deep within the hydrophobic interior of Cox1. In contrast, the binuclear, mixed-valent Cu_A center extends 8 Å above the surface of the inner membrane, being held by the IMS-located, C-terminal domain of Cox2. The two copper ions (Cu(I) and Cu(II)) of the Cu_A center are coordinated by two bridging cysteines of the CxxxC motif, two histidines, one methionine and a carbonyl oxygen of the peptide backbone of Cox2 (Tsukihara *et al.* 1995).

The Cu_A center serves as the primary acceptor of electrons channeled through the respiratory chain. The electrons donated by cytochrome *c* are rapidly distributed between the Cu_A center and heme *a*, further continuing to the catalytic site composed of high-spin heme a_3 and an electronically coupled Cu_B ion. This part of the catalytic cycle referred to as the reductive phase, ensures the reduction of heme a_3-Cu_B center, a prerequisite for binding of dioxygen (and CO, but not NO) to this site, and subsequent water formation (oxidative phase) (Michel *et al.* 1998, Brunori *et al.* 2005). Protons required for the reduction of dioxygen, together with those translocated through the complex to the IMS, are taken up from the matrix and transferred via two distinct pathways to the vicinity of the heme a_3-Cu_B catalytic site. The K-pathway, named after a conserved lysine residue, is responsible for one or two-proton supply during the reductive phase. The D-pathway, named after a conserved aspartic residue, transfers the remaining two or three "substrate" protons required for water formation, as well as four protons pumped to the IMS (Wikström *et al.* 2000). Recently, the fundamental mechanism of coupling of electron transfer with proton translocation was revealed (Belevich *et al.* 2006). It was shown that the electron transfer from heme *a* to the catalytic site is kinetically linked to an internal vectorial proton transfer, initiating the proton pump mechanism of the enzyme (Belevich *et al.* 2006). The free energy released during the electron-transfer reactions is thus transformed into the electrochemical transmembrane gradient of protons, that is utilized by F_1F_O-ATP synthase (complex V) to drive ATP synthesis.

Although no functional role for CcO dimerization has been suggested, catalytically active enzyme is believed to exist within the inner membrane as a dimer of two thirteen-subunit assemblies, with contact between monomers mediated merely by subunits Cox6a and Cox6b (Tsukihara *et al.* 1996, Musatov and Robinson 2002). In mammalian mitochondria CcO (one to four copies) is found associated with NADH:ubiquinone oxidoreductase (complex I) and dimeric cytochrome bc_1 complex (complex III), within a macromolecular assembly referred to as "supercomplex" (Schägger and Pfeiffer 2000). The functional relevance of such association of respiratory complexes is thought to reside in facilitating the electron flux between the complexes, by reducing the distance of diffusion of cytochrome *c*, and by substrate channeling (Schägger and Pfeiffer 2000). Recently, using electron microscopy and single particle image analysis, the molecular architecture of both predominant mammalian stoichiometric assemblies (I_1III_2 and $I_1III_2IV_1$) of respiratory complexes was characterized, suggesting that the $I_1III_2IV_1$ supercomplex of approx. 1.7 MDa represents a major physiological module of the mammalian respiratory chain (Schäfer *et al.* 2006).

2. Synthesis and insertion of heme moieties

Heme *a* is a unique heme derivative found exclusively in all eukaryotic and certain prokaryotic CcO enzymes. In contrast, many bacterial terminal oxidases utilize heme *b* or heme *o*, instead of heme *a* (Michel *et al.* 1998). Heme *a* differs from protoheme (heme *b* or ferroprotoporphyrin IX) in that the C2 vinyl side chain is replaced by an isoprenoid substituent and a methyl group is oxidized into a formyl group (Caughey *et al.* 1975). Heme represents a potentially toxic, hydrophobic iron

chelate, which may facilitate harmful cellular process through ROS formation, e.g. oxidative membrane damage (Ryter and Tyrrell 1999). Hence, the synthesis, delivery, and final incorporation of heme *a* into CcO must be carefully regulated. Despite this fact, almost nothing is known concerning the regulation of heme *a* homeostasis (Morrison *et al.* 2005). Recently, the possible role for copper as a regulator of heme *a* biosynthesis was investigated. However, no functional correlations could have been found (Morrison *et al.* 2005). Most of the yeast CcO accessory factors mutants characterized by blocked holoenzyme assembly and rapid turnover of intermediates is unable to accumulate heme *a*. Initially, this phenomenon was ascribed to increased turnover of free heme *a*, as a consequence of reduced Cox1 levels. However, this notion is inconsistent with the fact that some of the yeast mutants retain high heme *a* levels, even when Cox1 is almost undetectable (Barros and Tzagoloff 2002). Instead, preliminary studies have suggested that the synthesis of heme *a* is subject to either positive or negative regulation by intermediate/subunit of CcO at the level of heme *a* synthase (Cox15) (Barros and Tzagoloff 2002).

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

Two heme *a* moieties are found within the eukaryotic CcO. The bis-histidine low-spin heme *a* is a six-coordinate heme responsible for electron transfer. The second heme *a*, present in the complex, is a five-coordinate, high-spin heme a_3, that forms a heterobimetallic site with Cu_B ion, a place where dioxygen, CO or NO binds (Michel *et al.* 1998, Brunori *et al.* 2005). Both heme planes are oriented perpendicular to the membrane with their iron centers being 14 Å apart (Yoshikava *et al.* 1998). The insertion of heme *a* moieties into the Cox1 subunit has not been characterized so far. As both metal centers are enfolded within the hydrophobic interior of Cox1, buried 13 Å below the membrane surface, their incorporation is likely to occur either on nascent Cox1 or an early subassembly (Carr and Winge 2003). The Cox1·Cox4·Cox5a subcomplex, as structurally present in the mature CcO, constitutes an open channel through which it would be possible for both heme moieties to be incorporated into Cox1 from the IMS side (Cobine *et al.* 2006a). Recent evidence from *R. sphaeroides* suggests that Surf1 protein might facilitate the insertion of heme a_3 into CcO (Smith *et al.* 2005). Human Surf1 is a 30 kDa integral protein of the inner mitochondrial membrane, composed of two transmembrane domains with a central loop region facing the IMS (Yao and Shoubridge 1999). In *SCO2*-deficient heart mitochondria solubilized with 1.3 % lauryl maltoside, virtually no Surf1 exists as a monomer, but rather as a trimer, and as part of higher molecular weight complex that might involve some of the accumulated CcO subassembly species (L. Stiburek, unpublished observation). Mutations in human *SURF1* represent a common cause of CcO-deficient Leigh syndrome, a subacute necrotizing encephalomyopathy (Shoubridge 2001a, Pecina *et al.* 2004). Recently, we have demonstrated that this fatal neurological phenotype is associated with remarkable tissue pattern of CcO assembly impairment, pointing to rather tissue-specific character of regulation of CcO biogenesis (Stiburek *et al.* 2005).

3. Delivery and insertion of copper ions

Copper ions are required in mitochondria for formation of Cu_A and Cu_B sites in CcO and for incorporation into IMS-located fraction of Cu/Zn-

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

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

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

Like Cox17 and Cox19, yeast Cox23 is a small soluble protein containing four cystein residues within a specific helical hairpin conformation referred to as twin Cx_9C motif. Cox23 is localized both to IMS and cytoplasm (Barros *et al.* 2004). The CcO-specific, respiratory defect of Cox23 null mutant is rescued by increased concentrations of copper, but only when *COX17* is overexpressed simultaneously (Barros *et al.* 2004). The deletion of *COX23* does not affect mitochondrial copper level (Cobine *et al.* 2006a).

The copper-binding protein Sco1, member of the conserved Sco protein family appears to act downstream of Cox17 in copper delivery pathway to Cu_A site in Cox2. Sco1 is an integral inner-membrane protein containing a globular copper-binding domain that protrudes into the IMS (Buchwald *et al.* 1991). This domain consists of a thioredoxin fold composed of a central four stranded β sheet surrounded by four α helices (Williams *et al.* 2005). The protein is tethered to the membrane by a single N-terminal transmembrane helix that was shown to be

functionally important (Beers *et al.* 2002). The observation that yeast Sco1 physically interacts with Cox2 substantiates its postulated role in Cu_A site formation (Lode *et al.* 2000). Alternatively, based on its similarity with peroxiredoxin protein family, Sco1 was proposed to be involved in the maintenance of Cu_A site cysteines in the reduced state (Chinenov 2000, Balatri *et al.* 2003). More recently, based on the structural data, human Sco1 orthologue has been implicated as a redox switch in IMS (Williams *et al.* 2005). Despite the fact that the CcO defect of human *SCO1*-deficient cells is not reversed upon overexpression of human Cox17 (Leary *et al.* 2004), its expression is required for copper metallation of human Sco1 in yeast cytoplasm assay (Horng *et al.* 2005). Mutations in human *SCO1* result in neonatal hepatic failure associated with isolated, tissue-specific CcO deficiency (Valnot *et al.* 2000). In addition to severely reduced holoenzyme levels, human *SCO1*-deficient fibroblasts accumulate several CcO subassemblies, particularly the Cox1·Cox4·Cox5a subcomplex (Williams *et al.* 2004).

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

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

4. Import and membrane-insertion of constituent subunits

The vast majority of mitochondrial proteins, including ten of the thirteen CcO subunits, are encoded by nuclear genes and synthesized in cytoplasm as precursor proteins. The targeting of most of these proteins to mitochondria is mediated by a specific cleavable N-

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

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

The insertion of mitochondrial translation products, as well as a subset of conservatively sorted nuclear gene products, into the inner membrane ensures a conserved integral inner-membrane protein Oxa1, the founding member of the Alb3/Oxa1/YidC protein family (Herrmann and Neupert 2003). Members of this family possess a hydrophobic core domain containing five transmembrane helices that facilitate the membrane export of protein substrates (Herrmann *et al.* 1997, Kuhn *et al.* 2003). Unlike bacterial homologues, mitochondrial Oxa1 contain a C-terminal α-helical domain of approx. 100 residues that protrudes into the matrix (Jia *et al.* 2003, Preuss *et al.* 2005). This domain binds to 60S ribosomal subunit protein L41, located near the polypeptide exit tunnel, and physically recruits the mitochondrial translation apparatus to the translocation complex (Jia *et al.* 2003), represented by approx. 200 kDa homooligomeric assembly of four Oxa1 subunits (Szyrach *et al.* 2003). The yeast Oxa1 null mutant is respiratory deficient, with no detectable CcO activity and markedly reduced levels of the cytochrome bc_1 complex and F_1F_O-ATP synthase (Bonnefoy *et al.* 1994a, Altamura *et al.* 1996). The obligate aerobic fungi *N. crassa* and *S. pombe* are not viable in the absence of Oxa1 (Bonnefoy *et al.* 2000, Nargang *et al.* 2002). Cox1, Cox2 and Cox3 were shown to transiently interact with Oxa1 as nascent chains (Hell *et al.* 2001). The membrane translocation of both N- and C-termini of yeast Cox2 relies on Oxa1 function (Hell *et al.* 2001). The other mitochondrial translation products do not show such a strong dependency on Oxa1, suggesting the existence of an alternative insertion pathway (Stuart 2002). Mitochondrial Oxa1 proteins are functionally conserved since the homologues of humans, plants, *N. crassa* and *S. pombe* are able to rescue the respiratory deficiency of yeast Oxa1 null mutant (Bonnefoy *et al.* 1994b, 2000; Hamel *et al.* 1997; Nargang *et al.* 2002). The human Oxa1 orthologue, referred to as OXA1L, shares 33 % sequence identity with yeast polypeptide (Bonnefoy *et al.* 1994b, Rötig *et al.* 1997). The full-length, FLAG-tagged versions of both identified coding sequences of human OXA1L localize to mitochondria when expressed in HEK 293 cells (L. Stiburek, unpublished observation). Yeast Mba1, a protein associated with the matrix face of the inner membrane presumably functions as a ribosome receptor that cooperates with Oxa1 in the cotranslational

insertion process (Ott *et al.* 2006). The lack of Mba1 together with the C-terminus of Oxa1 results in association of mitochondrial translation products with mtHSP70 (Ott *et al.* 2006), a matrix chaperone known to specifically interact with unfolded polypeptides (Hartl and Hayer-Hartl 2002). Yeast Mba1 exhibits sequence similarity with mitochondrial ribosomal L45 proteins from higher eukaryotes (Ott *et al.* 2006).

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

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

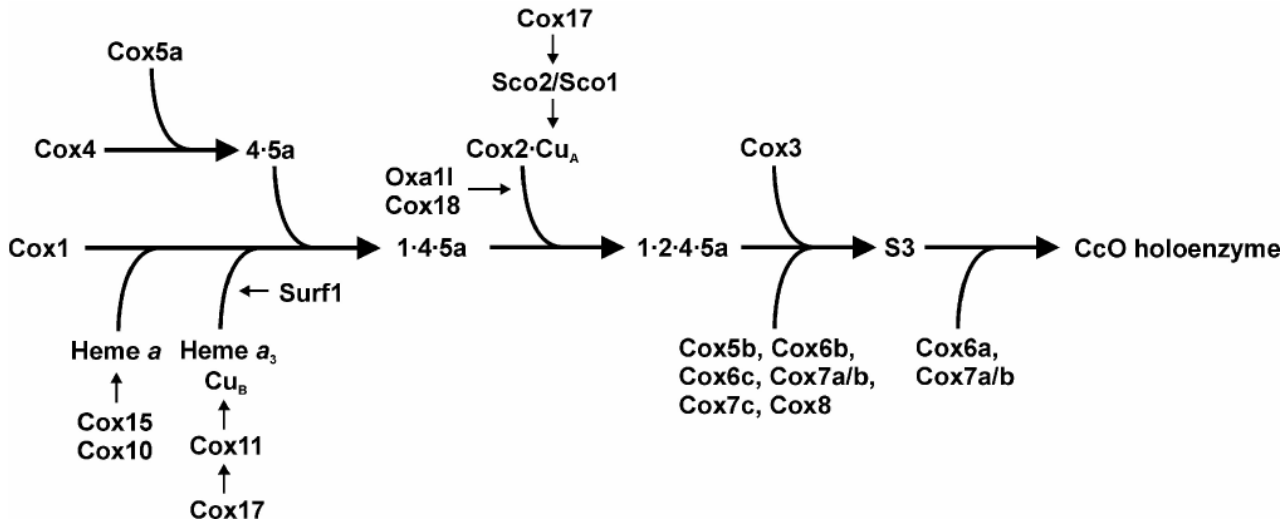

Fig. 1. Proposed model of the assembly pathway of human cytochrome *c* oxidase**.** The Arabic numerals denote subunits of the enzyme. Prosthetic groups and assembly factors are also indicated. S3 indicate previously identified assembly intermediate.

5. Assembly of mammalian CcO in the inner membrane

The spatiotemporal assembly of mammalian CcO within the inner mitochondrial membrane is a sequential and relatively slow process (Wielburski and Nelson 1983, Nijtmans *et al.* 1998). The half-life of the holoenzyme is thought to be about three days (Leary *et al.* 2002). Little is known about the sequential order in which prosthetic groups are delivered/synthesized and inserted, and constituent subunits are assembled to form the mature membrane-embedded complex. The fact that CcO subassemblies are allowed to accumulate in human mitochondria, have permitted the *bona fide* definition of several key stages of this intricate process (Fig. 1) (Nijtmans *et al.* 1998, Williams *et al.* 2004, Stiburek *et*

al. 2005). In contrast, yeast CcO subassemblies are difficult to detected as they undergo rapid proteolytic degradation (Horan *et al.* 2005). The nuclear encoded CcO subunits are imported into mitochondria upon synthesis on free cytoplasmic polysomes (Margeot *et al.* 2005). It is not known whether all of these subunits undergo conservative sorting or whether a subset of them is inserted from the IMS side (Cobine *et al.* 2006a). In contrast, most of the CcO accessory proteins are translated on outer membrane-attached polysomes, and might be imported through the TOM machinery in a cotranslational manner (Margeot *et al.* 2005). The intramitochondrial steady-state levels of various unassembled CcO subunits seem to differ considerably. Significant pools of free Cox1 and Cox5a appear to exist in mitochondria of various human tissues, whereas the levels of unassembled Cox4, and mainly of Cox2 are substantially lower (Stiburek *et al.* 2005). Cox1 appears to stably interact with several nonsubunit proteins before it associates with Cox4 and Cox5a, since it is readily detected as part of three 60-100 kDa complexes that apparently lack other CcO subunits. Subsequently, upon membrane insertion, Cox1 associates with the Cox4·Cox5a heterodimer, forming Cox1·Cox4·Cox5a subassembly (Stiburek *et al.* 2005). This subcomplex readily accumulates under conditions of blocked/retarded assembly, pointing to its high intrinsic stability. Two lines of evidence suggest that the insertion of heme *a* occurs either on unassembled Cox1 or during the formation of Cox1·Cox4·Cox5a subassembly. First, both heme moieties are buried deep within the transmembrane interior of Cox1, making the incorporation at the later stages unlikely (Tsukihara *et al.* 1996). Second, human cells deficient in heme *a* synthesis do not accumulate Cox1·Cox4·Cox5a subassembly (Antonicka *et al.* 2003b, Williams *et al.* 2004). The later finding also suggests that the presence of heme *a* within Cox1 might stabilize the binding of Cox4·Cox5a heterodimer to this subunit. In contrast, heme *a* is not required for assembly of the core subunits in *R. sphaeroides* CcO (Hiser and Hosler 2001). The insertion of active site heme might require the inner-membrane protein Surf1, since a significant fraction of CcO isolated from *R. sphaeroides* Surf1 null mutant is devoid of heme a_3 (Smith *et al.* 2005). Owing to the location of Cu_B site, its formation is likely to occur more or less concurrently with the insertion of heme groups. But the presence of Cu_B ion within Cox1 does not seem to be essential for stable incorporation of heme a_3 (Hiser *et al.* 2000). The intrinsic inner-membrane protein Cox11 might be responsible for the formation of Cu_B site (Hiser *et al.* 2000). Upon assembly of heme moieties and formation of Cu_B center, the Cu_A-containing Cox2 is believed to join the Cox1·Cox4·Cox5a subcomplex. The formation of Cu_A site in Cox2 appears to constitute a prerequisite for efficient association of this subunit with Cox1·Cox4·Cox5a subcomplex. Since the diminished formation of Cu_A site apparently leads to an accelerated turnover of Cox2 (Williams *et al.* 2004, Stiburek *et al.* 2005). The increased proteolytic degradation of such Cox2 might result either from the lowered intrinsic stability of the protein or its reduced binding to Cox1. Conversely, the proper assembly of Cox2 appears indispensable for subsequent association of Cox3, and hence for the stable binding of most of the remaining subunits. Indeed, transmitochonrial cell line (cybrid) with 100 % mutant load of a large C-terminal truncation in Cox3 was shown to lack the holoenzyme complex and accumulate subcomplex composed of Cox1, Cox2, Cox4 and Cox5a (Tiranti *et al.* 2000, Taanman and Williams 2001). Cox2 might be required to secure the incorporation of heme a_3, or whole active site, via capping the proposed heme-insertion channel formed in Cox1·Cox4·Cox5a subassembly (Bratton *et al.* 2000, Cobine *et al.* 2006a). Upon assembly of Cox2 and Cox3 the remaining nuclear encoded subunits, with the exception of Cox6a and Cox7a or Cox7b, are thought to join the complex (Nijtmans *et al.* 1998). The resulting assembly intermediate S3 represents a ubiquitous, although minor form of CcO in lauryl maltoside preparations. Subsequent association of the rest of the subunits completes the assembly of the holoenzyme complex (Nijtmans *et al.* 1998). In the next, maturation step a covalent bond is formed on assembled Cox1 bridging His^{240}, one of the three histidine ligands of Cu_B, with conserved Tyr^{244} located at the end of the proton translocation K-channel (Yoshikawa *et al.* 1998). This posttranslational modification is thought to secure the Cu_B ion in a certain configuration and distance from heme a_3, thus preventing the coordination of Cu_B via histidine ligands of the active site heme (Pinakoulaki *et al.* 2002). Finally, the mature holoenzyme complex associates with complex I and dimeric complex III, to form the 1.7 MDa respiratory supercomplex (Schägger and Pfeiffer 2001, Schäfer *et al.* 2006). The role of dimerization and cardiolipin in final maturation of CcO, as well as the function of cytochrome *c* during CcO assembly, remains elusive.

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Loss of function of Sco1 and its interaction with cytochrome c oxidase

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Loss of function of Sco1 and its interaction with cytochrome *c* oxidase

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Stiburek L, Vesela K, Hansikova H, Hulkova H, Zeman J. Loss of function of Sco1 and its interaction with cytochrome *c* oxidase. *Am J Physiol Cell Physiol* 296: C1218–C1226, 2009. First published March 18, 2009; doi:10.1152/ajpcell.00564.2008.—Sco1 and Sco2 are mitochondrial copper-binding proteins involved in the biogenesis of the Cu_A site in the cytochrome *c* oxidase (CcO) subunit Cox2 and in the maintenance of cellular copper homeostasis. Human Surf1 is a CcO assembly factor with an important but poorly characterized role in CcO biogenesis. Here, we analyzed the impact on CcO assembly and tissue copper levels of a G132S mutation in the juxtamembrane region of SCO1 metallochaperone associated with early onset hypertrophic cardiomyopathy, encephalopathy, hypotonia, and hepatopathy, assessed the total copper content of various *SURF1* and *SCO2*-deficient tissues, and investigated the possible physical association between CcO and Sco1. The steady-state level of mutant Sco1 was severely decreased in the muscle mitochondria of the *SCO1* patient, indicating compromised stability and thus loss of function of the protein. Unlike the wild-type variant, residual mutant Sco1 appeared to migrate exclusively in the monomeric form on blue native gels. Both the activity and content of CcO were reduced in the patient's muscle to ~10–20% of control values. *SCO1*-deficient mitochondria showed accumulation of two Cox2 subcomplexes, suggesting that Sco1 is very likely responsible for a different posttranslational aspect of Cox2 maturation than Sco2. Intriguingly, the various *SURF1*-deficient samples analyzed showed a tissue-specific copper deficiency similar to that of *SCO*-deficient samples, suggesting a role for Surf1 in copper homeostasis regulation. Finally, both blue native immunoblot analysis and coimmunoprecipitation revealed that a fraction of Sco1 physically associates with the CcO complex in human muscle mitochondria, suggesting a possible direct relationship between CcO and the regulation of cellular copper homeostasis.

mitochondria; copper; SCO2; SURF1

CYTOCHROME *C* OXIDASE (CcO) is the terminal heterooligomeric complex of the energy-transducing mitochondrial electron transport chain. This enzyme, which belongs to the superfamily of heme-copper containing aa_3-type terminal oxidases, is embedded in the inner mitochondrial membrane, where it catalyzes the transfer of electrons from reduced electron carrier cytochrome *c* to dioxygen, coupling this reaction with vectorial proton pumping across the inner mitochondrial membrane. The biogenesis of eukaryotic CcO is a complicated, sequential process requiring the coordinated synthesis and import of 3 mitochondria- and 10 nuclear-encoded structural subunits with subsequent assembly and insertion into the inner mitochondrial membrane. This process also requires the concurrent import/synthesis and incorporation of several prosthetic groups, including two heme *a* moieties, three copper ions, and zinc, magnesium, and sodium ions (16).

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A number of nuclear-encoded accessory factors essential for CcO biogenesis have been identified in eukaryotes, and mutations in six of them have been shown to be involved in human pathologies (16). Human Sco1 and Sco2 are closely related inner mitochondrial membrane copper-binding proteins encoded by paralogous genes. They have been demonstrated to exert nonoverlapping, cooperative roles in copper delivery to CcO (7). In addition, they have been shown to be involved in the maintenance of cellular copper homeostasis, presumably by controlling cellular copper export (6, 8). Mutations in both *SCO1* and *SCO2* cause severe tissue-specific CcO assembly impairment accompanied by marked copper deficiency (11, 17, 18). However, both genes have been shown to be ubiquitously expressed, displaying a similar expression pattern across human tissues. Mutations of *SCO1* have thus far only been reported in a single pedigree, where the two patients were compound heterozygotes carrying a nonsense mutation on one allele and a P174L missense mutation on the second allele (18). In contrast, mutations of *SCO2* are more common, with all reported patients carrying at least one E140K missense allele (19). Mutations in *SCO2* cause fatal infantile encephalomyopathy and hypertrophic cardiomyopathy, whereas the two reported *SCO1* patients presented with fatal infantile encephalomyopathy and hepatopathy. *SCO2* patients homozygous for the E140K substitution have a delayed onset and slightly prolonged course of the disease compared with compound heterozygotes.

Human Surf1 is an integral protein of the inner mitochondrial membrane composed of two transmembrane domains with a central loop region facing the mitochondrial intermembrane space (22). Mutations of *SURF1*, which account for the majority of nuclear-encoded, isolated CcO deficiencies in humans, are characterized by the development of Leigh syndrome, a subacute necrotizing encephalomyopathy (14, 23). Most of the identified *SURF1* mutations are predicted to lead to loss of protein function. Despite numerous attempts, the molecular function of human Surf1 in CcO assembly still remains to be elucidated (12, 13).

Here, we studied the impact on CcO biogenesis and tissue copper content of a novel homozygous missense mutation in SCO1 associated with early onset hypertrophic cardiomyopathy, encephalopathy, hypotonia, and hepatopathy. We also measured the total copper levels of various *SURF1* and *SCO2* tissues and investigated the possible physical association between CcO and Sco1. We found that the G132S substitution compromises the stability of the mutant protein, presumably by preventing its oligomerization, leading to a CcO assembly defect, accumulation of Cox2-containing subcomplexes, and severe copper deficiency in the patient's skeletal muscle. We further demonstrate that, similar to *SCO1* and *SCO2* tissues, the *SURF1* samples exhibited marked tissue-dependent copper deficiency, indicating that Surf1 is required, in a tissue-specific

manner, to maintain proper cellular copper homeostasis. The fact that the Cox2 subunit accumulates in *SCO1* muscle mitochondria in the form of low-molecular-weight subcomplexes suggests that Sco1 is very likely required for a different posttranslational step in Cox2 maturation than Sco2. Finally, both blue native (BN) electrophoresis and coimmunoprecipitation indicated that a fraction of Sco1 associates with fully assembled CcO in human muscle mitochondria. This is of particular interest since the Sco1-dependent metallation of Cox2 is thought to occur on membrane-embedded, yet unassembled subunit, and the functional relevance of this interaction might therefore relate to the role of Sco1 in copper homeostasis maintenance rather than CcO metallation.

MATERIALS AND METHODS

Ethics. The present study was carried out in accordance with the Declaration of Helsinki of the World Medical Association and was approved by the Committees of Medical Ethics at all collaborating institutions. Informed parental consent, in accordance with guidelines of the participating institutions, was obtained for all biopsies and autopsies.

Tissue samples and cell cultures. Tissue samples and/or cell cultures were obtained from one *SCO1* patient, five *SCO2* patients, and four *SURF1* patients. The three *SCO2* patients homozygous for the common g.1541G>A (E140K) substitution presented with progressive encephalopathy since the third month of age, and they died at <1 yr of age. The two *SCO2* compound heterozygotic patients (g.1280C>T/g.1541G>A and g.1518delA/g.1541G>A) presented with hypertrophic cardiomyopathy since birth and died at the ages of 7 and 16 wk, respectively (17, 19, 20). All *SURF1* patients (c.790_792delCT/c.845_846delCT, c.312insATdel10/c.821del18, c.688C>T/c.688C>T, and c.469C>T/c.845_846delCT) presented with failure to thrive and progressive hypotonia at the end of the first year of life, followed by a total arrest of psychomotor development. They all died between the third and fifth year of life (12).

Primary skin fibroblast cultures were established from forearm skin biopsies and grown in Quantum 333 complete medium for fibroblasts (PAA). Open muscle biopsies were obtained from the tibialis anterior muscle, frozen in liquid nitrogen, and stored at −80°C. Postmortem heart, liver, and brain (frontal cortex) tissue specimens were obtained at autopsy and frozen in liquid nitrogen at <2 h after death.

Mutation detection in the SCO1 gene. Genomic DNA was isolated from the peripheral blood. The six exons of the *SCO1* gene were amplified by PCR using *SCO1*-specific intronic primers. PCR products were purified using a QIAquick Gel Extraction Kit (QIAGEN) and directly sequenced using the ABI Prism 3100 AVANT genetic analyzer using the recommended chemistry. The detected mutation was confirmed by restriction fragment length polymorphism analysis using PCR with a mismatch primer changing the thymine at c.392 to cytosine to create a restriction site for *Hpa*II endonuclease (BioLabs). The mismatch primer corresponded to c.379-393. The 17-bp universal M13 primer was added to the 5′-end to enlarge the cleaved fragment. Thus, *Hpa*II cuts the wild-type allele, whereas the mutated allele retains its full length.

Copper content analysis. The total copper content of skeletal muscle, heart, brain (frontal cortex), and liver tissue specimens was measured by flame atomic absorption spectrometry on a Perkin-Elmer 3300 Atomic Absorption Spectrophotometer.

Enzyme activity assays. The enzyme activities of CcO and citrate synthase (CS) were measured spectrophotometrically in isolated muscle mitochondria essentially as previously described (17).

Immunoprecipitation. Immunoprecipitation of the CcO complex from skeletal muscle mitochondria and HEK-293 cell mitochondria was performed using the Complex IV Immunocapture Kit (Mitosciences, Eugene, OR). Mitochondria were solubilized with 1% *n*-dodecyl-β-D-maltoside (Sigma-Aldrich), and, after a clarifying spin (50,000 *g*), the respective supernatants supplemented with protease inhibitor cocktail (Sigma-Aldrich) were incubated overnight with antibody-loaded agarose beds under gentle agitation at 4°C. To partially correct for the lower CcO content in HEK-293 cell mitochondria, immunoprecipitation was performed at a protein concentrations of 6 mg/ml (HEK-293 cell mitochondria) and 2 mg/ml (muscle mitochondria). After an incubation step, immunocomplexes were washed five times with a buffer containing 0.05% (wt/vol) *n*-dodecyl-β-D-maltoside, and the bound material was then eluted with a buffer containing 1% SDS and routinely processed for SDS-PAGE.

Electrophoresis and immunoblot analysis. Tricine SDS-PAGE was carried out under standard conditions with 12% polyacrylamide-0.1% (wt/vol) SDS gels. Mitochondrial fractions were dissociated in 50 mM Tris·HCl (pH 6.8), 12% (vol/vol) glycerol, 4% SDS, 2% (vol/vol) 2-mercaptoethanol, and 0.01% (wt/vol) bromophenol blue for 30 min at 37°C, and ~10 μg of protein were loaded for each lane.

BN-PAGE was performed with 8–16% and 10–20% (wt/vol) polyacrylamide gradient gels using a MiniProtean 3 System (Bio-Rad Laboratories). Mitochondrial proteins were extracted with 1% (wt/vol) *n*-dodecyl-β-D-maltoside at a protein concentration of 2 mg/ml in a buffer containing 1.5 M aminocaproic acid, 2 mM EDTA, and 50 mM bis-Tris (pH 7.0) at 4°C. Serva Blue G (Serva) was added to solubilized mitochondrial proteins to a final concentration of 0.1 mg/mg of detergent, and 10–50 μg of protein were loaded for each lane. Electrophoresis was performed at 40 V and 4°C for 1 h and then at 100 V.

For two-dimensional BN-SDS-PAGE, strips of the first-dimension gels were incubated for 15 min in a buffer containing 1% 2-mercaptoethanol and 1% SDS and then for 2 × 10 min in 1% SDS, and denatured proteins were then resolved in the second dimension on 12% polyacrylamide-0.1% SDS gels.

Proteins were electroblotted from the gels onto Immobilon-P polyvinylidene difluoride membranes (Millipore) using the TE 70XP Semi-Dry Transfer Unit (Hoefer, Holliston, MA). Blots were finally developed using West Femto Chemiluminescent substrate (Pierce). Signal acquisition was performed using a VersaDoc 4000 imaging system (Bio-Rad Laboratories), and the resulting digital images were analyzed using the Quantity One application (Bio-Rad Laboratories).

Histology, immunohistochemistry, and electron microscopy. All analyses were performed in paraffin-embedded sections obtained from the autopsy of the *SCO1* patient. Paraffin sections were treated using routine histological stains. Mitochondria were detected essentially as previously described (20). For electron microscopy, paraffin-embedded heart, liver, and skeletal muscle samples were deparaffinized in xylol, gradually hydrated, osmicated, reembedded into Araldite Epon mixture, cut with a diamond knife, double contrasted, and examined using a Tesla BS-500 transmission electron microscope (Tesla, Brno, Czech Republic).

RESULTS

Clinical presentation of the SCO1 patient. A girl with intrauterine growth retardation was born to unrelated healthy parents at term with a birth weight of 2,200 g and length of 46 cm. The early postnatal adaptation was uneventful with an Apgar score of 10 in the fifth and tenth minutes. However, poor feeding, failure to thrive, progressive hypotonia, and hepatomegalia developed since the second month of life. Echocardiography revealed left ventricular hypertrophy, and sonography showed brain atrophy. Biochemical investigation revealed a mild increase in aminotransferase levels (alanine aminotransferase: 1.37–1.78 μkat/l and aspartate aminotransferase: 1.28–1.69 μkat/l) with normal levels of creatine kinase and blood copper (Cu: 18 μmol/l). The dominant metabolic finding since the age of 5 mo was hyperlactacidemia with a fasting B-lactate

level of 3.6 mmol/l (reference: <2.3 mmol/l) and a postprandial B-lactate level of 7.7 mmol/l. The fasting lactate-to-pyruvate ratio (L/P) was normal (L/P: 13; reference: 10–20) with a postprandial increase (L/P: 25). Blood alanine was markedly elevated at 738 μmol/l (reference: <500 μmol/l), the free B-carnitine level was reduced at 18 μmol/l (reference: <24 μmol/l), and the ratio between acylcarnitine and free carnitine was increased at 1.5 (reference: 0.23–0.50). The urinary organic acid profile showed increased excretion of lactate (103 mg/g creatinine; reference: <60 mg/g) and the Krebs cycle intermediates 2-oxoglutarate (1,150 mg/g creatinine; reference: <200 mg/g), fumarate (185–193 mg/g creatinine; reference: <15 mg/g), and malate (75 mg/g creatinine; reference: <15 mg/g).

The progressive course of the disease and pathological results of metabolic analyses suggested mitochondrial etiology; thus, the patient was recommended for muscle biopsy. The patient died at the age of 6 mo due to cardiac failure. The dominant finding at autopsy was marked cardiac hypertrophy (55 vs. 29 g), with uniform concentric thickening of the ventricles, predominantly the left ventricle.

Structural analysis of SCO1 tissues. Cardiocytes were hypertrophic with enlarged and often doubled nuclei (Fig. 1*A*). There was a uniform increase in the number of mitochondria, with many of them enlarged. Mitochondria were strongly stained with MU213-UC antibody and an antibody against prohibitin (Fig. 1*B*). Ultrastructurally, there was a numerical increase in the mitochondrial population, which displayed remarkable variation in the size of individual mitochondria (some reached 3–4 μm in diameter) with frequent intermitochondrial contacts and lucent vacuoles in the matrix. Occasionally, there were densified linear rigid cristae and discrete granular deposits of medium density mainly surrounded by a limiting membrane (Fig. 1, *C* and *D*).

The liver showed moderate microvesicular steatosis without other obvious structural abnormalities. Skeletal muscles were free of pronounced regressive or regenerative changes. The number of mitochondria showed a only slight tendency to increase, without evidence for ragged-red fibers. Ultrastructural findings of mitochondria in both locations were limited to slight variations in size. Megamitochondria were absent. There was no evidence of any histological changes in other organs (kidney, gastrointestinal tract, and adrenal glands).

Mutation detection in the SCO1 gene. Direct sequencing revealed the novel homozygous mutation c.394G>A in the third exon of the *SCO1* gene. This nucleotide substitution was not found in 200 healthy controls. The mutation is predicted to change the highly conserved glycine residue at position 132 to serine within a juxtamembrane region of Sco1.

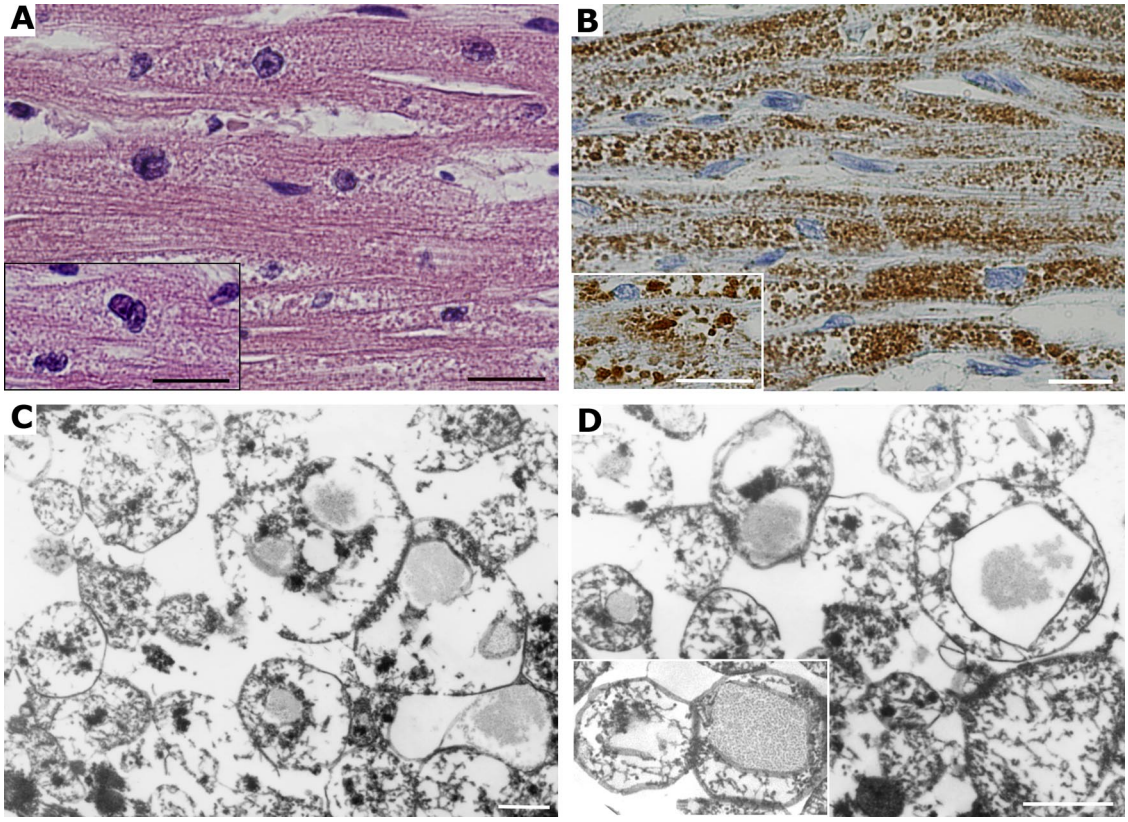

Fig. 1. Structural changes in the left cardiac ventricle of the *SCO1* patient. *A*: myocardium with enlarged hyperchromatic, often doubled (*inset*) nuclei reflecting cardiac hypertrophy. Note the increased number and size of mitochondria concentrated in the perinuclear space. Sample was stained with hemotoxylin and eosin. Bar = 20 μm. *B*: immunodetection of mitochondrial antigen with MU213-UC antibody showed a uniformly increased number of mitochondria in cardiocytes. Some mitochondria were remarkably enlarged (*inset*). Bar = 25 μm; bar in *inset* = 20 μm. *C* and *D*: electron micrographs of cardiocyte mitochondria showing the wide range of mitochondrial size, enlargement of individual mitochondria, variable degree of mitochondrial swelling, and close contact of external membranes. Intramitochondrial hemidense deposits were predominantly surrounded by a membrane that occasionally displayed an angular shape. Bars = 1 μm. The *inset* in *D* shows that the discretely granular character of these deposits corresponds most probably with their monoparticulate glycogen nature.

Steady-state level and oligomeric state of Sco1 in wild-type and SCO1-deficient muscle mitochondria. Skeletal muscle mitochondria were resolved using SDS-PAGE, and the resulting immunoblots were probed with rabbit polyclonal antiserum raised against human Sco1 (7) and with an antiserum against Sco2 (17). Equal loading was verified using an antibody targeting succinate dehydrogenase complex subunit A (SDHA) of respiratory complex II. The steady-state level of mutant Sco1 was decreased to ~10% of control values in the *SCO1* patient sample (Fig. 2*A*). Conversely, no significant alterations in Sco1 content were observed in the *SCO2* patient background. Probing of the immunoblots with the Sco2 antiserum revealed that the steady-state level of Sco2 was severely reduced in the *SCO2* patient sample but virtually unaffected in the Sco1 patient background (Fig. 2*A*). Consistent with the known functional involvement of SCO gene products, the levels of copper-containing mitochondrially encoded CcO subunits Cox1 and Cox2 were found to be severely reduced in both *SCO1* and *SCO2* patient backgrounds (Fig. 2*A*).

Since the *SCO1* mutation occurs within a region presumably involved in protein dimerization (1), we examined the oligomeric state of G132S mutant Sco1 in patient muscle mitochondria using two-dimensional BN-SDS-PAGE immunoblot analysis. Mitochondrial fractions were solubilized with 1% (wt/vol) *n*-dodecyl-β-D-maltoside and fractionated on a 10–20% (wt/vol) polyacrylamide gradient in the first (BN) dimension. Immunoblots were subsequently probed with an antibody targeting human Sco1. Wild-type Sco1 migrated as part of two minor high-molecular-weight and two predominant lower-molecular-weight assemblies (Figs. 2*B* and 3*A*). The 30- and 70-kDa estimated molecular weight of the faster-migrating predominant Sco1 complexes suggests that they represent monomeric and homooligomeric forms of Sco1 protein, respectively. Indeed, the 70-kDa apparent molecular weight of the larger, fast-migrating assembly suggests that it represents the homodimeric form of Sco1, which has been previously described as a 60-kDa complex (1, 7). The 10-kDa difference in apparent molecular weight likely reflects binding of dodecyl maltoside to the dimer. Furthermore, detection with antibodies against Cox1 and Cox2 revealed that the two minor high-molecular-weight (200–300 kDa) Sco1 complexes specifically comigrated with the CcO holoenzyme complex, suggesting a possible physical association of Sco1 with CcO (Fig. 3*A*).

Mainly due to the low abundance of residual mutant Sco1 in the sample, the complementary immunoblots of *SCO1* patient mitochondria produced relatively high levels of nonspecific signal in addition to the specific one. Nevertheless, it was possible to clearly recognize the presence of the monomeric form of Sco1 on the immunoblot. In contrast, no significant signals corresponding to the ~70-kDa complex as well as to higher-molecular-weight forms of Sco1 were discernible on *SCO1* patient immunoblots (Fig. 2*B*).

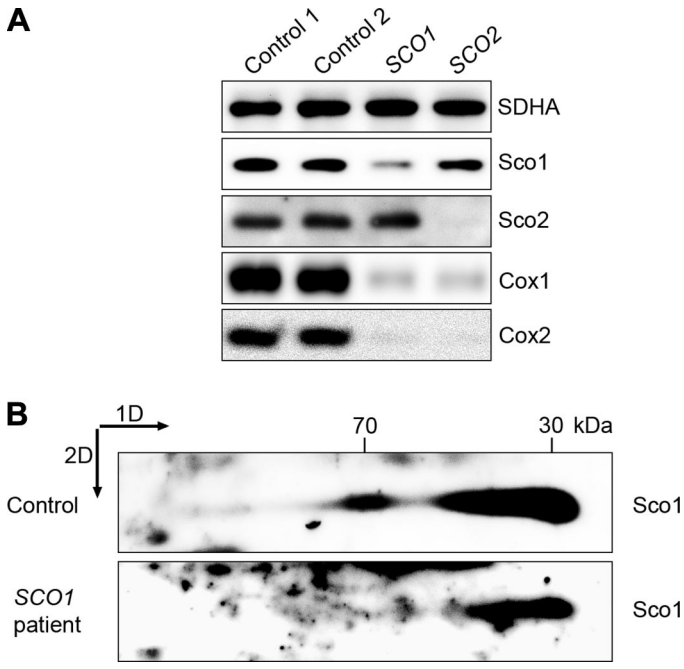

Fig. 2. The steady-state level of G132S mutant Sco1 is severely attenuated in the muscle mitochondria of the *SCO1* patient, and the protein migrates exclusively in the monomeric form on blue native (BN) gels. *A*: equal amounts of protein from isolated muscle mitochondria were fractionated using SDS-PAGE, and the resulting immunoblots were treated with rabbit polyclonal antiserum raised against human Sco1 and with an antiserum against human Sco2. SDHA, succinate dehydrogenase complex subunit A. *B*: isolated muscle mitochondria were solubilized with 1% dodecyl maltoside and fractionated (50 μg protein) using two-dimensional BN-SDS-PAGE on a 10–20% polyacrylamide gradient in the first dimension. The resulting immunoblot was treated with the antiserum against human Sco1.

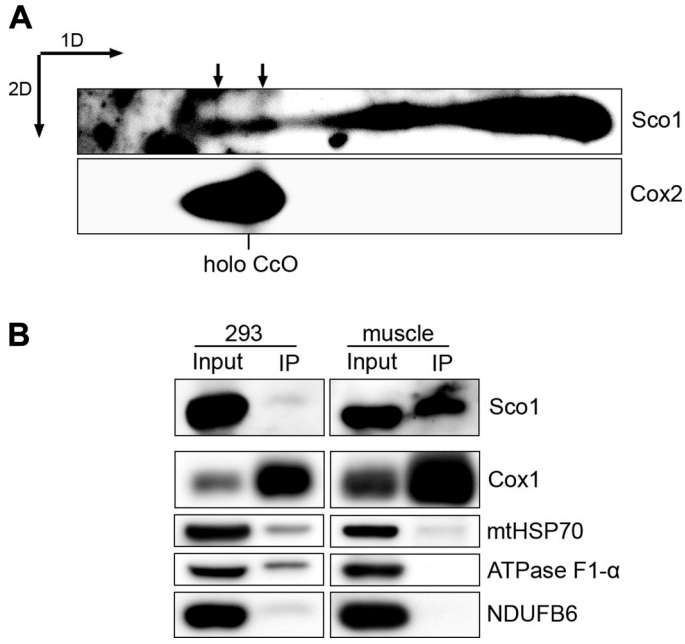

Fig. 3. A fraction of Sco1 physically associates with the cytochrome *c* oxidase (CcO) holoenzyme (holo) in human muscle mitochondria treated with dodecyl maltoside. *A*: human muscle mitochondria were solubilized with 1% dodecyl maltoside and fractionated (50 μg protein) using two-dimensional BN-SDS-PAGE on a 10–20% polyacrylamide gradient in the first dimension. The resulting immunoblots were treated with antibodies against Sco1 and Cox2. The arrows indicate the positions of Sco1 complexes that comigrated with the CcO holoenzyme in the BN dimension. *B*: mitochondria isolated from the HEK-293 cell line and human skeletal muscle were solubilized with 1% dodecyl maltoside, and CcO was immunoprecipitated from the samples using the Complex IV Immunocapture Kit (Mitosciences). The immunoprecipitates (IP) and original samples (input) were fractionated using SDS-PAGE, and the resulting polyacrylamide gels were immunoblotted using the indicated antibodies. mtHSP70, mitochondrial heat shock protein 70; NDUFB6, NADH-ubiquinone oxidoreductase β-subunit 6.

Coimmunoprecipitation of Sco1 with the CcO complex from human muscle mitochondria. To investigate the possible physical association of Sco1 with the CcO complex, we immunoprecipitated CcO from human muscle mitochondria and HEK-293 cell mitochondria solubilized with the nonionic detergent dodecyl maltoside and analyzed the immunoprecipitate using denaturing immunoblots. Consistent with the higher CcO content in muscle mitochondria compared with HEK-293 cell mitochondria, the former immunoprecipitate showed substantially higher CcO recovery (Fig. 3*B*). Immunodetection of Cox1 confirmed that immunoprecipitation was similarly effective for both samples (Fig. 3*B*). However, probably due to the higher protein concentration, slightly higher nonspecific binding was observed for the HEK-293 cell immunoprecipitate (Fig. 3*B*). Despite the high Sco1 content in HEK-293 samples, the muscle immunoprecipitate showed substantially increased recovery of Sco1 that matched the severalfold increase in the recovery of CcO (Fig. 3*B*). These results indicate that a fraction of Sco1 physically associated with the CcO complex in human muscle mitochondria. Furthermore, the fact that we were not able to precipitate Sco1 with CcO from HEK-293 cell mitochondria might suggests the tissue-specific nature of this interaction.

Steady-state level, activity, and assembly pattern of CcO in SCO1 muscle mitochondria. The residual content of CcO in mitochondria from muscle biopsy of the *SCO1* patient was assessed using BN-PAGE immunoblot analysis. Equal loading was verified using an antibody against the core 2 subunit of respiratory complex III. The content of the CcO holoenzyme was found to be reduced to ~10–20% of control values (Fig. 4*A*); this reduction was comparable with that observed in the *SCO2* patient sample.

In accord with the reduced holoenzyme content, the enzyme activity assay showed a marked reduction of the CcO-to-CS ratio in *SCO1* skeletal muscle mitochondria to 8% (0.047) of the mean reference value (0.6 ± 0.21). However, when the highly increased CS activity (240%) in the sample is taken into account, the normalized CcO activity was reduced to 19% (11.42 nmol·min^{-1}·mg^{-1}) compared with age-matched controls (58 ± 33 nmol·min^{-1}·mg^{-1}, n = 18).

Using two-dimensional BN-SDS-PAGE immunoblot analysis with a cocktail of antibodies against Cox1, Cox2, Cox4, and Cox5a subunits, mitochondria from the muscle biopsy of the *SCO1* patient were found to accumulate several CcO subcomplexes concomitantly with the reduction of the holoenzyme level. The highest increase in steady-state levels was observed for the previously identified Cox1-containing subcomplexes c, d, and e (17, 21). However, ~110-kDa subcomplex c was found to lack both Cox4 and Cox5a subunits. Unexpectedly, prolonged exposure of the immunoblot revealed that this subcomplex instead contained a significant amount of the Cox2 subunit. Moreover, the Cox2 subunit was found in a ~60- to 70-kDa assembly that comigrated with Cox1-containing subcomplexes e and f (Fig. 4*B*).

Distinct pattern of CcO subcomplexes in SCO2 and SURF1 primary fibroblasts. Mitochondria-enriched fractions from three different *SURF1* fibroblast cultures and one *SCO2* culture (E140K/Q53X) were resolved using BN-PAGE and subsequently probed with the antibody against Cox1. The relative amount of mitochondrial protein loaded as well as the molec-

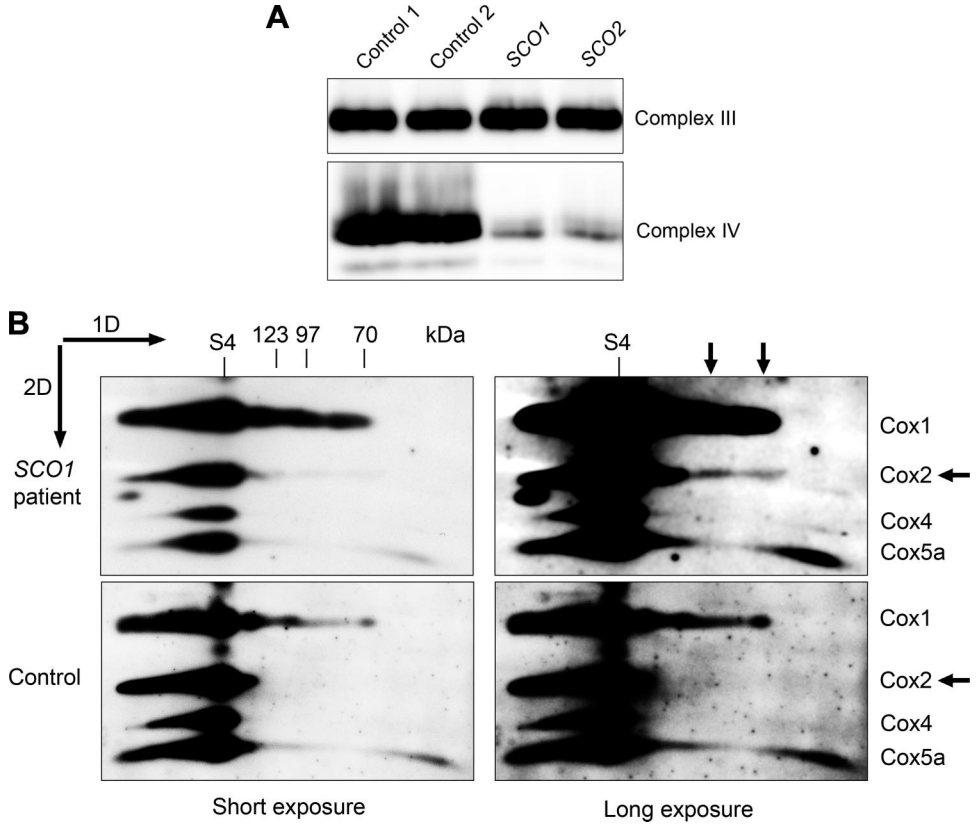

Fig. 4. The G132S amino acid substitution in Sco1 leads to severely decreased CcO levels and an accumulation of Cox2-containing subcomplexes in the muscle mitochondria of the *SCO1* patient. *A*: mitochondria from the muscle biopsy of the *SCO1* patient and control were solubilized with 1% dodecyl maltoside, and equal amounts of protein (10 μg) were fractionated using BN-PAGE. The resulting immunoblots were treated with antibodies against Core 2 and Cox2. *B*: muscle mitochondria were solubilized with 1% dodecyl maltoside and fractionated (50 μg protein) using two-dimensional BN-SDS-PAGE on a 8–16% polyacrylamide gradient in the first dimension. The resulting immunoblots were treated with a cocktail of antibodies against Cox1, Cox2, Cox4, and Cox5a subunits of CcO. The arrows indicate the positions of both identified Cox2-containing subcomplexes.

ular weight of detected CcO subcomplexes were estimated using an antibody targeting the SDHA subunit of respiratory complex II. All *SURF1*, and to a lesser extent *SCO2*, patient cells were found to accumulate distinct Cox1-containing subcomplexes in addition to showing reduced holoenzyme levels. As expected, all *SURF1* cultures showed an accumulation of the previously identified 110-kDa subcomplex S2 (21). However, the Cox1-containing assembly from *SCO2* cells displayed markedly distinct mobility, with an estimated molecular weight of at least 130–140 kDa (Fig. 5). This is intriguing since both *SCO2* and *SURF1* cells are thought to accumulate an identical subcomplex (S2) composed of Cox1, Cox4, and Cox5a subunits (7, 21).

Total copper content of various tissues harboring mutations in SCO1, SCO2, and SURF1. The total copper content of skeletal muscle, heart, brain (frontal cortex), and liver tissue specimens was measured by flame atomic absorption spectroscopy. *SCO2* and *SURF1* liver samples were the most severely affected, showing an average reduction in total copper levels to 23% (12.5 ± 5.4 μg/g, $n = 4$) and 20% (10.7 ± 10.3 μg/g, $n = 3$) of reference values (53.6 ± 26.8 μg/g, $n = 10$), respectively. This is contrasted by the lack of clinical liver involvement in these patients (12, 17). Moreover, all *SCO2* liver samples contained normal levels of the CcO holoenzyme (17). Consistent with their severe clinical and biochemical involvement, *SURF1* skeletal muscle samples showed an average copper content reduction to 26.5% (0.5 ± 0.1 μg/g, $n = 4$) of control values (1.9 ± 0.4 μg/g, $n = 7$). In contrast, despite marked CcO deficiency and severe clinical presentation (17), *SCO2* skeletal muscles displayed rather moderate copper deficiency (51.2% of control values, 1.3 ± 0.6 μg/g, $n = 3$). Indeed, an additional *SCO2* muscle sample from the E140K homozygote displayed completely normal copper content. In the skeletal muscle from the *SCO1* patient with profound CcO deficiency, the total copper level was found to be decreased to 21.2% (0.4 μg/g) of control values. Consistent with the severe clinical and biochemical presentation (19), *SCO2* heart specimens showed a marked reduction of total copper levels to 31.4% (1.03 ± 0.05 μg/g, $n = 3$) of control values (3.28 ± 0.76 μg/g, $n = 8$). In contrast, clinically unaffected *SURF1* heart samples with only moderately reduced CcO content showed a reduction of copper levels to 53.2% (1.75 ± 0.25 μg/g, $n = 2$) of control values. The copper content of *SCO2* frontal cortex specimens was reduced to an average of 58.2% (1.3 ± 0.3 μg/g, $n = 3$) of control values (2.23 ± 1.0 μg/g, $n = 6$). Finally, the two available *SURF1* frontal cortex samples showed moderately reduced (1.1 μg/g) and normal copper levels (Fig. 6). Intriguingly, all *SCO2* and *SURF1* patients showed severe central nervous system involvement, and all the studied frontal cortex specimens showed marked CcO deficiency (12, 17, 19).

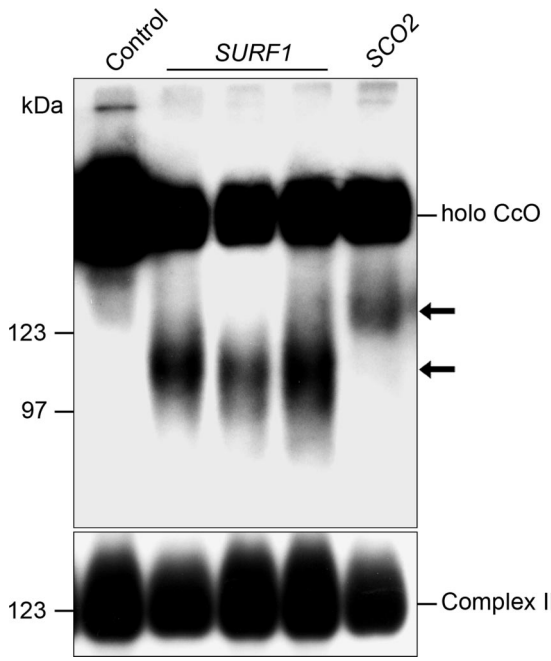

Fig. 5. Distinct pattern of CcO subcomplexes in *SCO2* and *SURF1* primary fibroblasts. Equal amounts of mitochondrial protein (10 μg) from cultured fibroblasts were fractionated using BN-PAGE on a 8–16% polyacrylamide gradient, and the resulting immunoblots were treated with antibodies against Cox1 and SDHA. The arrows indicate the positions of accumulated Cox1-containing subcomplexes.

DISCUSSION

In the present study, we showed that the G132S mutation in SCO1, which is associated with early-onset hypertrophic cardiomyopathy, encephalopathy, hypotonia, and hepatopathy, compromised the stability of the protein, presumably by preventing its oligomerization, leading to an impairment of CcO assembly, accumulation of Cox2-containing subcomplexes, and severe copper deficiency in the skeletal muscle of the *SCO1* patient. We further showed that, similar to *SCO1* and *SCO2* tissues, *SURF1* samples displayed a marked tissue type-dependent copper-deficient phenotype, indicating a role for Surf1 in copper homeostasis maintenance. Finally, we demonstrated that a fraction of Sco1 associates with fully assembled CcO in human muscle mitochondria, suggesting a possible direct relationship between CcO and the regulation of cellular copper homeostasis.

The described SCO1 mutation is predicted to change the highly conserved glycine residue at position 132 to serine within a juxtamembrane region separating the NH_2-terminal transmembrane helix from the globular domain. Substitution of the strongly hydrophilic serine for neutral and highly compact glycine is likely to significantly alter the character of this region. We showed that the steady-state level of G132S mutant Sco1 was substantially attenuated in the muscle mitochondria of the *SCO1* patient, indicating severely compromised stability and loss of function of the protein. In contrast, the only SCO1 missense mutation reported to date (P174L) does not affect the stability of the protein but leads to altered functional properties, namely, compromised copper loading by the Cox17 metallochaperone and transduction of an aberrant copper overload signal (2, 6). Based on our inability to detect mutant Sco1 in its ~70-kDa, most likely homodimeric, form on BN immunoblots, we speculate that the mutation might abrogate the ability of Sco1 to oligomerize, eventually leading to reduced stability of the monomeric form. This is supported by the fact that the amino acid substitution occurs within a region shown to be required for dimerization of Sco1 (1), which exists exclusively in the homooligomeric form in human cells (7).

Two lines of experimental evidence indicate that a fraction of Sco1 physically interacts with fully assembled CcO in

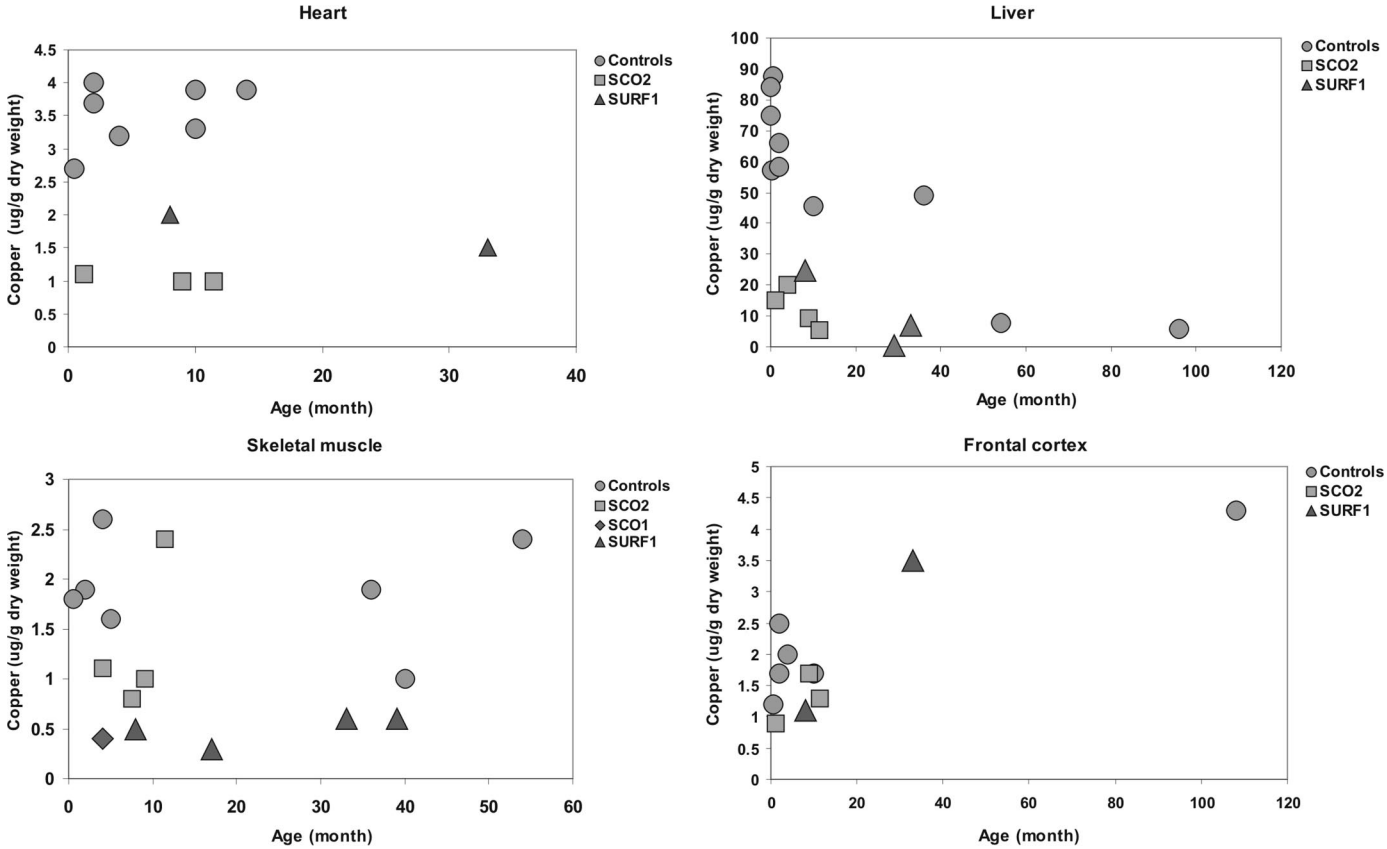

Fig. 6. Tissue-dependent copper deficiency of various *SCO1*, *SCO2*, and *SURF1* tissues. The total copper content of the indicated tissue specimens was measured by flame atomic absorption spectroscopy on a Perkin-Elmer 3300 Atomic Absorption Spectrophotometer.

human muscle mitochondria. First, two Sco1 complexes were found to specifically comigrate with the CcO holoenzyme in human muscle mitochondria solubilized with 1% dodecyl maltoside, and, second, a fraction of Sco1 was specifically coimmunoprecipitated with the CcO complex from an identical sample (Fig. 3). Using size-exclusion chromatography, Leary et al. (7) showed that in the presence of 1% sodium deoxycholate, wild-type Sco1 is exclusively found in the mitochondria of HEK-293 cells as a complex of ~60 kDa. Consistent with this result, we were not able to coimmunoprecipitate Sco1 with CcO from dodecyl maltoside-treated mitochondria isolated from HEK-293 cells despite their relatively high Sco1 content. This might suggest a tissue-dependent nature of this interaction that should be further investigated in detail. The fact that, in contrast to immunoprecipitation, only a relatively minor fraction of Sco1 remained associated with CcO after native electrophoresis suggests that the interaction is relatively weak. Given that the Sco1-dependent metallation of Cox2 presumably occurs during an early stage of CcO assembly, most likely in the unassembled polypeptide, we speculate that the functional relevance of this interaction might relate to the role of Sco1 in copper homeostasis signaling rather than in Cu_A site formation (8). The association of Sco1 with the holoenzyme complex might enable CcO, as an important cellular copper recipient, to participate in the maintenance of cellular copper homeostasis. Together with the potential tissue-specific nature of this interaction, this effect might provide an alternative mechanistic explanation for the severe, tissue-dependent copper deficiency of tissues with CcO defects arising from mutations in CcO assembly factors such as Cox10, Cox15, and Surf1 (Fig. 6) (6). Recently, it has been reported that Shy1, the yeast homolog of human Surf1, physically interacts with different assembly forms of CcO, including its supercomplexes (9). Intriguingly, similar to Sco1, human Surf1 also appears to play a role in cellular copper homeostasis maintenance (Fig. 6).

Consistent with the crucial role of Sco1 in CcO biogenesis, mitochondria from the muscle biopsy of the *SCO1* patient showed the accumulation of several CcO subcomplexes in addition to severely reduced holoenzyme levels. However, the subunit composition and relative abundance of these species markedly differed from those found in P174L *SCO1* fibroblasts and myoblasts and in *SCO2* muscle mitochondria (17, 21). We could not detect the common Cox1-Cox4-Cox5a subcomplex (S2) in *SCO1* muscle mitochondria, as ~110-kDa Cox1-containing subcomplex c apparently lacked both nuclear-encoded subunits. Intriguingly, a minor but significant amount of subunit Cox2 was found in G132S *SCO1* mitochondria in the form of two distinct, faster-migrating assemblies. The larger one contained at least Cox1 and Cox2 subunits, whereas the precise subunit composition of the faster-migrating species remains elusive. Indeed, it appeared too small to contain a Cox1-Cox2 heterodimer and was probably too large to represent free, unassembled apoCox2.

Newly synthesized Cox2 was shown to undergo accelerated turnover in P174L *SCO1* fibroblasts, presumably due to a failure to incorporate the Cu_A-lacking subunit into the Cox1-

Cox4-Cox5a subcomplex (2). This is thought to cause an impairment of enzyme assembly, characterized by the accumulation of the S2 subcomplex, which appears to be resistant to inner membrane proteases. Indeed, consistent with a cooperative role for Sco2 in copper trafficking to CcO, a subcomplex pattern identical to that of P174L *SCO1* fibroblasts was observed in both heart and skeletal muscle mitochondria carrying loss-of-function mutations of *SCO2* (17). Given the inability to detect even trace amounts of free unassembled Cox2 in these samples, it was deduced that the function of Sco2 is strictly required for the assembly/stability of Cox2 (5, 17). Therefore, the accumulation of Cox2 in the form of oligomeric subcomplexes in Sco1-deficient mitochondria indicates that Sco1 is very likely responsible for a different posttranslational aspect of Cox2 biogenesis than Sco2. This is further supported by the fact that the steady-state level of Sco2 was virtually unaffected in the G132S Sco1 patient background (Fig. 2). The rather unusual character of CcO subcomplexes found in G132S *SCO1* mitochondria suggests that they might represent misassembled off-path products. Indeed, it is possible that Cox2 prematurely associates with Cox1, thereby compromising the subsequent association of the Cox4-Cox5a subcomplex and leading to stalled CcO assembly. This may result either directly from misfolding of Cox2 or from a lack of specific assembly chaperone activity.

Based on a single report of two affected siblings, encephalopathy, hypotonia, and hepatopathy have been considered to be the primary symptoms of *SCO1* deficiency (18). Here, we report that, similar to *SCO2* compound heterozygotes, our *SCO1* patient presented with early onset hypertrophic cardiomyopathy, in addition to hypotonia, encephalopathy, and hepatopathy. Given the similar functional involvement of SCO proteins and their ubiquitous expression with a similar expression pattern across human tissues, such clinical phenotypes were rather expected. The lack of apparent cardiac involvement in previously published *SCO1* cases likely resulted from either the considerably reduced survival time of both siblings or the distinct nature of the missense allele expressed in these patients. Indeed, P174L mutant Sco1 shows markedly altered functional properties and almost normal polypeptide levels (6), whereas the G132S allele appears to lead to a simple, yet almost complete loss of protein and function.

Mitochondrial ultrastructural abnormalities in the *SCO1* heart resembled those described in the myocardium of *SCO2* compound heterozygotes (20). The finally granular hemidense deposits occasionally seen in mitochondria are suggestive of glycogen origin (4). They were not lipid containing, as they resisted dehydration during embedding in paraffin. They did not resemble any of the crystalloid inclusions described mainly in skeletal muscles in a variety of mitochondrial disorders (10).

Finally, the *SCO1* and *SCO2* tissues analyzed displayed a marked copper-deficient phenotype, the extent of which significantly varied according to tissue type. Intriguingly, the various *SURF1* samples studied also showed marked tissue type-dependent copper deficiency, suggesting a role for human Surf1 in copper homeostasis maintenance. Interestingly, yeast cells lacking Shy1, the yeast homolog of human Surf1, are deficient in mitochondrial copper, whereas the total cellular copper content remains normal (15). In addition, supplementation of *shy1*Δ cultures with exogenous copper partially rescues the respiratory capacity of these cells (3, 15). Recently, it has been reported that both Sco1 and Sco2 play important roles in the regulation of cellular copper export in addition to their roles in copper trafficking to CcO. It was further demonstrated that the corresponding copper defect was fully separable from CcO deficiency, at least in immortalized human fibroblasts (6). Consistent with these results, we found that the residual copper content was negatively correlated with the extent of CcO deficiency in the livers and brains of *SCO2* and *SURF1* patients. Intriguingly, a similar discrepancy between residual copper and CcO levels was also found in all studied *SCO2* muscles. In contrast, a positive relationship was observed in *SCO2* and *SURF1* hearts and in *SCO1* and *SURF1* muscles. Collectively, these results once again indicate tissue-specific functional differences of these ubiquitously expressed proteins that likely evolved to meet the profound tissue-specific requirements of mitochondrial function and biogenesis.

In summary, we demonstrate here that the G132S substitution in Sco1 compromises the stability of the protein, presumably by preventing its oligomerization, leading to severe copper deficiency and CcO assembly impairment. We further show that a fraction of Sco1 physically interacts with the CcO complex in human muscle mitochondria and, based on a dissection of the Sco1-deficient CcO assembly pattern, suggest distinct functional involvement of SCO proteins in posttranslational Cox2 maturation. Finally, based on the marked tissue-specific copper deficiency of analyzed *SURF1* tissues, we hypothesize that this CcO assembly factor plays an important role in cellular copper homeostasis maintenance.

NOTE ADDED IN PROOF

Since the submission of this article, we have performed immunoprecipitation of Sco1 from dodecyl maltoside-solubilized HEK-293 and skeletal muscle mitochondria using the Sco1 antiserum. Surprisingly, a significant amount of CcO was found to copurify with Sco1 from HEK-293 mitochondria in this experiment. Our inability to pull down a significant amount of Sco1 from HEK-293 mitochondria using the anti-CcO antibody was thus likely caused by the low CcO content in HEK-293 mitochondria and its resulting low recovery.

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The impact of mitochondrial tRNA mutations on the amount of ATP synthase differs in the brain compared to other tissues

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Abstract

The impact of point mutations in mitochondrial tRNA genes on the amount and stability of respiratory chain complexes and ATP synthase (OXPHOS) has been broadly characterized in cultured skin fibroblasts, skeletal muscle samples, and mitochondrial cybrids. However, less is known about how these mutations affect other tissues, especially the brain. We have compared OXPHOS protein deficiency patterns in skeletal muscle mitochondria of patients with Leigh (8363G>A), MERRF (8344A>G), and MELAS (3243A>G) syndromes. Both mutations that affect mt-tRNA^{Lys} (8363G>A, 8344A>G) resulted in severe combined deficiency of complexes I and IV, compared to an isolated severe defect of complex I in the 3243A>G sample (mt-tRNA$^{Leu(UUR)}$). Furthermore, we compared obtained patterns with those found in the heart, frontal cortex, and liver of 8363G>A and 3243A>G patients. In the frontal cortex mitochondria of both patients, the patterns of OXPHOS deficiencies differed substantially from those observed in other tissues, and this difference was particularly striking for ATP synthase. Surprisingly, in the frontal cortex of the 3243A>G patient, whose ATP synthase level was below the detection limit, the assembly of complex IV, as inferred from 2D-PAGE immunoblotting, appeared to be hindered by some factor other than the availability of mtDNA-encoded subunits.

Keywords: Brain; COX — cytochrome *c* oxidase; Leigh syndrome; MELAS syndrome; MERRF syndrome; Tissue specificity

1. Introduction

The mammalian organism fully depends on the oxidative phosphorylation system (OXPHOS) as the major energy (ATP) producer of the cell. Disturbances of OXPHOS may be caused by mutations in either mitochondrial DNA (mtDNA) or nuclear DNA, and environmental factors have also been shown to have important effects on OXPHOS. In human mitochondria, a small circular DNA molecule (16 659 bp) codes for 13 polypeptides that form, together with nuclear-encoded subunits, five inner-membrane OXPHOS complexes. For the translation of these 11 mRNAs (nine monocistronic and two bicistronic), mitochondria contain a separate translational system made of protein components, encoded exclusively by nuclear genes and RNA components encoded by the mitochondrial genome (two ribosomal RNA (rRNA) genes and 22 transfer RNA (tRNA) genes). Mitochondrial translation is precisely controlled to meet tissue-specific demands for mtDNA-encoded structural subunits of the OXPHOS complexes [1]. Although the basal components of the mitochondrial expression system are known, the mechanism of regulation of the system in response to the metabolic needs of the cell is poorly understood [2,3].

Since the late 1980s, large-scale deletions and point mutations in mtDNA (approximately 120 in tRNAs and 11 in rRNAs) have been identified to cause disorders of mitochondrial protein synthesis, which is associated with defects of mitochondrial bioenergetics in tissues that most depend on OXPHOS. Despite numerous clinical studies on patients with mt-tRNA mutations,

Abbreviations: BN-PAGE, Blue-Native PAGE; COX, cytochrome *c* oxidase, complex IV; CS, citrate synthase; mt-tRNA, mitochondrial tRNA; mtDNA, mitochondrial DNA; OXPHOS, oxidative phosphorylation system; SQR, succinate:coenzyme Q_{10} reductase, complex II

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which usually involve morphological and functional characterization of OXPHOS insufficiency in skin fibroblast cultures or skeletal muscle, little is known regarding tissue specificity of OXPHOS deficiencies.

The aim of this study was to determine the steady-state levels of OXPHOS protein complexes in the mitochondria of various tissues (skeletal muscle, heart, frontal cortex and liver) of patients with mt-tRNA mutations. We chose (i) a patient with an 8363G>A mutation in mt-tRNA^{Lys}, who died of Leigh syndrome; (ii) a patient with an 8344A>G mutation in the same tRNA, who suffers from MERRF syndrome (myoclonic epilepsy with ragged-red fibers); and (iii) a patient with a 3243A>G mutation in mt-tRNA$^{Leu(UUR)}$, who died of MELAS syndrome (mitochondrial encephalomyopathy, lactic acidosis and stroke-like episodes). In the skeletal muscle of these patients, the mutations manifested themselves as described in previous studies [4–11]; however, these results disclose new aspects of OXPHOS deficiencies in the brain, particularly in the case of ATP synthase. Furthermore, the 3243A>G frontal cortex mitochondria showed a marked loss of the complex IV holoenzyme, accompanied by accumulation of assembly intermediates, which might be caused by the virtual absence of complex V in this sample. A similar phenomenon was described in yeast ATP synthase mutants [12–15].

2. Patients, materials and methods

2.1. Ethics

This study was carried out in accordance with the Declaration of Helsinki of the World Medical Association, and was approved by the Committee of Medical Ethics of the Faculty of Medicine and General Faculty Hospital. Informed parental consent, in accordance with the guidelines of the Faculty of Medicine, Charles University, was obtained for all biopsies and autopsies.

2.2. Case reports

2.2.1. Patient 1, with an 8363G>A mtDNA mutation

The boy was born at term with a birth weight of 2590 g (5th percentile) and a length of 48 cm. The early postnatal adaptation was uneventful, but a failure to thrive and progressive hypotony developed beginning in infancy, and he was wheelchair-dependent from the age of 8 years. Psychological investigations revealed moderate mental retardation, which later became severe. At the age of 8 he had his first epileptic paroxysm, and at the age of 11, an acute stroke-like encephalopathy developed, resulting in generalized weakness and respiratory failure requiring ventilatory support. The liver function was unaffected and aminotransferases were always within the reference range. The EEG pattern was abnormal; an EMG demonstrated peripheral neuropathy, echocardiography showed hypertrophic cardiomyopathy, and MRI revealed bilateral necrotic basal ganglia lesions typical of Leigh syndrome. The boy died at the age of twelve. Metabolic analyses revealed hyperlactacidemia (2.5–5.5 mmol/l, controls <2.1 mmol/l) with an increased lactate/pyruvate ratio (L/P 28).

2.2.2. Patient 2, with a 3243A>G mtDNA mutation

The girl was born at term with a birth weight of 3140 g and a length of 50 cm. Her early postnatal adaptation was uneventful, but a failure to thrive and growth retardation were observed beginning in infancy. At the age of 16 years, her weight and height were 30 kg and 140 cm (<3rd percentile). Cardiac evaluation revealed dilated cardiomyopathy and Wolf–Parkinson–White syndrome. At the age of 18 years, blurred vision, emesis and tiredness developed, and subsequently, seizures and coma. An MRI study showed symmetrical lesions within the basal ganglia, compatible with Leigh syndrome, in addition to several hypodensities in the subcortical regions of the frontal and occipital parts of the brain, which are consistent with a diagnosis of acute ischemia. Metabolic analyses revealed metabolic acidosis (BE-10 to -25 mmol/l, controls±2 mmol/l), as well as increased levels of lactate in the blood (4–10 mmol/l, controls <2.1 mmol/l), cerebrospinal fluid (12 mmol/l, controls <2.1 mmol/l), and urine (4976 mM/mol creatinine, controls <60 mM/mol creatinine). Despite intensive treatment, the girl died of progressive encephalomyopathy and respiratory failure due to acquired infection during the terminal phase of the disease, at the age of 18 years.

2.2.3. Patient 3, with an 8344A>G mtDNA mutation

The boy was born at term with a birth weight of 3500 g and length of 51 cm, with normal postnatal adaptation. Beginning at the age of 11 years, he developed progressive muscle weakness and paresthesia. Now, at the age of 15, he has generalized muscle hypotrophy and hyporeflexia. No mental deterioration has been observed. An EMG disclosed diffuse myopathic abnormalities and normal nerve conduction velocities. The EEG and cardiac evaluation were normal. Metabolic analyses revealed an increased level of lactate in the blood (5.6–7.88 mmol/l, controls <2.1 mmol/l) and lactate in the urine (5148 mM/mol creatinine, controls <60 mM/mol creatinine).

2.3. Tissues

All studied tissues were obtained from three patients harbouring one of two mutations in mt-tRNA^{Lys} (8363G>A and 8344A>G) or one mutation in mt-tRNA$^{Leu(UUR)}$ (3243A>G), as well as from age-related controls. Open muscle biopsies from the tibialis anterior muscle were frozen at -80 °C. Post-mortem tissue specimens obtained at autopsy of 8363G>A and 3243A>G patients, and controls were frozen less than 2 h after death. The studies were performed in available stored material from the muscle (tibialis anterior), heart, liver, and brain (frontal cortex).

2.4. mtDNA analysis

Total genomic DNA was isolated by phenol extraction from available tissues. The DNA sample from the muscle biopsy of patient 1 was used for the sequencing of the whole mtDNA molecule on an AbiPrism 3100 Avant Genetic Analyser (Applied Biosystems).

To determine the amount of mtDNA containing the mutation, PCR/RFLP analysis was performed. PCR products (8279–8485) were radioactively labelled with [α-^{32}P]dCTP in the final cycle of PCR, and run on a non-denaturing 10% (w/v) polyacrylamide gel after complete digestion with Tsp*RI* (New England BioLabs). The mutation abolishes one of two Tsp*RI* restriction sites on the fragment. The proportions of wild-type to mutant mtDNA were measured using a PhosphorImager and ImageQuant software (Molecular Dynamics). The levels of heteroplasmy of the 3243A>G and 8344A>G mutations were determined as described elsewhere [16].

2.5. Electrophoresis

BN-PAGE (Blue-Native PAGE) [17] was used for the separation of mitochondrial membrane protein complexes on polyacrylamide 6–15% (w/v) gradient gels using a MiniProtean® 3 System (Bio-Rad Laboratories). 5–50 μg of protein, which was prepared as described previously [18], was loaded in each lane. Two-dimensional BN/SDS/PAGE [17] was performed as described previously [18]. The protein content was measured by the Bio-Rad Protein Assay (Bio-Rad Laboratories), using BSA as a standard.

2.6. Immunoblot analysis

Proteins were electroblotted from the gels onto Immobilon™-P PVDF membranes (Millipore) using semi-dry transfer for 90 min at a constant current of 0.8 mA/cm^2. Membranes were air-dried overnight, rinsed twice with 100% (v/v) methanol, and blocked in TBS and 10% (w/v) non-fat dried milk for 1–2 h. Primary detection of BN/PAGE-blots was performed with mouse monoclonal antibodies raised against the complex I subunit NDUFA9 (2 μg/ml), ATP synthase subunit alpha (2-3 μg/ml), complex III subunit Core 2 (0.5 μg/ml), complex IV subunit COX2 (0.5–1 μg/ml), and complex II subunit 70 kDa protein (1 μg/

ml) (Mitosciences), at indicated dilutions. Primary detection of two-dimensional BN/SDS/PAGE-blots for the COX assembly was performed according to Stiburek et al. [18]. Blots were incubated with primary antibodies in TBS, 0.3% (v/v) Tween 20, and 2% non-fat dried milk for 2 h. Secondary detection was carried out with a goat anti-mouse IgG-horseradish peroxidase conjugate (1:1000–1:4000) (Sigma–Aldrich) in TBS, 0.1% Tween 20, and 2% non-fat dried milk, for 1 h. The immunoblots were developed with SuperSignal West Femto Maximum Sensitivity Substrate (Pierce). The immunoblot-images of chemiluminescence signals were captured using the VersaDoc Imaging System, Model 4000 (Bio-Rad Laboratories), and analysed by the Quantity One application (Bio-Rad Laboratories). All blotting experiments were repeated with independently isolated mitochondrial samples. Duplicate experiments yielded consistent results.

2.7. Spectrophotometric assays

The activities of respiratory chain complexes in isolated muscle mitochondria were measured spectrophotometrically by standard methods at 37 °C. NADH: coenzyme Q10 reductase (NQR, complex I), succinate:coenzyme Q10 reductase (SQR, complex II), succinate:cytochrome *c* reductase (SCCR, complex II+III), NADH:cytochrome *c* reductase (NCCR, complex I+III), coenzyme Q10:cytochrome *c* reductase (QCCR, complex III), and cytochrome *c* oxidase (COX, complex IV) were measured according to Rustin et al. [19], and citrate synthase (CS) according to [20]. Total protein amount was determined by the method of Lowry [21].

2.8. High resolution oxygraphy in muscle fibers

Muscle fibers were separated mechanically according to [22], and oxygen consumption by saponin-skinned muscle fibers was determined using multiple substrate inhibitor titrations as described previously [23].

3. Results

3.1. Heteroplasmy of mt-tRNA mutations in investigated tissues

Analyses of heteroplasmy by radioactive PCR-RFLP showed 80 – 97% heteroplasmy of mt-tRNA mutations in the tissues of all patients (Table 1). Despite the narrow range of the mutation load found in patient tissues, the severity of the clinical/functional phenotype did not correlate with the level of heteroplasmy.

3.2. Steady-state levels of OXPHOS complexes in 8363G>A, 8344A>G and 3243A>G skeletal muscle

To investigate the impact of mtDNA mutations on the system of oxidative phosphorylation, immunoblots of mitochondrial fractions resolved by BN-PAGE were prepared. Dilutions of samples from control mitochondria were loaded on the same gels in order to express the residual steady-state levels of OXPHOS complexes in the patient tissues as a percentage of control values.

In the 8363G>A skeletal muscle sample obtained at autopsy, profoundly decreased levels of complex I (5% of control) and IV (<10% of control) were detected. The sample also revealed a diminished amount of complex V holoenzyme (35% of control), along with accumulated sub-complexes, most likely V* (F1-ATPase with several c-subunits) and F1-ATPase (Fig. 1A). The same sub-complexes were also detected in a bioptic muscle sample from the 8363G>A patient using BN-PAGE with Coomassie staining, followed by the second denaturing electrophoretic dimension with silver staining (Fig. 2).

A very similar pattern, although less severe, was found in the 8344A>G skeletal muscle sample. The amount of complex I was reduced to approximately 25% of control, the level of complex IV holoenzyme was <15% of control, and the holoenzyme level of complex V was decreased to 60% of the control value. Immunodetection of complex V further showed sub-complexes similar to those observed in the 8363G>A sample (Figs. 1A, 2A).

In 3243A>G skeletal muscle, relative levels of OXPHOS holoenzymes were normal, except for a decrease in the content of complex I to 30% of control and complex IV to 60% of control (Fig. 1A).

3.3. Steady-state levels of OXPHOS complexes in other 8363G>A and 3243A>G tissues

Only the 8363G>A and 3243A>G samples of heart, frontal cortex, and liver tissue were available for comparison of the

Table 1
Summary of clinical consequences, mtDNA mutation loads and character of OXPHOS deficiencies in investigated tissues of the patiens

Skeletal muscle	8363G>A	3243A>G	8344A>G
Clinical phenotype	Myopathy (+++)	Myopathy (++)	Myopathy (++)
Heteroplasmy	80%	90%	89%
OXPHOS protein deficiencies*	I and IV (↓↓↓), V (↓↓), V^{sub}	I (↓↓)	I and IV (↓↓↓), V (↓), V^{sub}
ADP-stimulated respiration after pyruvate/glutamate/succinate	↓↓/↓↓/↑↑	nd	↓/N/↑↑↑
Heart	8363G>A	3243A>G	8344A>G
Clinical phenotype	$Cardiomyopathy^{H}$(++)	$Cardiomyopathy^{D}$(++)	–
Heteroplasmy	89%	89%	nd
OXPHOS protein deficiencies	I and IV and V (↓↓↓), V^{sub}	I (↓↓↓)	nd
Frontal cortex	8363G>A	3243A>G	8344A>G
Clinical phenotype	Encephalopathy (++)	Encephalopathy (++)	–
Heteroplasmy	87%	89%	nd
OXPHOS protein deficiencies	V (↓↓↓), I and IV (↓↓)	V (↓↓↓), I and IV (↓↓↓), IV^{sub}	nd
Liver	8363G>A	3243A>G	8344A>G
Clinical phenotype	–	–	–
Heteroplasmy	97%	87%	nd
OXPHOS protein deficiencies	I (↓↓)	N	nd

–, absent; ++, moderate; +++, severe; ↓↓↓, <30% of control; ↓↓, 30 – 50% of control/mean control value; ↓, >50% of control/mean control value; ↑↑, 160% of mean control value; ↑↑↑, >200% of mean control value; N, normal; nd, not done; ^{sub}-assembly intermediates of indexed complex were present; ^{D}- dilated; ^{H}- hypertrophic.
* relative steady-state protein levels (Western blot) correlated with activities of respiratory chain complexes (spectrophotometry), (Fig. 3).

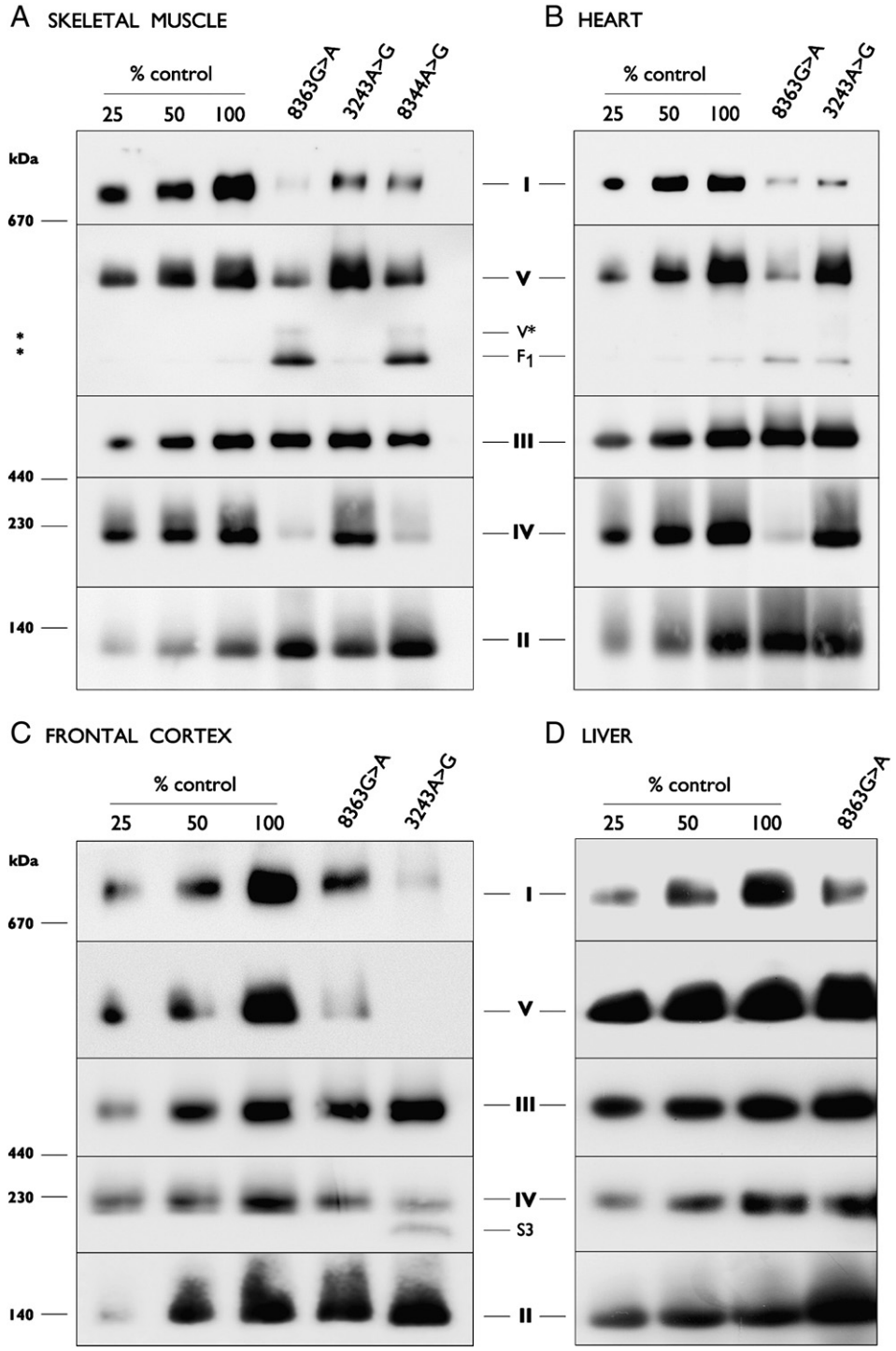

Fig. 1. Analysis of the assembly of OXPHOS complexes by immunoblotting of BN-PAGE. BN-PAGE of lauryl maltoside-solubilised mitochondria isolated from autoptic (8363G>A, 3243A>G) and bioptic (8344A>G) muscle (A) and from autoptic (8363G>A, 3243A>G) heart (B), frontal cortex (C) and liver (D) was electroblotted onto PVDF membranes and probed with monoclonal antibodies that detect the native forms of the OXPHOS complexes. Three aliquots of control mitochondria corresponding to the indicated dilutions of control samples were loaded on the same gels. The migration of holoenzymes (I–V) and molecular mass standards (kDa) are indicated; the actual position of sub-complex V* and/or F1 of complex V (*) in skeletal muscle (A) and heart (B) is directly below complex III (around 370–470 kDa).

impact of mtDNA mutations on the system of oxidative phosphorylation in skeletal muscle and other tissues (Table 1).

The 8363G>A heart sample showed the same considerable reduction in complexes I (5% of control) and IV (<10% of control) content as skeletal muscle, but despite the greater reduction of complex V holoenzyme (15% of control), there was a significantly lower accumulation of F1-ATPase. The 3243A>G heart sample had a pronounced reduction of complex I (20% of control), similar to skeletal muscle (Fig. 1B).

In the 8363G>A frontal cortex, the assembly of complexes I and IV were less affected than in the above-mentioned tissues. The amount of complex I was decreased to 40% of control, and

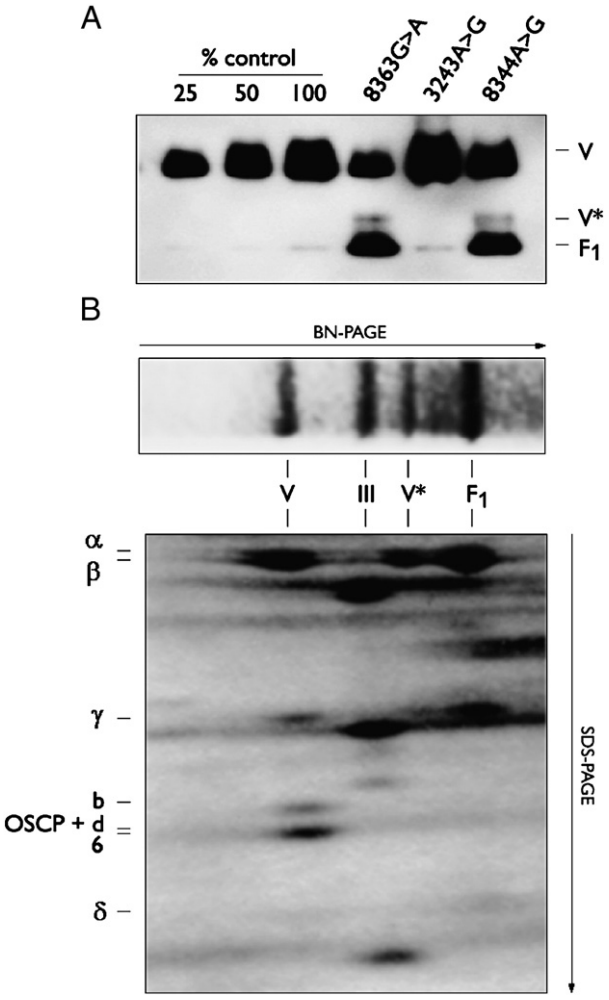

Fig. 2. Complex V (V) subassemblies V* and F_1-ATPase (F_1), compatible with those described previously for T8993G mitochondria, ρ^0 cells, or where mitochondrial translation has been inhibited. Long exposure of complex V immunodetection with the monoclonal antibody against the ATP-alpha subunit from Fig. 1A (A). Coomassie staining of BN-PAGE (above) and silver staining of two-dimensional BN/SDS/PAGE (below) of the 8363G>A sample obtained from skeletal muscle (bioptic sample) (B).

the holoenzyme level of complex IV was reduced to 50% of control. Conversely, complex V (<20% of control) appeared to be the most severely affected member of the OXPHOS system. The decrease was more substantial than in skeletal muscle; nevertheless, no detectable sub-complexes could be found. The 3243A>G frontal cortex sample showed a dramatic reduction of complex V to below the detection limits of the method, and of complex I to 10% of the control value. Probing of the immunoblots with an anti-COX2 antibody showed a reduction of complex IV to 20% of control, as well as the presence of a high

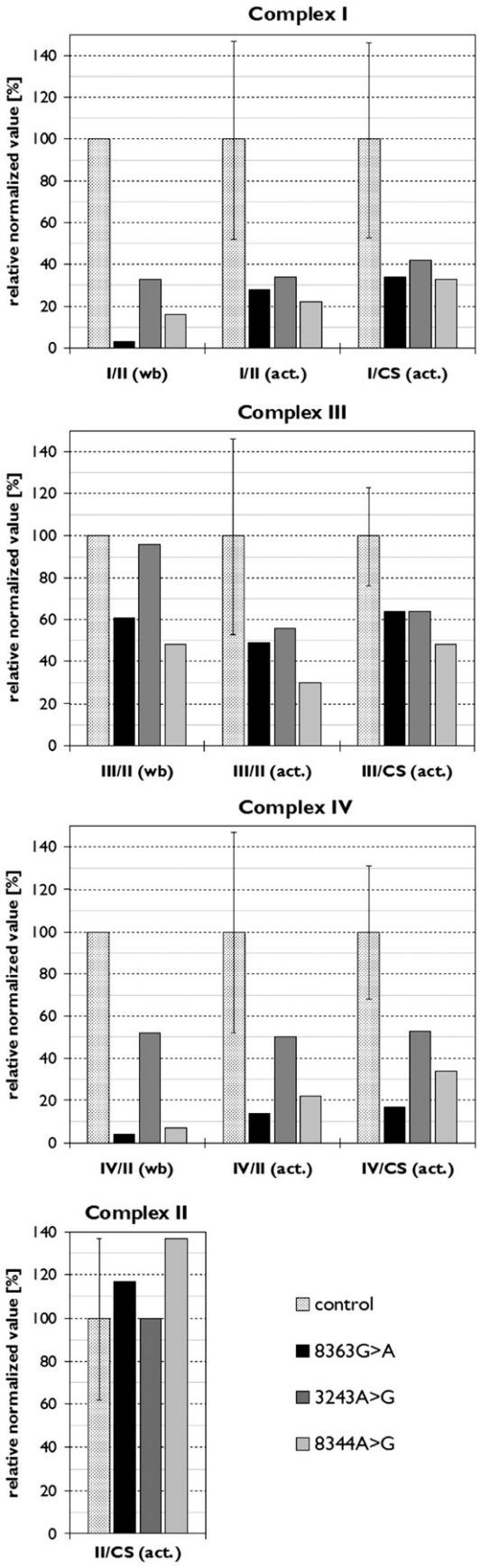

Fig. 3. Relative steady-state protein levels and activities of respiratory chain complexes normalized to complex II (II) or citrate synthase (CS) in isolated muscle mitochondria. The ratios of specific activities are expressed as the percentage of the mean of the control values (reference ranges are shown). Fractions by the x-axis indicate which ratios are concerned; I — complex I, III — complex III, IV — complex IV, (wb) — the ratio of holoenzyme levels obtained from a Western blot, (act.) — the ratio of specific activities obtained using spectrophotometry.

molecular weight sub-complex (Fig. 1C). Due to the unexpected OXPHOS deficiency pattern observed in brain tissue, particularly in the 3243A>G patient, the immunoblotting was also performed on the frontal cortex sample from another 3243A>G patient. In the only available sample with a 65% level of heteroplasmy, the immunoblotting analysis revealed normal levels of OXPHOS complexes in comparison to control (data not shown).

Despite the highest level of heteroplasmy (Table 1), the 8363G>A liver sample only showed an isolated deficiency of complex I (40% of control) (Fig. 1D). Steady-state levels of OXPHOS complexes in the 3243A>G liver mitochondria were comparable to control (data not shown).

3.4. Activities of respiratory chain complexes in 8363G>A, 8344A>G and 3243A>G skeletal muscle

To characterize the impact of the 8363G>A, 8344A>G and 3243A>G mutations on the function of respiratory chain complexes, the specific activities of the respective enzymes and citrate synthase (CS, control enzyme) were measured spectrophotometrically. The values expressed as relative ratios of the activities of complexes I, III and IV normalized to complex II (II, SQR) or CS were compared to the relative ratios of steady-state protein levels normalized to complex II (Fig. 3). Consistent with immunoblotting results, the spectrophotometry revealed lower activity ratios for complex I (I/II and I/CS) in the isolated muscle mitochondria of all three patients, and a severe deficiency of complex IV in 8363G>A and 8344A>G isolated muscle mitochondria. The COX/SQR (IV/II) and COX/CS (IV/CS) activity ratios in the 3243A>G patient were just below the control range.

3.5. Oxygraphic analysis in 8363G>A and 8344A>G skeletal muscle fibers

The functional consequences of the 8363G>A mutation in comparison to the 8344A>G mutation were analysed by high-resolution oxygraphy of the patients' skeletal muscle fibers permeabilized by a low concentration saponin treatment (Table 2).

Table 2
Oxygen consumption by saponin-permeabilized skeletal muscle fibers with 8363G>A and 8344A>G mutations

	8363G>A	8344A>G	Controls (n=9)
Mitochondrial respiration (nmol O_2 min^{-1} per mg of wet weight)			
Pyruvate/malate	6.9	14.7	16–26
Glutamate/malate	5.1	16.5	10–22
Succinate	21.4	30.0	9–18
Ascorbate/TMPD	66.0	57.0	43–83
Value normalized to respiration after succinate addition			
Pyruvate/malate	0.32	0.49	0.96–1.85
Glutamate/malate	0.24	0.55	0.83–1.72
Ascorbate/TMPD	3.08	1.90	3.50–5.98

Polarographic measurements were performed as multiple substrate-inhibitor titration in the presence of 1 mM ADP with subsequent additions of pyruvate (10 mM)/malate (5 mM) or glutamate (10 mM)/malate (5 mM); succinate (10 mM); ascorbate (2 mM)/TMPD (500 μM).

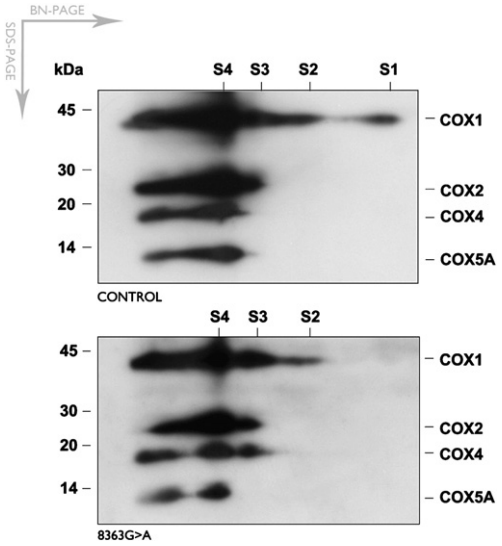

Fig. 4. Subunit composition of complex IV subassemblies in skeletal muscle obtained from the 8363G>A patient (bioptic sample). Two-dimensional BN/SDS/PAGE of lauryl maltoside-solubilised mitochondria isolated from the skeletal muscle of a control or the 8363G>A patient, obtained at biopsy, were electroblotted onto PVDF membranes and probed with monoclonal antibodies specific for the subunits COX1, COX2, COX4 and COX5A. The positions of the holoenzyme (S4) and subassemblies (S1–S3) are indicated, along with the migration of the molecular mass standard (kDa).

In both samples, a decrease of ADP-stimulated oxygen consumption compared to control fibers was observed using pyruvate as a substrate, and an increase was found after succinate addition. A pronounced reduction in ADP-stimulated respiration was found after pyruvate (33% of the mean control value) as well as after glutamate (32% of the mean control value) additions in 8363G>A muscle fibers. Indeed, ADP-stimulated respiration after succinate treatment increased to 158% of the mean control value. In 8344A>G muscle fibers, ADP-stimulated respiration after pyruvate addition was 70% of the mean control value, but after glutamate, it was within the reference range. Similarly to 8363G>A muscle fibers, the ADP-stimulated respiration after succinate addition increased to 222% of the mean control value in the 8344A>G sample. The absolute oxygen consumption after ascorbate+TMPD treatment was, in both samples, within control levels; however, normalization of the data to the level of respiration after succinate addition revealed decreased ratios after treatment with all of the substrates (Table 2).

3.6. Mitochondrial content in skeletal muscles with mt-tRNA^{Lys} mutations

In isolated muscle mitochondria, Western blotting showed an increased level of complex II holoenzyme, which is entirely encoded by nuclear DNA, up to 150% and 160% of control in 8363G>A and 8344A>G patient samples, respectively (Fig. 1A). After normalization, this leads to artificially decreased amounts of complex III in the skeletal muscle mitochondria of both patients (Fig. 3, Complex III). Accordingly, the II/CS activity ratio remained on the upper border of

the reference range (Fig. 3, Complex II), indicating a higher mitochondrial membrane content (altered mitochondrial morphology).

In tissue homogenates, the specific activity of CS was increased to 380% and 285% of the mean control value in the 8363G>A and 8344A>G patient samples, respectively. Histochemistry revealed increased amounts of SDH product in 8363G>A skeletal muscle and ragged-red fibers in 8344A>G skeletal muscle.

Based on the above-mentioned observations and on the increased ADP-stimulated oxygen consumption after succinate treatment (antimycin A-sensitive) of patients' skeletal muscle fibers (Section 3.5), a proliferation of mitochondria with altered morphology could be expected as a result of cellular energetic imbalance.

3.7. Immunoblots of two-dimensional native/denaturing gels

Immunoblotting of BN/SDS/PAGE (native in the first dimension and denaturing in the second dimension) with COX1, COX2, COX4 and COX5A antibodies was used to analyze the assembly of complex IV in 8363G>A skeletal muscle, and to confirm the alignment of the high molecular weight band identified in the 3243A>G frontal cortex after immunoblotting of BN/PAGE with COX2 antibody.

In 8363G>A skeletal muscle, the level of free S1 sub-complex was found to be below the detection limit of the method (Fig. 4). This probably reflects the limiting character of the COX1 subunit in the holoenzyme assembly.

In 3243A>G frontal cortex, significantly increased levels of all known complex IV assembly intermediates were observed, including free apoCOX1 (S1 sub-complex), apoCOX2, and apoCOX5A (Fig. 5). The high molecular weight band just below the COX holoenzyme, which was detected with anti-COX2 antibody (Fig. 1C), very likely represents the S3 assembly intermediate (Fig. 5). Long exposure of the same immunoblot also revealed the band representing free apoCOX2 subunit (data not shown). This finding further supports the assumption that the final reduction in the content of COX holoenzyme in the 3243A>G frontal cortex mitochondria is not caused solely by the limiting character of mtDNA-encoded subunits of COX (translational defect, Fig. 4).

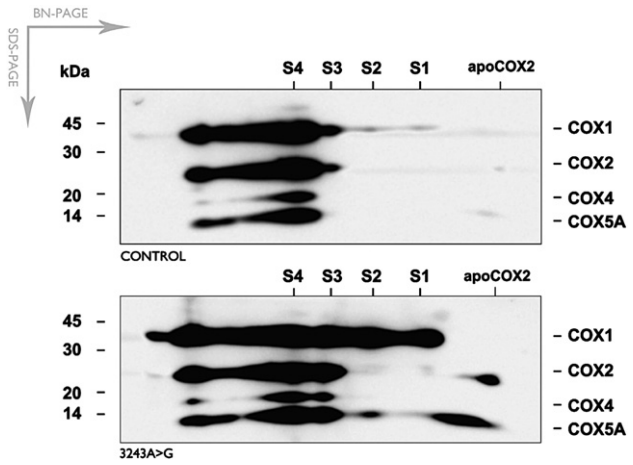

Fig. 5. Subunit composition of complex IV subassemblies in the frontal cortex obtained at autopsy of the 3243A>G patient. Two-dimensional BN/SDS/PAGE of lauryl maltoside-solubilised mitochondria isolated from the frontal cortex of a control or the 3243A>G patient, obtained at autopsy, were electroblotted onto PVDF membranes and probed with monoclonal antibodies specific for the subunits COX1, COX2, COX4 and COX5A. The positions of holoenzyme (S4) and subassemblies (S1–S3, apoCOX2) are indicated, along with the migration of the molecular mass standard (kDa).

4. Discussion

This paper has presented a detailed analysis disclosing the tissue-specific impact of the 8363G>A mtDNA mutation on the amount, stability, and function of the OXPHOS complexes in a patient who died of Leigh syndrome. Furthermore, these data were compared with that from two other mtDNA mutations; the first one in the same tRNA (8344A>G) and the other one in mt-tRNA$^{Leu(UUR)}$ (3243A>G), both of which are at least twice as common as the total number of other mtDNA point mutations known to cause disorders affecting the central nervous system [24,25]. Although just one patient with a comparable level of heteroplasmy was available per studied mutation, the observed data demonstrate intriguing tissue-specific patterns of OXPHOS protein deficiencies, with the most unexpected findings in the 3243A>G frontal cortex.

In the skeletal muscle of patient 1, the lowest level of heteroplasmy but the most severe OXPHOS defect suggests a more profound impact of the 8363G>A mutation on the translational system than that of the 8344A>G mutation. Concerning the 3243A>G mutation, the relatively proportional levels of heteroplasmy in 8344A>G and 3243A>G skeletal muscles, the more significant decrease in the content of complex I, the severe decrease in the amount of complex IV, and the slightly lower level of complex V in patient 2 all indicate a less pronounced impact of the 3243A>G mutation on mitochondrial translation in this tissue. However, the different nuclear backgrounds [26–30], distributions of heteroplasmy levels in cells and mitochondria [25,31,32], and environmental factors [33,34] which have been shown to influence expression of mitochondrial respiratory insufficiency, prevent the reduction of these results to any simple quantitative trait.

The frequency of UUR (Leu) codons in mitochondrially-translated subunits of OXPHOS implies decreased steady-state levels of complex I subunits, namely ND6, ND3, ND2 and ND5 (14–9 UURs) in 3243A>G mitochondria. In 8363G>A and 8344A>G mitochondria, the distribution of AAR (Lys) codons anticipates diminished levels of ND5, ND2, ND4 and COX1 (21–10 AARs). Moreover, all these subunits contain two X/Lys/Lys/X motifs, or one X/Lys/Lys/X and one Lys/X/Lys motif (http://www.mitomap.org), which are apparently strong stalling points for the ribosome. The particular abundance and distribution of codons in mitochondrially-translated subunits of respiratory chain complexes appears to be a plausible explanation for the isolated defect of complex I in the patient with a mutation in mt-tRNA$^{Leu(UUR)}$, along with the combined deficiency of complexes I and IV in patients with mutations in mt-tRNA^{Lys}.

Furthermore, the complete absence of unassembled apoCOX1 (S1) in the skeletal muscle of the 8363G>A patient, as revealed by BN/SDS/PAGE immunoblotting, conforms to the limiting character of the COX1 subunit in holoenzyme assembly. On the contrary, the OXPHOS deficiency patterns found in the frontal cortex mitochondria of the 8363G>A and 3243A>G patients could suggest, similarly to [18], a specific character for brain OXPHOS. First, in both frontal cortex samples, the decrease in the content of complex V was more profound than in that of complexes I and IV. Second, despite such a pronounced defect of complex V, no sub-complexes similar to those observed in skeletal muscle and heart could be detected, even when the immunoblot exposure was prolonged (data not shown). Unfortunately, no 8344A>G frontal cortex specimen was available for the analysis. However, a selectively decreased expression of COX2 subunit was previously reported in frontal cortex and cerebellum of a MERRF patient [35]. F1-ATPase, which was observed in heart, was found along with a sub-complex denoted V* in skeletal muscle. The sub-complex V* is likely composed of F1-ATPase and several c-F_0 subunits. These sub-complexes were described previously in 8993T>G mitochondria [36,37], ρ^0 cells [37], and in cells with inhibited mitochondrial translation [38]. The observed steady-state levels of OXPHOS complexes suggest that the brain ATP synthase is most sensitive to disturbances of the mitochondrial translational system caused by the studied mt-tRNA mutations. Such a tissue-specific impact of mt-tRNA mutations with comparable tissue heteroplasmy is likely to result from tissue-specific variations in the nature of mitochondria. Indeed, it was shown that the brain, liver and kidney OXPHOS system is mainly controlled at the phosphorylation level by ATP synthase and a phosphate carrier, in contrast to the muscle and heart, where it is essentially controlled at the level of the respiratory chain [39]. On the other hand, instead of diminished energy provisions, an insufficient discharge of mitochondrial membrane potential leading to reactive oxygen species (ROS) production was proposed as the underlying pathogenic mechanism of ATP synthase deficiency [40]. Accordingly, the complete lack of complex V from 3243A>G frontal cortex may be, apart from the translational defect, responsible for the unusual assembly pattern of COX in this sample. Indeed, it was shown in yeast that (i) cells deficient in ATP synthase have a severe reduction of COX holoenzyme [12–15] and (ii) no decrease in COX synthesis is observed in uncoupled ATP synthase mutants, where the maintenance of mitochondrial potential is severely compromised by a massive proton leak through the F_0 sector [41,42]. Although the COX1 subunit was shown to be a key regulatory target for COX reduction in yeast cells [12], some other mechanism is likely to be involved in the hindered assembly of COX in the 3243A>G frontal cortex mitochondria, since this sample had high accumulated levels of all three mitochondrially encoded subunits, either free or partially assembled.

Although it is necessary to analyze considerably more samples with high levels of heteroplasmy (such samples are difficult to obtain), these data show new effects of mt-tRNA mutations on the brain which differ substantially from those described for skeletal muscle, heart, and liver tissues.

Acknowledgements

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