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Inherited Disorders of Cytochrome *c* Oxidase Biogenesis

PhD Thesis

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CONTENTS

ABBREVIATIONS

AD	Alzheimer's disease
ADP	adenosine diphosphate
ATP	adenosine triphosphate
BOX	bovine cytochrome c oxidase
cAMP	cyclic adenosine monophosphate
CCRC	clear cell renal carcinoma
COX	cytochrome *c* oxidase
DHA	docosahexaenoic acid
Δm_H+	electrochemical proton gradient
Δp	protonmotive force
ΔpH	gradient of protons
ΔY_m	mitochondrial membrane potential
EPA	eicosapentaenoic acid
ERR alpha	estrogen-related receptor alpha
EST	expressed sequence tag
$FADH_2$	flavin adenine dinucleotide, reduced form
HIF	hypoxia-inducible transcription factor
KSS	Kearns-Sayre syndrome
LHON	Leber hereditary optic neuropathy
LRPPRC	leucine rich pentatricopeptide repeat-containing protein
LSCOX	Leigh syndrome associated with cytochrome c oxidase deficiency
LSFC	Leigh syndrome - French Canadien type
MELAS	mitochondrial myopathy, encephalopathy, lactic acidosis, stroke-like episode
MERRF	myoclonical epilepsy and ragged red fibers
MERRF	myoclonus epilepsy with ragged red fibers
mtDNA	mitochondrial deoxyribonucleic acid
mTERF	mitochondrial transcription termination factor
NADH	nicotinamide adenine dinucleotide, reduced form
nDNA	nuclear deoxyribonucleic acid
NRF-1, NRF-2	nuclear respiratory factor 1, 2
OXPHOS	oxidative phosphorylation
p50	partial pressure of oxygen at half-maximal respiration
PEO	progressive external ophtalmoplegia
pet	respiratory defective yeast mutant
PGC1α	peroxisome proliferator activated receptor γ coactivator 1α
PGC1β	peroxisome proliferator activated receptor γ coactivator 1β
PKCε	protein kinase C isoform ε
pO2	partial pressure of oxygen
POX	cytochrome c oxidase from Paracoccus denitrificans
PRC	PGC-1 related coactivator

PUFA	polyunsaturated fatty acid
RCI	respiratory control index
ROS	reactive oxygen species
rRNA	ribosomal ribonucleic acid
Tfam	mitochondrial transcription factor A
TFB1M	mitochondrial transcription factor B1
TFB2M	mitochondrial transcription factor B2
TMPD	N,N,N′,N′-tetramethyl-p-phenylenediamine dihydrochloride
tRNA	transfer ribonucleic acid
UCP	uncoupling protein
VHL	von Hippel-Lindau tumor suppressor protein

1. INTRODUCTION

1.1 Mitochondria

Mitochondria are localized in the cytoplasm of nearly all eukaryotic cells. They are usually depicted as stiff, elongated cylinders with a diameter of 0.5 – 1 μm. Time-lapse microcinematography of living cells, however, shows that mitochondria are remarkably mobile and plastic organelles, constantly changing their shape and even fusing with one another and then separating again. In the context of a eukaryotic cell, these dynamic organelles integrate numerous metabolic pathways and are the major producers of ATP – the universal energetic fuel. Without them, eukaryotic cells would be dependent on the relatively inefficient process of glycolysis, which yields only 2 molecules of ATP per molecule of glucose compared to 30 molecules produced, when glucose oxidation is completed in mitochondria (Alberts 2002).

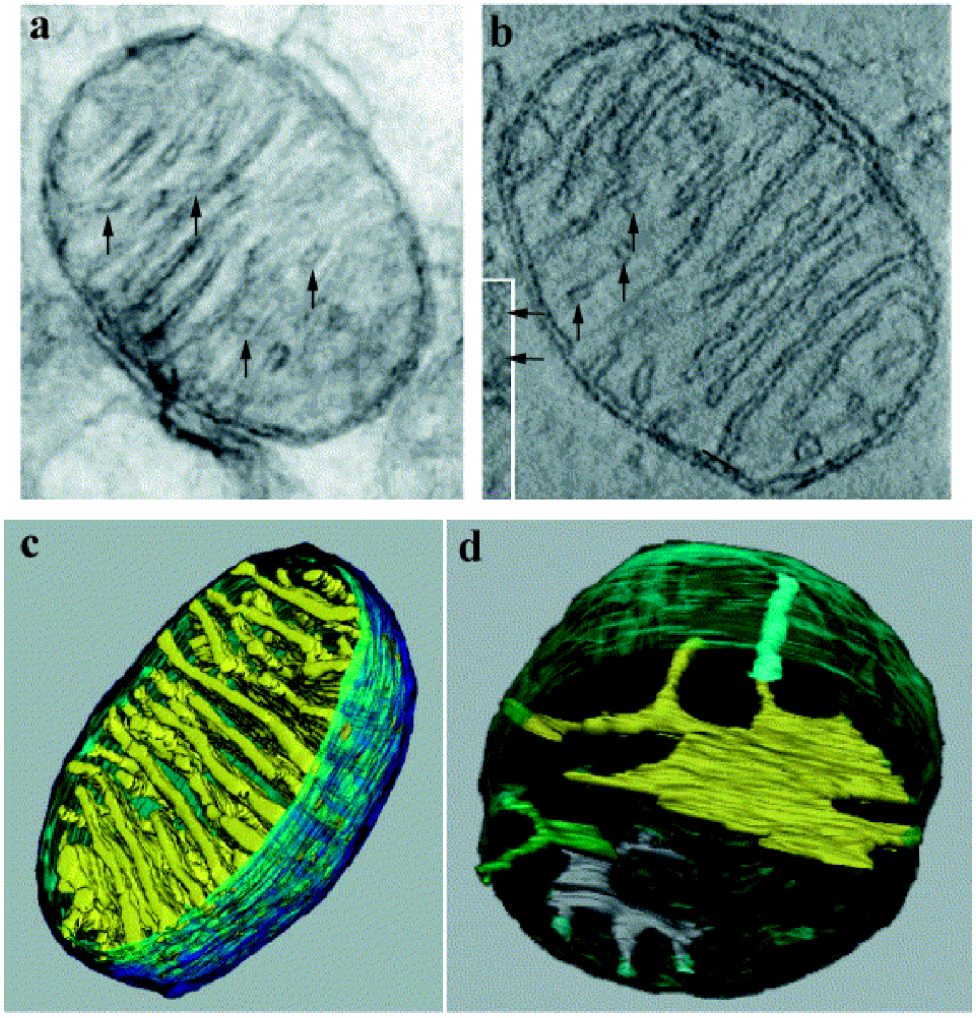
The research of the past 30 years has consolidated the hypothesis of mitochondria as endosymbionts of a primitive eukaryote. The serial endosymbiosis theory postulates that a protoeukaryotic cell without mitochondria evolved first, and this organism then captured a proteobacterium by endocytosis. After establishing a symbiotic relationship, the loss of redundant genes and the transfer of genes from the bacterium to the nucleus lead to the currently observed distribution of genes between the two genomes (Gray et al. 1999).

Mitochondria are surrounded by the inner and outer membranes that create two separate mitochondrial compartments, the internal matrix space, and a narrow intermembrane space. The smooth outer membrane contains proteins essential for import of nuclear-encoded mitochondrial proteins and the protein called porin, which forms aqueous channels, permeable to all molecules with molecular mass less than 5 kDa. The inner membrane is folded into numerous cristae, which greatly increases its total surface area. This membrane contains proteins with three major types of functions: the enzymes of the respiratory chain, ATP synthase complexes, and specific transport proteins that regulate the passage of metabolites and macromolecules across the inner mitochondrial membrane.

The standard model of cristae structure has been reevaluated in recent years. Electron tomography has provided detailed three-dimensional models of mitochondria that have redefined the concept of mitochondrial structure (Fig. 2.1). The models reveal an inner membrane consisting of two components, the inner boundary membrane closely

apposed to the outer membrane and the cristae membrane that projects into the matrix compartment. These two components are connected by tubular structures of relatively uniform size called cristae junctions (Frey et al. 2002).

Figure 1.1 The Recent Model of Mitochondrial Structure *(a) Electron micrograph of a 0.5 μm thick section of chick cerebellum. Arrows point out circular profiles observed in this orientation corresponding to circular cristae junctions. (b) One section of the electron tomogram calculated from a tilt series of the mitochondrion in (a). The vertical arrows point to cross sections of cristae junctions, horizontal arrows point to two circular cristae junctions in the inset. (c) A three-dimensional model of a segmented electron tomogram. The outer membrane is displayed in translucent dark blue, the inner boundary membrane in translucent light blue, and the cristae in yellow. (d) A three-dimensional model of the tomogram in (b) with only four cristae segmented separately and displayed in different colors. Adapted from (Frey et al. 2002).*



1.1.1 The Oxidative Phosphorylation Apparatus

The electrons from oxidized substrates stored in the form of redox equivalents NADH and FADH are utilized by the oxidative phosphorylation apparatus (OXPHOS) in

the inner mitochondrial membrane. The OXPHOS consists of the complexes of the respiratory chain proteins - complex I, NADH-CoQ oxidoreductase; complex II, succinate-CoQ oxidoreductase; complex III, bc_1 complex, and complex IV, cytochrome *c* oxidase. These protein complexes are associated with a variety of redox-active prosthetic groups with successively increasing reduction potentials that transport electrons from NADH and FADH to oxygen (Fig 2.2). In addition to the traditional components mentioned above, the respiratory chain contains yet other dehydrogenases, e.g. the glycerophosphate dehydrogenase (GPDH) (Houstek et al. 1975) and flavoprotein-linked acetyl CoA-dehydrogenase (Liang et al. 2001). The free energy released during the electron transport is stored in the form of proton gradient ($\Delta\mu_H^+$) across the inner mitochondrial membrane (see section 2.1.2). The proton gradient is consequently used by the F_oF_1 ATPase for phosphorylation of ADP. It also drives transport of metabolites and macromolecules across the membrane and can be dissipated in the form of heat by the uncoupling proteins (Fig. 2.3).

The respiratory chain complexes have been thought to be laterally mobile in the phospholipid bilayer of the inner mitochondrial membrane, but recent studies convincingly show that the complexes are organized in supramolecular structures (Schagger 2001).

Figure 1.2 The Scheme of the Redox Components of the Respiratory Chain. *Iron–sulfur clusters in the complex I and complex II segments are distinguished with suffixes Nx and Sx, respectively. Q_N, Q_S, and (Q_o and Q_i) denote specific UQ binding sites in complex I, complex II, and complex III segments, respectively. Specific inhibitor binding sites are indicated by arrows. Adapted from (Ohnishi 1998).*

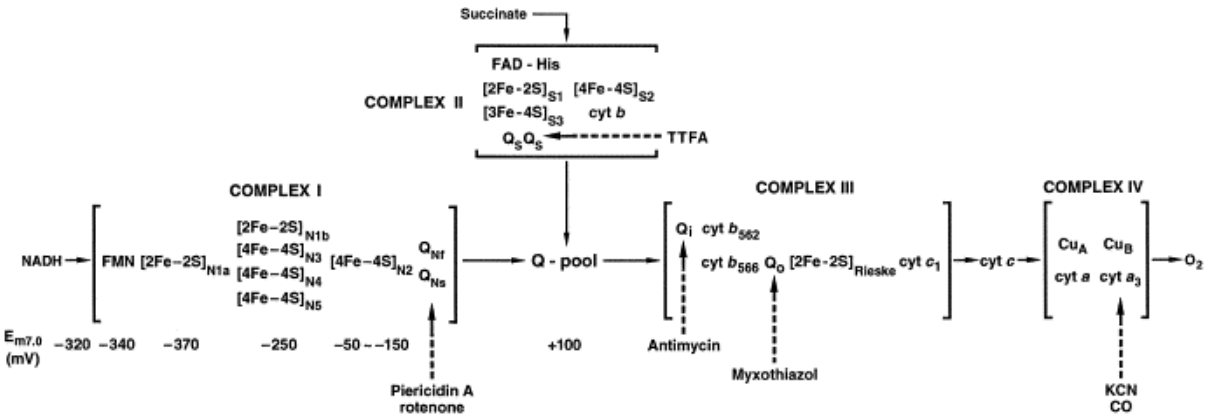
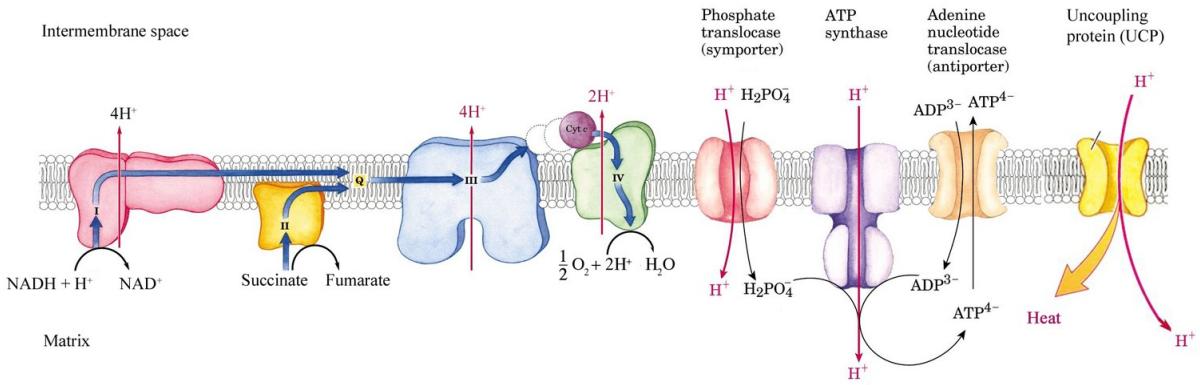


Figure 1.3 The Oxidative Phosphorylation Apparatus. *The apparatus comprises the respiratory chain enzymes (complexes I, II, III and IV), which transport electrons from NADH or FADH to oxygen and concomitantly generate proton gradient across the inner mitochondrial membrane. The proton gradient is subsequently used by F_OF_1 ATPase for ADP phosphorylation, drives the ADP/ATP exchange and transport of inorganic phosphate into mitochondria or it can be dissipated by uncoupling proteins. Adapted from (Lehninger et al. 2000).*



1.1.2 The Chemiosmotic Theory

The chemiosmotic theory of energy coupling has been postulated by Peter Mitchell in 1961 (Mitchell 1961) and it was awarded the Nobel Prize for Chemistry in 1978, because of fundamental contribution to understanding the basis of energy conversion in living organisms.

The mitochondrial respiratory chain in the inner membrane is proton translocating; it pumps H^+ out of the matrix space when electrons are transported along the chain. The mitochondrial ATPase also translocates protons across the inner membrane. Being reversible, it can use the energy of ATP hydrolysis to pump H^+ across the membrane, but if a large enough electrochemical proton gradient is generated by the respiratory chain, protons flow in the reverse direction through the complex and drive ATP synthesis.

The movement of protons across the inner mitochondrial membrane has two major consequences. First, it generates a pH gradient (ΔpH) across the membrane, with the pH higher in the matrix than in the cytosol, where the pH is generally close to 7. Second, it generates a membrane potential ($\Delta\psi_m$), negative inside, as a consequence of the uncompensated net movement of positive ions (H^+).

Both components constitute an electrochemical proton gradient ($\Delta\mu_{H^+}$):

$$\Delta\mu_{H^+} = F\Delta\psi_m - 2.303\ RT\,\Delta pH$$

where R is the gas constant, T is the absolute temperature and F is the Faraday constant. Mitchell defined the term proton motive force (Δp), which is the electrochemical proton gradient expressed in units of millivolts (mV):

$$\Delta p = \frac{\Delta\mu_{H^+}}{F} = \Delta\psi_m - 2.303\,\frac{RT}{F}\Delta pH$$

At 37°C the value of 2.303 RT/F is about 60 mV, so the equation above could be re-written in a simple form:

$$\Delta p = \Delta\psi_m - 60\ \Delta pH$$

The proton motive force is a measure of the free energy change for the transfer of protons from one side of the membrane to the other. To quantify the magnitude of Δp, it is necessary to know both ΔpH and $\Delta\psi_m$. Various studies on mammalian mitochondria revealed that the membrane potential (~150 - 170 mV) represents the main component of Δp in comparison with the pH gradient (~ 0.5 - 1 pH units, which corresponds to 30-60 mV) (Nicholls and Ferguson 2002).

The tightness of coupling between respiration and phosphorylation can be evaluated by measuring the oxygen consumption using an oxygen electrode. If mitochondria are incubated in the medium containing substrate and phosphate, then addition of ADP causes a sudden burst of oxygen uptake as the ADP is converted into ATP. The actively respiring state is referred to as "state 3" respiration, while the slow rate after all the ADP has been phosphorylated to form ATP is referred to as "state 4". The ratio of the respiration rates in states 3 and 4 is called the respiratory control index (RCI) and indicates the degree of coupling between respiration and ATPase. Typical RCI values range from 3 to 10, varying with the substrate and the quality of the mitochondrial preparation. Another important parameter is a *P/O* ratio - the amount of ATP synthesized when two electrons are transferred from a substrate through a respiratory chain to reduce one oxygen atom (1/2 O_2). It can be calculated by measuring the increase of oxygen consumption rate during the rapid state 3 respiration after addition of defined amount of ADP.

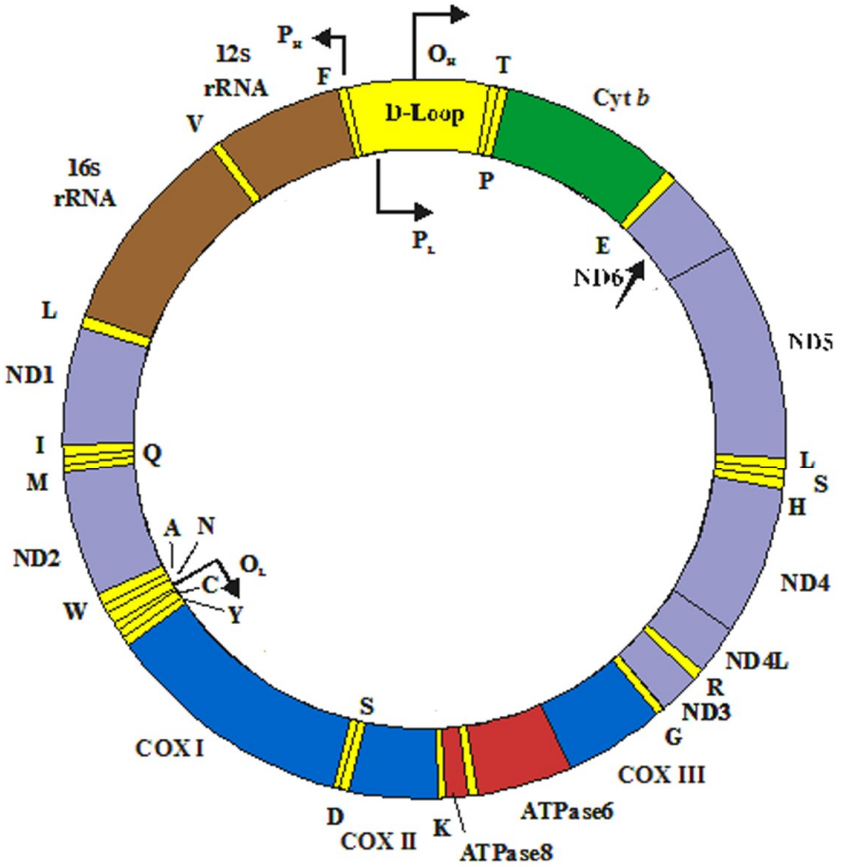
According to the best current estimates, the oxidation of NADH to NAD^+ and water is associated with the net export of 10 protons from the mitochondrial matrix to the intermembrane space (Illingworth 2002). ATP synthesis by the ATPase is thought to require 3 protons to re-enter the matrix space. Since mitochondria synthesize ATP in the matrix, it is necessary to export the nucleotide to the cytosol. Two carriers are involved: the adenine nucleotide translocator for the uptake of ADP and export of ATP, and the phosphate carrier for the uptake of inorganic phosphate. The transport of ATP, ADP and phosphate across the inner mitochondrial membrane costs 33 % additional energy over the minimum required for the synthesis of ATP within the mitochondrial matrix compartment. One additional proton is used to drive both of these transport systems: the positive charge on this single proton drives adenine nucleotide translocator, while its acidity drives the phosphate uptake (Nicholls and Ferguson 2002).

Thus the overall requirement is 4 protons for each ATP delivered to the cytosol. This implies that the *P/O* ratio is 2.5 for NAD^+-linked substrates. The *P/O* ratio for succinate is only 1.5. This lower value indicates that electrons from succinate (and also acyl CoA and glycerophosphate) enter part of the way along the respiratory chain, bypassing the first coupling site where energy is stored for ATPase (complex I that moves 4 protons across the inner membrane).

1.1.3 Mitochondrial Biogenesis and Genetics

The biogenesis of mitochondria, as the only organelle of mammalian cell, depends on the coordinated expression of two genomes, nuclear and mitochondrial. The human mitochondrial genome is a 16,569 base pairs long circular DNA (Anderson et al. 1981). Proteins encoded by mtDNA are absolutely essential for mitochondrial energetics. mtDNA has a prokaryotic character, is highly compact and contains only 37 genes: 2 genes encode ribosomal RNAs, 22 encode transfer RNAs, and 13 encode OXPHOS polypeptides. mtDNA codes for 7 subunits of Complex I (ND1-6 and ND4L), cytochrome *b* of Complex III, 3 subunits of Complex IV (COX I-III) and 2 subunits of Complex V (ATPase A6 and A8) (Fig. 2.4).

Figure 1.4 Human mitochondrial DNA map. *The scheme shows the genes for the 12S and 16S ribosomal RNAs, the subunits of Complex I (ND1, ND2, ND3, ND4, ND4L, ND5, and ND6), Complex III (cyt b), Complex IV (COX I, COX II, and COX III), Complex V (ATPase6 and ATPase8), and 22 tRNAs (1-letter amino acid nomenclature). The origins of heavy-strand (O_H), light-strand (O_L) replication, and the promotors for initiation of transcription from the heavy-strand (HSP) and light-strand (LSP), are shown by arrows.*



Most of the proteins that reside in the mitochondrion are nuclear gene products. These proteins are translated in the cytoplasmic compartment of eukaryotic cells and subsequently transported into the mitochondrion. Three properties characterize protein import into mitochondria: (1) all the information required to target a precursor protein from the cytosol to the mitochondrial matrix is contained within its targeting sequence which is N-terminal or cryptic (2) only unfolded protein can be imported; and (3) translocation of precursors to the matrix occurs at the contact sites where the outer and inner membranes are close together. These nuclear-encoded proteins play key roles in mitochondrial transcription and translation, mitochondrial lipid and heme synthesis, substrate oxidation by the tricarboxylic acid cycle, and OXPHOS system. All together more than 500 different nuclear proteins are present in all four mitochondrial compartments (inner and outer mitochondrial membranes, matrix and intermembrane space) and they account for > 90 % of the protein mass of the mitochondrion.

Transcriptional regulators that act on both nuclear and mitochondrial genes have been implicated in the bi-genomic expression of the respiratory chain. Mitochondrial transcription is directed by a small number of nucleus-encoded factors (Tfam, TFB1M, TFB2M, mTERF). The expression of these factors is coordinated with that of nuclear respiratory proteins through the action of transcriptional activators and coactivators. In particular, environmental signals induce the expression of PGC-1 family coactivators (PGC-1alpha, PGC-1beta, and PRC), which in turn target specific transcription factors (NRF-1, NRF-2, and ERR alpha) in the expression of respiratory genes. This system provides a mechanism for linking respiratory chain expression coordinately from both genomes according to environmental conditions, and for integrating it with other functions related to cellular energetics (Scarpulla 2006).

1.1.4. Mitochondrial Diseases

Deficiencies in oxidative phosphorylation are associated with diverse array of multisystem disorders that are often referred to as mitochondrial encephalomyopathies because of the prominent involvement of the nervous system and striated muscle. Primary OXPHOS defects can be caused by mutations in mtDNA or nuclear genes.

Mitochondria follow the rules of population genetics. Six aspects of their behavior are critical for understanding the etiology and pathogenesis of mitochondrial disorders: (1) they are maternally inherited; (2) cells typically contain hundreds of organelles and thousands of mitochondrial genomes; (3) mutations can arise in a mtDNA population,

resulting in the coexistence of two or more mtDNA genotypes within a single cell, organ or individual (heteroplasmy); (4) if the mutation is pathogenic, the proportion of mutated molecules in the heteroplasmic population (mutational load) affects the severity of the biochemical defect, but not necessarily in a linear fashion; (5) mtDNA replication and inheritance in lineages of somatic cells is stochastic, often resulting in changing mutational loads during the life of the patient, and in different mutational loads in different cells and tissues (mitotic segregation); (6) because different cell types have different minimal oxidative energy requirements (thresholds), the level of heteroplasmy and the dynamics of the mitotic segregation play a critical role in determining the clinical presentation and outcome.

Owing to the fact that more than 98 % of genes for mitochondrial proteins are coded in the nucleus, much attention has recently turned to the investigation of the nuclear OXPHOS gene defects. The majority of these are inherited as autosomal recessive traits, producing severe and usually fatal diseases in infants. Defects in approximately 20 different nuclear genes have now been identified which encode structural components of the OXPHOS complexes, assembly/maintenance factors and factors necessary for the maintenance of mtDNA integrity.

1.2 Cytochrome c Oxidase

Cytochrome *c* oxidase (COX; EC 1.9.3.1) is the terminal enzyme of the respiratory chain. It is localized in the inner mitochondrial membrane where it transfers electrons from ferrocytochrome *c* to molecular oxygen, the ultimate acceptor of all reducing equivalents. It belongs into superfamily of heme-copper oxidases which is defined by high sequence similarity within the largest subunit I and a binuclear active site consisting of a high-spin heme (heme a_3) and a closely associated copper ion (Cu_B) (Abramson et al. 2001).

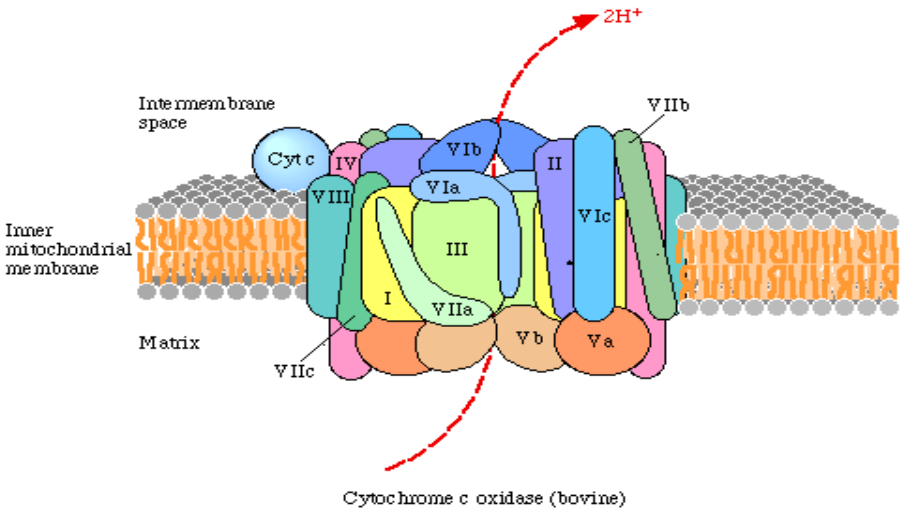
The eukaryotic cytochrome *c* oxidase is a two-face enzyme. On one hand it is extremely conservative in performing its role of transporting electrons from cytochrome *c* to molecular oxygen in essentially all aerobic organism, thus allowing them to extract a substantial portion of free energy and store it in the form of electrochemical gradient of protons. The three core catalytic subunits, the enzyme prosthetic groups, both the electron transport and proton pumping mechanisms, and the elegant way it handles such potentially toxic compound – oxygen, have virtually not changed from the simple oxidase of mitochondrion's procaryotic ancestor to the multisubunit enzyme complexes present in

modern eukaryotes. It is the addition of novel subunits during the evolution of eukaryotic cytochrome *c* oxidase that represents the other, rather unconservative face of the enzyme. The new components seem to allow the eukaryotic cell to modulate the cytochrome oxidase activity according to its energy demand. In mammals these novel subunits outnumber the original ones ten to three, and they confer the enzyme many interesting features such as tissue specifity, modulation of proton transport efficiency, regulation by reversible phosphorylations and undoubtedly many other. This way cytochrome c oxidase becomes fully intertwined within the fascinating regulatory network of the cell and perhaps, as many believe, one of the key regulators of cell metabolism.

1.2.1 Structure of COX

The mammalian COX is composed of 13 subunits (Fig. 2.5). The three largest subunits (I, II, and III) are encoded by mitochondrial DNA. They are homologous to subunits of prokaryotic COX and form the remarkably conserved catalytic core. The remaining 10 subunits (IV, Va, Vb, VIa, VIb, VIc, VIIa, VIIb, VIIc, and VIII) adopted into the enzyme structure during the eukaryotic evolution are encoded by the nuclear genome. They do not participate directly either in electron transport or proton translocation, but numerous studies performed on yeast and mammalian models show that they are involved in the assembly and regulation of activity of the enzyme (Poyton and McEwen 1996; Kadenbach et al. 2000).

Figure 1.5 The Topology of COX Subunits. *The positions of individual COX subunits of the COX dimer from bovine heart are indicated.*

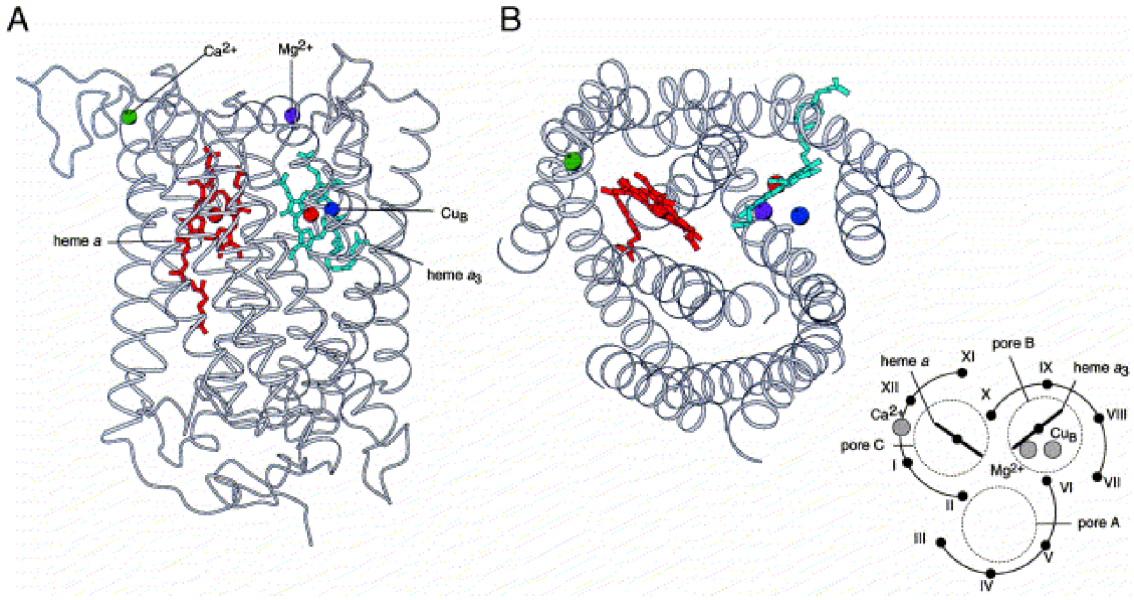


A major advance in cytochrome *c* oxidase research was achieved with the X-ray structures of *Paracoccus denitrificans* (POX) and bovine (BOX) enzymes. The POX structure (four-subunit enzyme) was initially determined to a resolution of 2.8 Å (Iwata et al. 1995); this was followed by the structure of the two-subunit oxidase (subunits I and II) at 2.7 Å (Ostermeier et al. 1997) and then an improved structure of the four-subunit enzyme (Harrenga and Michel 1999). The BOX structure was also initially reported to a 2.8 Å resolution (Tsukihara et al. 1996) but has been largely improved with the structures of fully oxidized (2.3 Å), fully reduced (2.35 Å), azide bound (2.9 Å) and carbon monoxide-bound (2.8 Å) forms (Yoshikawa et al. 1998).

The crystallographic data helped to elucidate the detailed structural framework of COX. The transmembrane part of this enzyme is composed of 28 α–helices. Subunit I contains 12 closely packed transmembrane helices (Fig. 2.6). Both the N and C termini of subunit I are located on the matrix side of the membrane.

Subunits II and III interact with the transmembrane domain of subunit I without having any contact with each other. Subunit II contains two transmembrane helices, closely associated with the transmembrane domain of subunit I, and a large polar domain at the cytosolic side of membrane. The polar domain contains a ten-strand ß barrel structure that binds the Cu_A redox center. Both the N and C termini of subunit II are on the outer side of the membrane. Subunit III contains seven transmembrane helices organized in two bundles, (I, II and III to VII), which form a V-shaped cleft. Helices I and III form contact with subunit I. The N terminus of subunit III is on the inner side of the membrane. Then seven transmembrane helices of subunit III are connected to the catalytic center of the enzyme. Nuclear-encoded subunits IV, VIa, VIc, VIIa, VIIb, VIIc and VIII are located inside the membrane, each contributing to the enzymatic structure with one helix. Subunit VIb is associated on the cytosolic side of the membrane and subunits Va and Vb to the matrix side (Fig. 2.5).

Figure 1.6 Structure of Subunit I of Cytochrome c Oxidase. *(A) View parallel to the membrane. The subunit contains 12 transmembrane helices. Heme a (red), heme a_3 (cyan), Cu_B (blue sphere), magnesium ion (magenta sphere) and calcium ion (green sphere) are also shown. (B) View along the membrane normal from outside of the membrane. Only transmembrane helices are shown with hemes and metal centers. Adapted from (Abramson et al. 2001).*



X-ray crystallographic structure analysis further revealed 8 phospholipid (5 phosphatidyl ethanolamines and 3 phosphatidyl glycerols) molecules that are closely bound to the protein by salt bridges or hydrogen bonds. Interestingly, the inner mitochondrial membrane lipid cardiolipin was not found, even though it was described as an essential factor for the catalytic activity of the enzyme (Robinson 1993). Nevertheless, the electron density distribution of unsaturated bonds positioned in between the two monomers remains to be characterized in detail and two cardiolipin molecules are the most probable candidates to occupy this site (Tsukihara et al. 1996).

1.2.2 The Functional Properties of Cytochrome c Oxidase

Cytochrome c Binding to COX

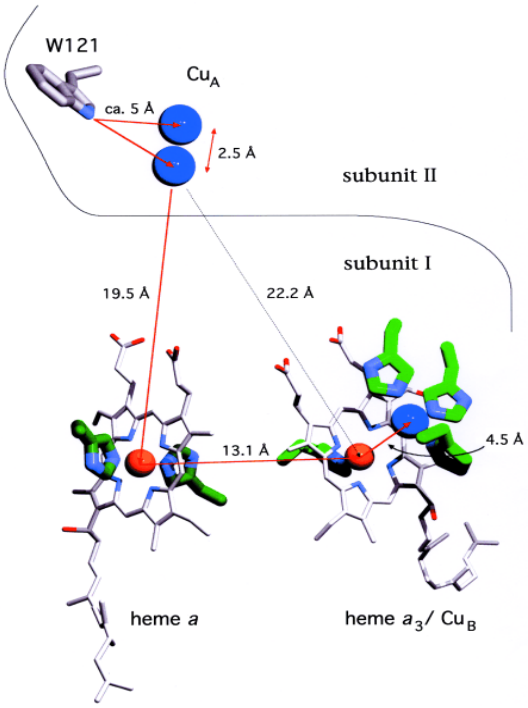
Cytochrome *c* binds with high affinity to the extramembraneous domain of the subunit II. The binding is mediated by electrostatic interactions between the positively charged lysine residues of cytochrome *c* and the negatively charged aspartate and glutamate residues at the binding site. The binding cleft is located in the site between the

COX monomers. A second, low affinity binding site is proposed to bind second molecule of cytochrome *c* (Capaldi 1990).

The Catalytic Cycle of COX

The electrons donated from cytochrome *c* are transferred to oxygen through its four internal redox centers to form water. The Cu_A center, composed of two electronically coupled, mixed-valence (Cu^I/Cu^{II}) copper ions, is located at the hydrophilic domain on the cytosolic side of subunit II. The electron is subsequently transferred from the Cu_A center to heme *a* in subunit I. This low-spin heme is closer to the Cu_A center than heme a_3, and therefore heme *a* is the kinetically preferred electron acceptor (Fig. 2.7).

Figure 1.7 The Electron Pathway through Four Redox Centers of COX. *See text for description. Adapted from (Ludwig et al. 2001).*

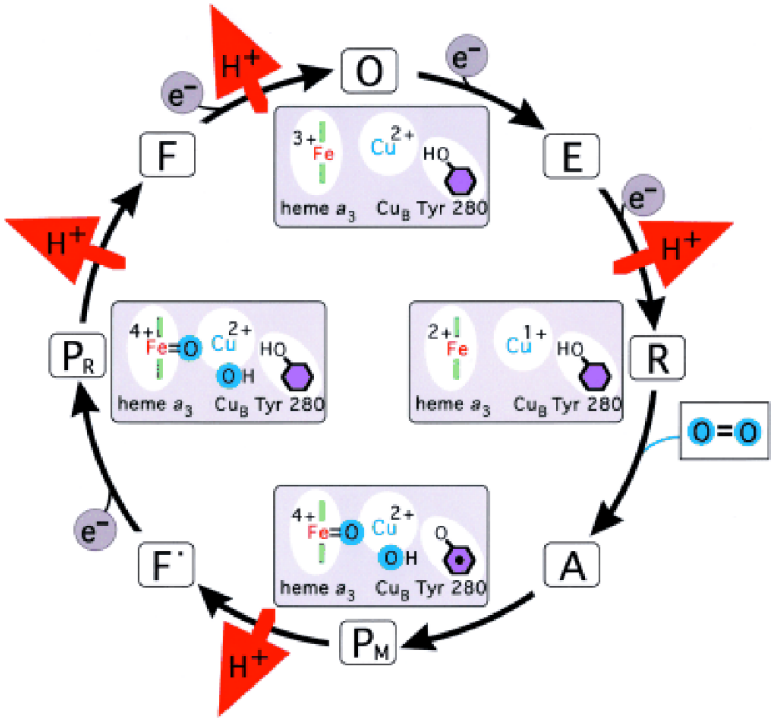


Both heme planes are oriented perpendicular to the membrane, at an angle of 108° to each other. Their iron centers are 13 Å apart, but their closest edge-to-edge distance amounts to only 5 Å; two of their histidine ligands (H 411 and H 413) are spaced by only one amino acid on the same transmembrane helix X. The high-spin heme a_3 and an electronically coupled Cu_B ion form the binuclear center of the enzyme. This center, buried about one third into the depth of the membrane, is the site of oxygen binding and water formation, requiring free access for its substrates (oxygen and protons) and probably is the key player in energy transduction.

In the oxidized state ("O"), the binuclear center metal ions carry a +3 (heme iron) and +2 charge (Cu). With the first two electrons entering the binuclear site, both metal centers are reduced (+2/+1) in two steps ("E" to "R" in Figure 2.8). With two redox equivalents available at the binuclear site ("R"), oxygen is able to bind, leading to the formation of species "A". On a fast time scale, this compound reacts directly to state "P_M", in which the

dioxygen bond is already cleaved and both oxygen atoms are present in the formal oxidation state of water. Two further electrons are provided transiently from within the site: the heme iron center is formally oxidized to the ferryl state (+4), and the nearby cross-linked tyrosine is able to donate the fourth electron, yielding a tyrosine radical. The unique structural setup at the binuclear site, including the atypical side chain cross-link, seems to allow the enzyme, once oxygen is bound, to immediately split the O-O bond even if the supply of further electrons is stalled, thus avoiding the formation of reactive oxygen species such as superoxide anion or hydrogen peroxide. The reaction scheme in the second half of the cycle requires resolution of the two unusual electronic states at the binuclear center, reduction of the tyrosine radical by transfer of the third electron, and the fourth electron to resume the oxidized state also for the iron center of heme a_3 (+3) (Ludwig et al. 2001).

Figure 1.8 The Oxygen Reduction Cycle. *Key intermediates (O, E, R, A, P_M etc.) are listed clockwise along with the electron input steps (gray circles), dioxygen binding (blue) and proton translocation steps (red). Adapted from (Ludwig et al. 2001).*



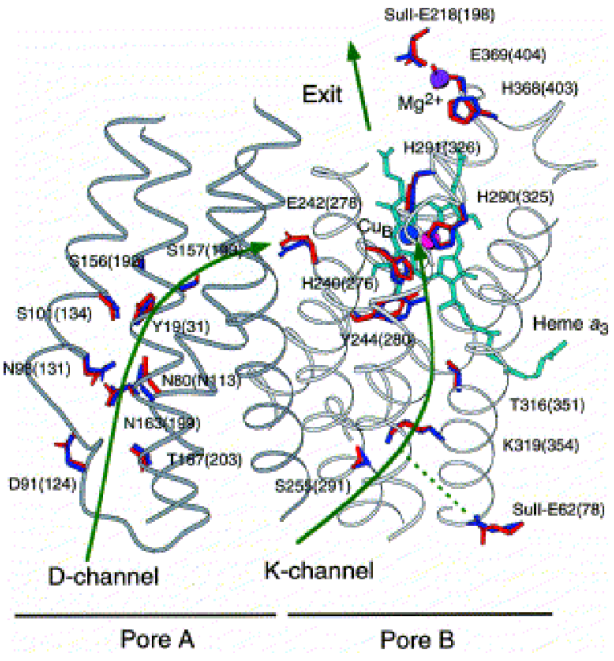
ProtonTranslocation by COX

Since the discovery of the vectorial proton translocation in COX by Wikstrom (Wikstrom and Saari 1977) the details of coupling between electron transport and proton pumping remain a matter of discussion. In earlier studies (Casey et al. 1979; Sigel and Carafoli 1980; Wikstrom et al. 1981) a constant H^+/e^- stoichiometry of about 1.0 was found. Other investigators, however, measured higher H^+/e^- ratios of up to 2 (Azzone et al. 1979; Reynafarje et al. 1982; Lemasters 1984; Hendler and Shrager 1987). After numerous discussions it was generally accepted that the H^+/e^- ratio is 1 and that different ratios found previously were explained by inconsistencies of the experimental setup (Babcock and Wikstrom 1992). In later studies the variable stoichiometry was explained by "slippage" of proton pumping, as measured in mitochondria (Azzone et al. 1985) or with the isolated and reconstituted enzyme (Steverding and Kadenbach 1991; Steverding et al. 1993). There has been reported a variable H^+/e^- stoichiometry depending on the turnover rate of the enzyme (Papa et al. 1991). The mechanisms of translocation in COX including the variation of the H^+/e^- stoichiometry in order to control the flow of electrons in the respiratory chain, and/or to vary the extent of thermogenesis in different tissues (Ludwig et al. 2001) will be discussed in the next section.

During a complete oxygen cycle four protons are taken up from the matrix side for the formation of water ("chemical or substrate protons"), and four additional protons are assumed to be translocated from the matrix across the inner mitochondrial membrane to the cytosolic side ("pumped protons"). The system is designed such that the introduction of negative charge (electrons) into the buried heme a, heme a_3, Cu_B centers is the driving force for all of the protons taken up from the inner surface of the oxidase. Pumped protons are directed to a site where they cannot protonate the O_2 intermediates and provide the charge repulsion that expels the pumped protons to the outside (Hosler et al. 2006). Two proton pathways, the D- and the K-channel, named after key aspartate and lysine residues, respectively, were suggested theoretically and according to mutational studies and were later confirmed by the crystallographic studies (Abramson et al. 2001) (Fig. 2.9). Early experiments seemed to indicate that the K channel is used to allow access of the four protons to the binuclear site for water formation, while the D channel was assumed to translocate protons across the membrane. From later kinetic approaches, and a separation of the reaction cycle into reductive and peroxidase half-reactions, it was concluded that the first (or the first two) protons would pass through the K pathway, while the remaining

majority would use the D pathway in a dual-purpose function, requiring some kind of distributing device (Ludwig et al. 2001). A current controversy relates to the question of which steps in the COX catalytic cycle are linked kinetically to the proton translocation. Direct measurements of proton translocation during a single turnover, initiated by adding O_2 to the fully reduced enzyme, revealed pumping of two protons during the oxidative phase of the catalytic cycle (one proton pumped each in the P⟶F and F⟶O steps), and pumping of two more protons during the reductive phase (Verkhovsky et al. 1999). This finding was surprising in relation to the earlier view that all proton translocation would take place in conjunction with the oxidative phase, which was based on equilibrium titrations in intact mitochondria at high protonmotive force (Wikstrom 1989). Another surprising finding was that proton pumping during the reductive phase was observed only when this phase was allowed to take place immediately after the oxidative phase. Reduction of the enzyme without an immediately preceding oxidation by O_2 therefore failed to yield proton pumping (reviewed in (Wikstrom and Verkhovsky 2002)). Thus, despite the evident progress in our understanding of the proton pumping mechanism, which was greatly enhanced by solution of the enzyme structure by X-ray crystallography, the consensus about the microscopic details of the process has not yet been completely achieved (for detailed discussions see these recent reviews (Hosler et al. 2006; Papa et al. 2006).

Figure 1.9 The Proton Pathways of COX*. The residues in bovine cytochrome c oxidase (BOX, red) and P. denitrificans oxidase (POX, blue) are superimposed. Structures of helices, heme a_3 and metal centers are based on the POX structure. Adapted from (Abramson et al. 2001).*



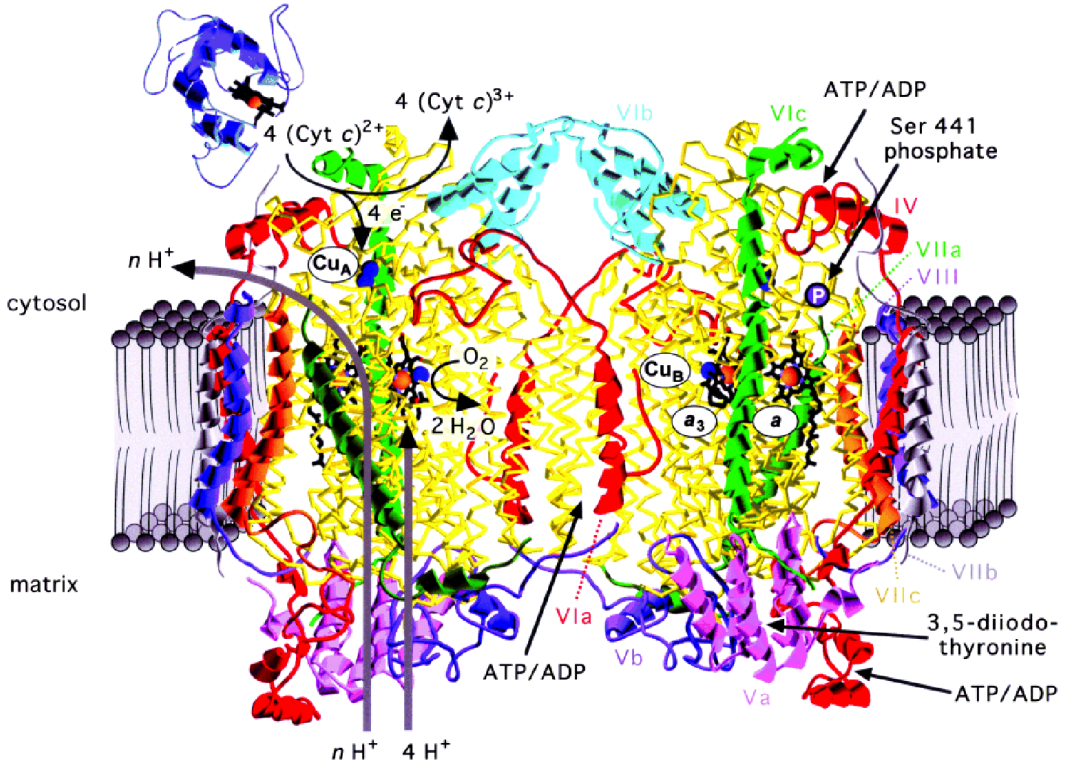
1.2.3 Role of Cytochrome c Oxidase in Mitochondrial Physiology

Mitochondria can not be further considered to be merely the "cellular powerplants". Our progress in understanding of the complexity of eukaryotic cellular physiology underscores the view of mitochondria as important players in the regulatory network of the cell. In addition to their major and indispensable function in energy provision, mitochondria play key role in calcium homeostasis, triggering of apoptosis, thermogenesis, sensing and adaptation to hypoxia, reactive oxygen species homeostasis to name just the major issues. The cytochrome c oxidase – as a key enzyme of the respiratory chain - has been recently found to be involved in most of the mitochondrial physiological processes. The following chapters review some of the most exciting and diverse reports of the powerful physiological capabilities of COX.

Regulation of COX by Allosteric Factors and Role of ncDNA Encoded Subunits

The evolution equipped the mitochondrial cytochrome *c* oxidase with a number of accessory subunits that are absent in its bacterial counterparts. This fact is most striking when encountering the mammalian COX, which consists of 13 subunits - the maximum number of subunits known so far. In addition, some of these subunits are expressed as tissue-specific isoforms. Long has it been thought, that the ncDNA encoded subunits might be involved in the regulation of the enzyme, and indeed, the regulatory role was recently confirmed for subunits IV, Va, Vb, VIaH, and VIaL. Consequently, new theory of COX regulation was postulated featuring the allosteric binding of adenine nucleotides, hormone dependent phosphorylation cycles, and changes in the H^+/e^- ratio (Ludwig et al. 2001). Thus, COX represents perhaps the most tightly regulated enzyme of the respiratory chain (Fig. 2.10).

Figure 1.10 Structure of COX Dimer from Bovine Heart Mitochondria. *Indicated are: binding sites for substrates (cyt c and O_2) and allosteric regulatory factors (ATP/ADP, diiodothyronine) and phosphorylation epitope at subunit I (Ser 441). Adapted from (Ludwig et al. 2001).*



A decrease of the H^+/e^- ratio from 1.0 to 0.5 with increasing intraliposomal ATP/ADP ratio was measured with reconstituted cytochrome *c* oxidase from bovine heart. The decrease was half-maximal at an ATP/ADP ratio of 100 and correlated to the exchange of bound ADP by ATP at the matrix domain of subunit VIaH, since preincubation of the enzyme with a monoclonal antibody against subunit VIaH prevented the decrease (Frank and Kadenbach 1996; Huttemann et al. 1999). The nucleotide-binding site at subunit VIaH was verified in the crystal structure of the enzyme by identification of a cholate molecule at this site, since cholate is structurally very similar to ADP (Tsukihara et al. 1996). With reconstituted cytochrome *c* oxidase from bovine liver (and kidney) an H^+/e^- ratio of 0.5 was measured under the same conditions as the ones applied to the bovine heart enzyme. The enzymes from heart or skeletal muscle (VIaH) and from liver or kidney (VIaL) contain different isoforms of subunit VIa (and also subunits VIIa and VIII). The lower H^+/e^- ratio of the liver enzyme was neither influenced by the intraliposomal ATP/ADP ratio nor by a monoclonal antibody against subunit VIaH.

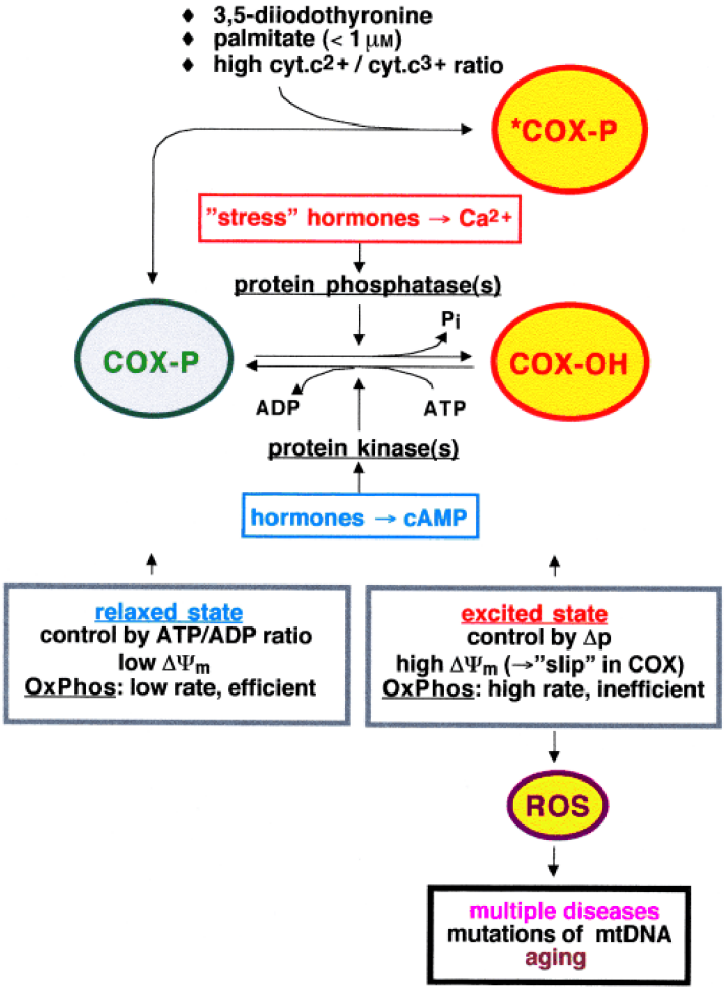
Interestingly, in liver, heart, and skeletal muscle of a bird (turkey) only the liver-type isoform (VIaL) was found, and the reconstituted enzymes from turkey liver and heart showed H^+/e^- ratios of 0.5, which were independent of the intraliposomal ATP/ADP ratio (Huttemann et al. 2001). It was postulated that the decreased H^+/e^- ratios for the enzyme from skeletal muscle tissue (subunit VIaH) at high ATP/ADP ratios (e.g. during sleep), and for the enzyme from nonskeletal muscle tissues, participate in thermogenesis (Huttemann et al. 1999).

Second Mechanism of Respiratory Control

In addition to the classical respiratory control exerted by the proton motive force (Δp), a "second mechanism of respiratory control" was found, which is independent of Δp and based on the inhibition of cytochrome *c* oxidase activity at high intramitochondrial ATP/ADP ratios. The nucleotides bind to the matrix domain of the transmembraneous subunit IV. This was concluded from preincubation of the enzyme with a monoclonal antibody against subunit IV, which prevented the allosteric ATP inhibition, and from the requirement of high ATP/ADP ratios inside the vesicles for the inhibition of the ascorbate respiration of mitochondrial particles (Arnold et al. 1997). Half-maximal inhibition of activity was obtained at an intramitochondrial ATP/ADP ratio of 28 (Arnold and Kadenbach 1999).

Hormones, acting through cAMP as second messenger, switch on the allosteric ATP inhibition by cAMP-dependent phosphorylation of cytochrome *c* oxidase (COX-P), and hormones increasing the intracellular Ca^{2+} concentration switch it off by calcium-induced dephosphorylation (COX-OH). The site of cAMP-dependent phosphorylation was determined for bovine heart cytochrome *c* oxidase by incubation with protein kinase A, cAMP, and [γ -^{32}P]ATP. The autoradiography showed labeling of subunits I, II (or III), and Vb, the Ser 441 of subunit I was proposed as the phosphorylation target (Bender and Kadenbach 2000).

Figure 1.11 Schematic Representation of the Postulated Regulation of OXPHOS in vivo. *See text for description. Adapted from (Ludwig et al. 2001).*



Based on these experimental data, it was postulated that in cells a hormonally controlled dynamic equilibrium exists between two states of energy metabolism: a relaxed state with efficient oxidative phosphorylation according to ATP utilization, and associated with low $\Delta\Psi_m$ and low ROS formation (COX–P); and an excited state with increased $\Delta\Psi_m$, but less efficient oxidative phosphorylation, associated with increased ROS formation (COX–OH). The relaxed state is switched on by cAMP-dependent phosphorylation of cytochrome *c* oxidase, and switched off by calcium ion induced dephosphorylation (Fig.2.11). Without dephosphorylation, COX-P can be switched to the excited state by high cyt c^{2+}/cyt c^{3+} ratios, allosteric binding of 3,5-diiodothyronine or palmitate (Fig. 2.11). It was suggested that *in vivo* cytochrome *c* oxidase is mostly phosphorylated, and respiration is mainly controlled (except for control by substrates) by the intramitochondrial ATP/ADP

ratio. This mechanism converts cytochrome *c* oxidation into the rate-limiting step of the respiratory chain. On the other hand, "stress hormones" lead to an increase in $\Delta\Psi_m$ (150-200 mV) through calcium-induced dephosphorylation of cytochrome *c* oxidase, and thus turn on the excited state of energy metabolism. The dephosphorylated enzyme no longer represents the rate-limiting step of the respiratory chain (Ludwig et al. 2001).

Reserve Capacity and Flux Control of COX

In recent years, the metabolic control of oxidative phosphorylation has received growing attention, and the approach based on the control of flux theory has been increasingly applied to the study of mitochondrial metabolism. The discovery of mitochondrial disorders manifesting as reduced activities of the respiratory chain complexes have raised fundamental question as to the degree of control that the individual steps of oxidative phosphorylation exert on the rate of mitochondrial respiration. Most of the experimental work aiming to answer these questions has been carried out by parallel inhibitor titrations of the overall respiratory rate and the isolated activity of the particular complex studied (Villani et al. 1998).

Concerning COX, the original and even some recent studies performed on isolated mitochondria revealed large excess capacity (2 - 4 fold) of COX over the upstream complexes of the respiratory chain with evidence for a tissue-specific pattern (Letellier et al. 1993; Rossignol et al. 1999). Thus, the control strength of COX was considered negligible. However, studies performed on cultured cells (Villani and Attardi 1997; Villani et al. 1998) or permeabilized muscle fibers (Kunz et al. 2000; Kudin et al. 2002) showed much tighter control of COX over the respiratory rate. Based on the cell type and the method used (e.g. TMPD concentration), the COX excess capacity varies between 10 and 250 percent over the overall respiratory capacity. Fibroblasts, which are used in this study of functional manifestation of Leigh syndrome, were estimated to have just 20 percent excess COX capacity (Villani et al. 1998).

Another strong argument supporting the view of COX as having relatively tight control over respiration is the concept of allosteric regulation by adenine nucleotides described in the previous section. Such regulation would be much more effective if COX was nearly limiting relative to the overall respiration.

The concept of COX tight control has already found its applications in the research of mitochondrial disorders. When the biochemical phenotype of transmitochondrial cybrids constructed by transferring mitochondria from patient carrying a nonsense mutation in the

gene for COX subunit I was analyzed, cells carrying 35 % mutant DNA exhibited 55 % residual COX activity and 75 % of residual overall respiration, while cells carrying 65 % mutant DNA had only 15 % of COX and 10 % of overall respiratory activity (Bruno et al. 1999).

Cytochrome c Oxidase and its Role in Regulation of Oxygen Homeostasis

The "ancient invention" of the unique design of COX reaction centre and the elegancy with which it handles its potentially toxic substrate oxygen was undoubtedly one of the major evolutionary events that determined the character of today's biosphere. In eukaryotes, COX is by far the major oxygen consumer and therefore shapes the oxygen enviroment in cells and whole organisms.

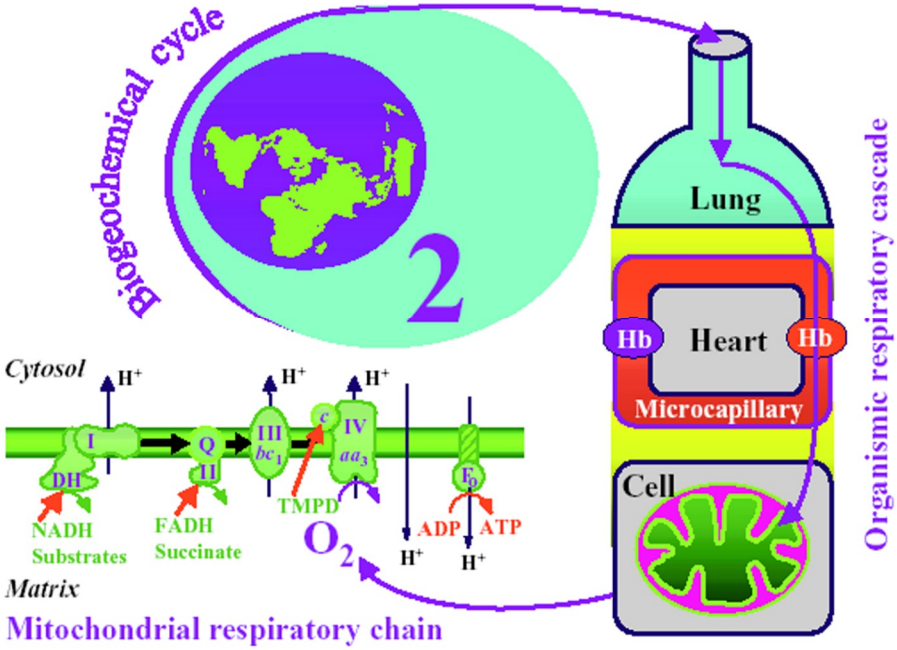
In mammals, the oxygen partial pressure decreases by more than one order of magnitude during the transport through the respiratory cascade from 20 kPa in the environment to less than 1 kPa inside the cells (Fig. 2.12). Most data on the intracellular oxygen concentrations are available for the myocytes, where p_{02} was estimated 0.3 kPa (reviewed in (Gnaiger et al. 1995)). The long standing question whether COX activity (and thus the respiratory rate) is limited at these intracellular oxygen concentrations has been solved in recent years due to development of high-resolution respirometry. This technique provides enough sensitivity and time resolution for kinetic measurements at low oxygen tension in both cultured cells and isolated mitochondria (Gnaiger et al. 1995).

In the measurements with cultured cells, a hyperbolic oxygen-dependence of the respiratory rate is observed. This dependence can be described by equation:

$$J_{02} = (J_{max} \cdot p_{O2})/(p_{50} + p_{O2})$$

where the oxygen flux (respiratory rate) J_{02} is characterized by two parameters, p_{50}, the oxygen partial pressure at which respiratory flux is 50 % of maximum, and J_{max}, the maximum oxygen flux at saturating level of oxygen (Gnaiger et al. 1998). The p_{50} can be referred to as the apparent K_M for oxygen and is thus a measure of COX oxygen affinity. The published p_{50} values vary between 0.02 – 0.16 kPa depending on the type of the cells and conditions of the measurement. In myocyte, for example, the p_{50} was found to be 0.32 kPa and so the myocyte intracellular p_{02} of 0.3 kPa itself would limit the respiratory oxygen flux to 90 % of maximum (Gnaiger et al. 1998).

Figure 1.12 The Respiratory Cascade. *The p_{O2} of air at sea level is approximately 20 kPa, alveolar air contains oxygen of cca. 13 kPa. Oxygen diffuses to the blood, which enters the lung at a mixed venous p_{O2} of 5 kPa which drops to about 3 kPa in the capillary. Steep extracellular diffusion gradient results in p_{O2} of less than 1 kPa inside the cells and relatively shallow diffusion gradient spans from the cell surface to the inner mitochondrial membrane. Adapted from (Gnaiger et al. 1998).*



Eventhough the p_{O2} limits the respiratory rate to some extent, COX still displays remarkable affinity for oxygen. This may reflect the fact, that mitochondria became associated with cells early during evolution, when atmospheric oxygen levels were probably 0.1 % of the present and so the COX possessing high oxygen affinity was preferred by selective pressure. This quality is well paid off in multicellular organisms, where oxygen diffusion gradients result in similarly low intracellular p_{O2} (Gnaiger et al. 2000). The apparent p_{50} is further decreased by the excess COX capacity, which provides the necessary diffusion channel area for oxygen to the COX binuclear centre, which is necessary to keep up with the electron flux through the respiratory chain (Gnaiger et al. 1998). The *in vivo* significance of oxygen limitation was confirmed in a study showing that flux control of COX in muscle depends on p_{O2} (Wiedemann and Kunz 1998).

Oxygen dependence of respiration was also shown in yeast where two isoforms of subunit V are expressed according to p_{O2} (Burke and Poyton 1998). The expression of the isoform Vb, which enhances the COX oxygen affinity is switched on at 0.05 kPa, which well corresponds with the published values of p_{50}. Only one form of its mammalian

counterpart, subunit IV, had been known only until recently, when its second isoform – COX IV-2 - was discovered (Huttemann et al. 2001). While its significant expression was originally detected solely in lung, it was difficult to postulate, whether it may have such functional significance as the yeast homolog. Last year, however, COX IV-2 expression was shown to increase dramatically in astrocytes and cerebellar granule cells upon entering hypoxia (Horvat et al. 2006). Moreover, this isoform switch led to abolition of the allosteric inhibition of COX by ATP. This mechanism sets an example how COX can actively adapt to low subtrate levels.

Oxygen is not the only gaseous ligand to be so passionately attracted to the COX binuclear centre. While for example carbon monoxide (CO) inhibition of the inhibitory was demonstrated so long ago by Warburg, this fact has been largely ignored. (reviewed in (Brunori et al. 2006). Reappraisal of this concept was brought up last year in a study from Moncada's lab, which shows that endogenously produced CO by heme oxygenase is sufficient to inhibit 70% of COX activity under hypoxic conditions (D'Amico et al. 2006). Much more attention and also physiological significance, however, is attributed to nitric oxide (NO) binding to cytochrome *c* oxidase. The inhibition of COX by NO has been convincigly documented in the 1990s, the mechanism of the inhibition is competitive and is based on the reversible NO binding to both R and E catalytic intermediates of the binuclear centre (Brunori et al. 2006). The physiological relevance of this inhibition was supported by the discovery of a mitochondrial isoform of nitric oxide synthase (mtNOS) (Bateson et al. 1996). During first years of the new millenium, we have been witnessing increasing efforts to constitute a specific role for NO binding to COX (for review see (Giulivi et al. 2006) Moreover, mtNOS was recently reported to interact directly with COX subunit Va (Giulivi et al. 2006). Owing to the proximity of the enzymes, the fact that mtNOS activity is triggered only after calcium binding, and the limited lifetime of NO, its binding to COX is under precise temporospatial regulation. By decreasing oxygen consumption of COX, NO release can effectively contribute to redistribution of intracellular oxygen, making it available for other enzymes with higher K_M for oxygen to be active. At the same time, upstream electron transporters in the respiratory chain become more reduced, which increases the chance of electron leak in the form of oxygen radicals (ROS) production. Specifically, the ROS production at complex III is thought to be critical for stabilization of HIF-1α - the major transcription factor for "hypoxic genes" (Guzy and Schumacker 2006). These reports indicate, how could be the COX function related to oxygen sensing. To make the situation even more complex, cytochrome *c* oxidase itself

seems to be capable of feeding electrons to nitrite (NO_2^-) to form NO (Castello et al. 2006). This reaction occurs only in hypoxia and was demonstrated both in yeast and rat liver mitochondria. Authors suggested its role in hypoxic signalling, possibly via a pathway that would involve protein tyrosine nitration.

One of the most thoroughly studied phenomena concerning hypoxia is the cardiac preconditioning. It is a paradoxical response in which one or more brief cycles of oxygen deprivation and reperfusion lead to protection against injury induced by prolonged oxygen deprivation. Unsurprisingly, COX seems to be involved in this phenomenon, too. Last year, COX subunit IV was shown to interact with the ε isoform of protein kinase C (PKCε) in neonatal cardiac myocytes during short exposure to hypoxia (Ogbi and Johnson 2006). PKCε is considered to be one of the major signalling proteins in preconditiong, the COXIV phosphorylation by this kinase led to 4-fold increase in COX activity and helped to maintain the COX content during prolonged hypoxia (Ogbi and Johnson 2006).

Obviously, our knowledge of mitochondrial physiology is still far from complete. For certain, in near future we will be encountered with more fascinating reports in this field, which will hopefully provide the basis for a comprehensive integative model of mitochondrial function in the eukaryotic cell.

1.2.4 Assembly of COX Holoenzyme

Assembly of the functional multisubunit enzyme cytochrome *c* oxidase requires coordinated interplay of both nuclear and mitochondrial genome. In addition to expression of 3 structural subunits encoded by mtDNA and 10 by ncDNA, numerous "assembly factors" are necessary for successful biogenesis of the complex. These assembly factors take part in multiple levels of COX biogenesis, namely mRNA processing, mRNA translation, synthesis and incorporation of prosthetic cofactors, insertion of proteins into mitochondrial membrane and their assembly, and quality control and proteolytic degradation of misfolded proteins.

Our knowledge of the components that participate in the biosynthesis of COX is mostly based on studies of yeast respiratory defective (*pet)* mutants. Screens of nuclear *pet* mutants revealed a group of more than 30 genes that selectively affect expression of COX (Tzagoloff and Dieckmann 1990). The function of most of these genes was identified during the last ten years (reviewed in (Fontanesi et al. 2006)). Proteins encoded by genes *COX10*, *COX15* and *YAH1* are involved in the synthesis of heme *a*. Genes *COX11*,

COX17, *SCO1* and *SCO2* code for proteins responsible for the incorporation of the copper ions into COX. Products of genes *SHY1*, *COX14*, *COX16*, *COX20*, *COX22*, *COX23*, *COX24*, *PET100* and *PET 117* participate in subunit assembly (Fig. 2.13).

Numerous homologs of these yeast genes have been found in human genome, which led to an optimistic suggestion that the COX biogenesis is evolutionary so much conserved among eukaryotes that the results from the studies in the yeast model of COX assembly provide generally relevant information. One of the original approaches used for identification of the human homologs is screening of an expressed sequence tags database (EST), which led to discovery of *COX15*, *COX11*, *SCO1* and *PET112* (Petruzzella et al. 1998). With the progress of the human genome research, the searching techniques have become easier and generally available and screening for homologs is usually performed concomitantly with the discovery of a *pet* gene. Indeed, the factors involved in maturation of prosthetic centres have demonstrated strong functional homology between yeast and mammals. The differences in mtDNA genes expression between these taxa, however, underscore the low sequence similarity between yeast ancillary proteins functioning in regulation of mtDNA expression and their mammalian counterparts, if they are found at all (Fontanesi et al. 2006). More detailed description of the role of assembly factors relevant to human diseases is included in the following section, which is devoted to COX pathologies.

Using two-dimensional electrophoresis, Nijtmans et al. identified four assembly intermediates (S1 – S4) that accumulate during COX assembly and most probably reflect the rate-limiting steps of the sequential incorporation of the 13 subunits of mammalian COX (Fig. 2.14) (Nijtmans et al. 1998). The intermediates could not be found in yeast mitochondria even in assembly deficient strains, probably due to efficient clearance by mitochondrial proteolytic enzymes. The assembly sequence is, however, thought to follow the scheme proposed in mammalian cells (Fontanesi et al. 2006). Since 1998, the scheme has been only slightly refined – subunit COXVa was shown to assemble at S2 level along with COXI and IV (Williams et al. 2004) and also that COXVa interacts with COXIV prior to association with COX I (Stiburek et al. 2005).

Figure 1.13 Schematic Depiction of Different Steps in COX Assembly. *Only some of the COX-specific genes are listed. Events catalyzed by functionally related gene products are boxed. Subunit 2 is depicted with its two transmembrane helices in the box showing its insertion and processing. The mature subunit 2 is shown as a shaded subunit in the partially assembled and mature COX. The two bars in the central subunit 1 represent two heme a groups. Adapted from (Barrientos et al. 2002).*

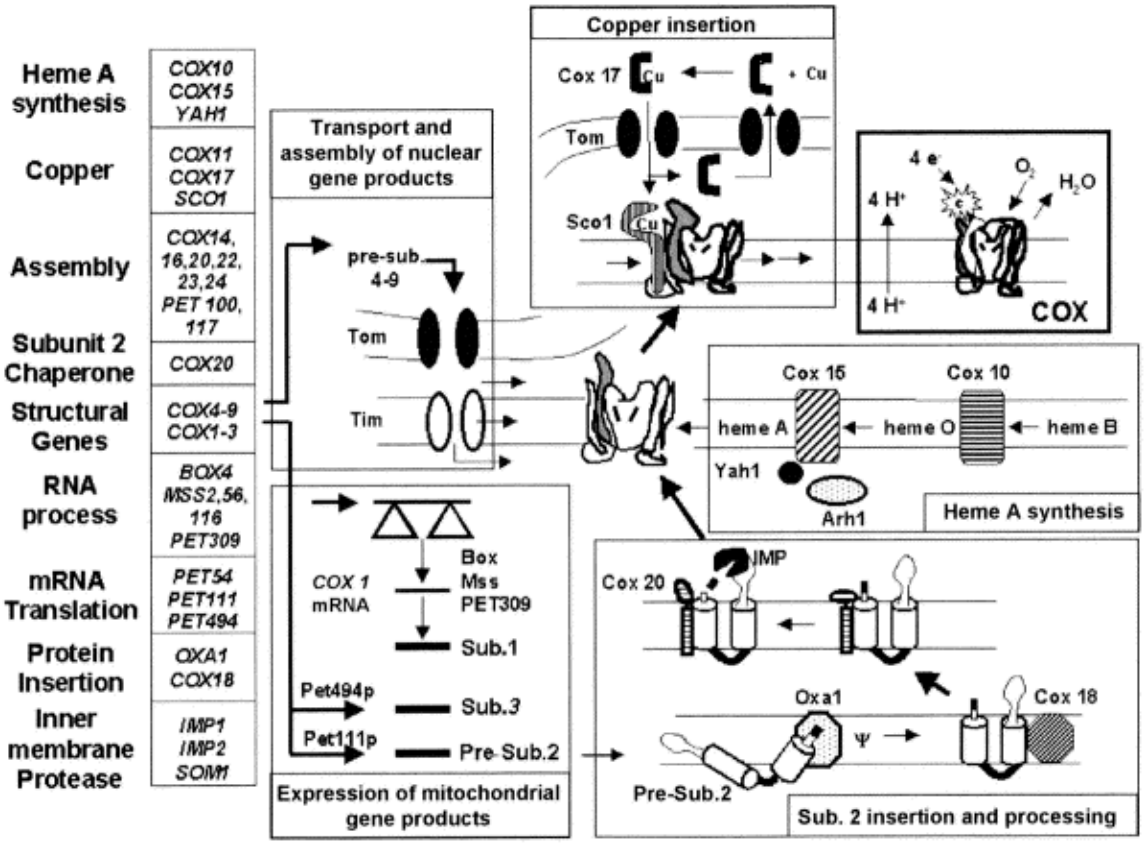
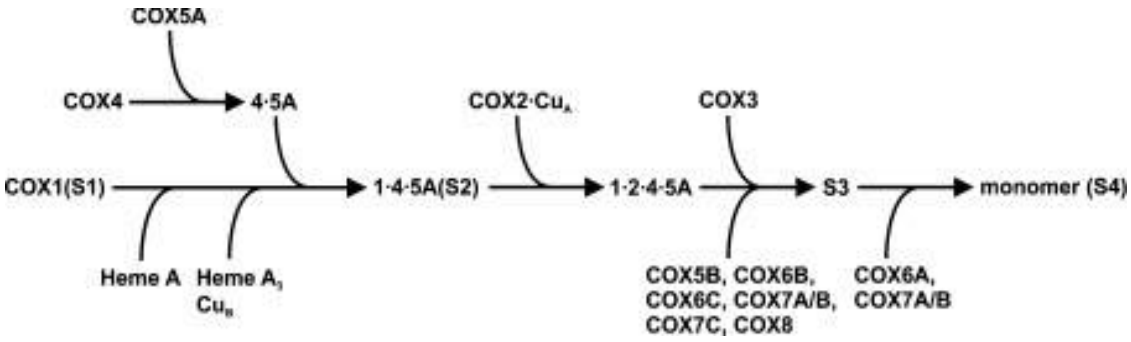


Figure 1.14 Model of the Assembly Pathway of Human COX. *The assembly intermediate S1 comprises just subunit COX I, S2 subcomplex is formed after incorporation of heterodimer of subunits IV and Va. Incorporation of subunit II starts the sequential assembly of ncDNA encoded subunits Vb, VIb, VIc, VIIb, VIIc, and VIII resulting in the S3 subcomplex. The assembly is finished with the addition of subunits VIa and VIIa, making possible the COX dimerization. Adapted from (Stiburek et al. 2005).*



1.2.5 COX assembly into respiratory supercomplexes

Classicaly, the organization of inner mitochondrial membrane was described by the fluid state model. According to this model, complexes I to IV would diffuse freely in the membrane and the electron transfer would be based on random collisions of the involved enzymes. Recent lines of evidence, however, refute the fluid state model and suggest the existence of higher structural organization of OXPHOS: (i) many isolation procedures for OXPHOS complexes result in the co-purification of more than one oxidoreductase, (ii) reconstitution experiments reveal highest electron transfer activities if different OXPHOS complexes are present at defined stoichiometries, (iii) point mutations within genes encoding subunits of one OXPHOS complex affect the stability of other OXPHOS complexes, (iv) flux control experiments indicate functional units larger than the single OXPHOS complexes, (v) supercomplexes including more than one type of OXPHOS complex are displayed by native gel electrophoresis procedures or sucrose gradient centrifugation, and (vi) analyses by single particle electron microscopy prove very defined associations of different OXPHOS complexes within respiratory supercomplexes (reviewed in (Boekema and Braun 2007). The supercomplexes of respiratory chain were found in mitochondria of generally all studied eukaroytes as well as in bacterial membranes using Blue-Native Electrophoresis, the relative abundance and stoichiometric composition of the supercomplexes is variable in different organisms. The most thoroughly characterized supercomplexes containing COX are the III_2IV_2 (two copies of complexes III and IV) and III_2IV_1 from yeast mitochondria, and the $I_1III_2IV_{1-4}$ supercomplex isolated

from bovine heart mitochondria (Boekema and Braun 2007). The structure of the yeast III_2IV_2 supercomplex was very recently obtained by electron microscopy at 15 Å resolution – two complex IV monomers are specifically attached to dimeric complex III. An electron density detected on the interface between the complexes was identified as cytochrome *c*. The authors proposed that complexes III and IV behave as a single functional unit in yeast (Heinemeyer et al. 2007). The bovine $I_1III_2IV_4$ supercomplexes are thought to further associate into „respiratory strings" of approximately 30 nm lenght.

1.3 COX Pathologies

Cytochrome *c* oxidase and complex I deficiencies are the most frequent cause of respiratory chain defects in humans. Patients affected with COX defects present heterogeneous clinical phenotypes. This clinical heterogeneity is probably due to a large number of genes influencing COX expression and possible tissue-specific differences existing in the cellular abundance of COX-related gene products (Barrientos et al. 2002).

1.3.1 Mutations in Structural COX Genes

Since 1995 when the first mutation in mtDNA gene coding for COX subunit was identified, four mutations have been reported in COX subunit I, three in subunit COX II and four in subunit COX III (Shoubridge 2001). The mutations resulted in isolated COX deficiency. Common feature in these patients was that the mutation load was found higher in COX negative than positive fibers. This fact provides support for the threshold theory stating that an enzymatic defect is present only after the mutation load reaches a threshold level.

A 5-bp microdeletion located at the 5' end of *COI* gene, leading to truncated protein, was identified in patient with motor neuron-like degeneration. Decreased levels of subunits I, II, III and IV were found, suggesting a defective COX assembly (Comi et al. 1998). A heteroplasmic G→A transition at position 6930 in the *COI* gene, changing a glycine to stop codon resulting in a loss of 170 amino acids, was detected in patient with multisystem mitochondrial disorder (Bruno et al. 1999). Another nonsense mutation in *COI* gene, the G5920→A, was identified in a patient suffering from a mitochondrial myopathy associated with recurrent myoglobinuria (Karadimas et al. 2000). A heteroplasmic missense mutation C6489→A changing the highly conserved leucine 196 in COX I to isoleucine was detected in patient with epilepsia partialis continua. Analysis of mitochondrial function in saponin-permeabilized muscle fibers containing approximately

90 % mutated DNA showed decreased respiration rate and increased sensitivity to cyanide due to a 2-fold increase of COX flux control on muscle fiber respiration (Varlamov et al. 2002).

A T7587→C transition changing the initiation codon of *COII* gene was identified in two members of family with encephalopathy. A decrease of COX activity was observed when the mutant load reached 55 %. The COX II subunit was not detectable in fibroblasts with more than 95 % mutated mtDNA (Clark et al. 1999). T→A transversion at position 7617 changing the methionine of the first transmembrane segment of COX II into lysine was found in patient with proximal myopathy and lactic acidosis. Severe reduction of the levels of subunits II, III, Vb, VIa, VIb and VI c was reported, while COX I showed milder reduction. However, a dramatic decrease of COX I associated heme *a3* suggested that a structural association of subunits I and II is necessary to stabilize the heme binding (Rahman et al. 1999).

A frame-shift truncation of COX II caused by 8042delAT mutation, resulting in the loss of cytochrome *c* binding domain, was reported in an infant with fatal course of apnea, bradycardia and severe lactic acidosis (Wong and Clayton 1986).

Patient with mitochondrial encephalomyopathy, lactic acidosis and stroke-like episodes (MELAS) harbored a missense mutation in *COIII* gene at position 9957. The T→C transition converted a highly conserved phenylalanine into leucine (Manfredi et al. 1995). A 15-bp microdeletion in *COIII* gene was identified in patient with severe isolated COX deficiency and recurrent myoglobinuria. Immunoblot analysis suggested deficient COX assembly or complex instability (Keightley et al. 1996). A G9952→A transition resulting in the loss of the last 13 amino acids of COX III subunit was detected in an adult patient suffering from proximal myopathy and episodes of encephalopathy. Muscle immunocytochemistry revealed a defect in either COX stability or assembly (Hanna et al. 1998). A frameshift mutation 9537C(ins) in *COIII* gene was reported in patient with severe lactic acidosis and brain lesions characteristic for Leigh syndrome. While the *COIII* transcription was not impaired, no COX III protein was detected in translation assay. Using two-dimensional electrophoresis, the accumulation of COX early assembly intermediates was observed. Several smaller subunits could not be detected in COX III lacking complexes (Tiranti et al. 2000).

Interestingely, not a single mutation in any of the ten nuclear-coded COX subunits has ever been reported, despite several thoroughful screenings of large patient groups

(Adams et al. 1997; Jaksch et al. 1998; Coenen et al. 2006). It can't be ruled out that some mutations may have passed unnoticed because since the discovery of mutations in COX assembly factors, most attention in the clinical sequencing services is paid to them, and the genes for nuclear-encoded COX subunits are not sequenced routinely. Or, perhaps, the putative mutations result in such severe COX defect that would lead to extremely early embryonic lethality, leaving no subject for examination.

1.3.2 tRNA Mutations, Large Scale Deletions of mtDNA

Mutations in tRNA coding genes or large scale deletions in mtDNA result in several clinically defined phenotypes and can usually affect the function of more respiratory enzymes including COX.

MELAS - mitochondrial epilepsy, lactic acidosis and stroke-like episodes is probably the most common of the maternally inherited mitochondrial encephalomyopathies (Hirano et al. 1992). This phenotype was found to be associated with 8 tRNA mutations (the $tRNA^{Leu}$ comprised 5 of those), a 16S rRNA mutation, and 7 mutations in structural genes (MITOMAP: A Human Mitochondrial Genome Database. http://www.mitomap.org). While in most mitochondrial disorders associated with tRNA mutations, COX histochemistry in muscle shows a mosaic pattern: some fibers have normal activity whereas others are COX-deficient, in MELAS, on the other hand, the mosaic pattern could still be seen, but there are few, if any, COX-negative fibers and the intensity of the histochemical reaction is much lower than what would be expected.

MERRF - myoclonus epilepsy with ragged red fibers is in most cases caused by the A→G transition at nt-8344 in the $tRNA^{Lys}$ gene. Three other mutations in this gene have also been reported to be associated with MERRF (MITOMAP: A Human Mitochondrial Genome Database. http://www.mitomap.org). Detailed genetic analyses of these mutations in transmitochondrial cybrids show that these mutated tRNAs cause both a severe reduction in mitochondrial protein synthesis and the production of a discrete set of aberrant translation products of unknown functional or pathogenic significance (Chomyn et al. 1991). An altered COX kinetics, with a decreased V_{max} for both the low-affinity and high-affinity phases and unchanged K_M, a 50 % decrease of ATP synthesis and 80 % decrease of

mitochondrial membrane potential at state 4 was reported in patient fibroblasts harboring 89 % of the 8344 mutation (Antonicka et al. 1999).

PEO - progressive external opthalmoplegia is characterized by weakness of the extraocular muscles and ptosis and it is often accompanied by limb weakness. PEO has been associated with different types of mtDNA mutations. About one half of patients harbor large-scale deletions of mtDNA, 14 different types of point mutations in tRNA genes were also described (MITOMAP: A Human Mitochondrial Genome Database. http://www.mitomap.org).

In contrast to muscle biopsies from MELAS patients, which contain mostly COX-positive ragged red fibers (RRF), the muscle in PEO patients contains numerous COX-negative RRF.

Kearn-Sayre Syndrome (KSS) - is a sporadic multisystem disorder due to a defect of OXPHOS associated with clonally expanded rearrangements of mitochondrial DNA, deletions and/or duplications. It is clinically characterized by progressive external opthalmoplegia, pigmentary degeneration of the retina and at least one of following symptoms: cardiac conduction defect, ataxia, increased cerebrospinal fluid protein content (Berenberg et al. 1977). Due to often overlapping phenotypes, it has been suggested that PEO and KSS describe different degrees of the same disease (Hammans 1994).

Pearson's syndrome (Pearson Marrow/Pancreas Syndrome) - represents the most serious disease caused by mtDNA deletions. It is a generally fatal, childhood disorder associated with pancytopenia, pancreatic fibrosis, and splenic atrophy. Individuals who do survive often progress to a KSS phenotype (McShane et al. 1991).

1.3.3 Mutations in ncDNA Genes Encoding COX Assembly Factors

Numerous cases of isolated COX deficiency of nuclear origin could not have been associated with any pathogenic mutations for a long time. In recent years, sequencing of candidate genes encoding homologs of yeast COX assembly factors identified etiologic mutations for COX deficiencies with Mendelian inheritance. Interestingly, no mutations in any of the ten ncDNA encoded subunits have been found and the mutations of assembly factors include six genes - *SURF1*, *SCO1*, *SCO2*, *COX 10*, *COX 15*, and *LRPPRC*.

Homozygous C→A transversion corresponding nucleotide 612 of ***COX10*** gene was identified in an infant presenting with ataxia, poor eye contact, severe muscle weakness,

hypotonia, ataxia, ptosis, pyramidal syndrome and status epilepticus. This transversion results in the change of an uncharged asparagine into a basic lysine residue (N204K) at a conserved position in the protein functioning as farnesyltransferase, which catalyzes the first step in the synthesis of heme *a* from protoheme *b*. Unlike to wild-type, the mutated protein could not complement the COX-deficient phenotype of yeast strain with disrupted *COX10* locus. The steady-state levels of COX subunits were only mildly decreased, with the exception of subunit II decreased to just 3 % of control value. The relatively unchanged level of subunit I suggested that the presence of hemes aa_3 is not necessary for COX I stabilization (Valnot et al. 2000). Two different combinations of compound heterozygous missense mutations in COX10 were identified in a patient with Leigh syndrome, and other patient with hypertrophic cardiomyopathy, respectively. COX10 mutations can, therefore, cause various clinical phenotype. Fibroblasts of both these patients had dramatically decreased levels of COX holoenzyme, the cells, however, lacked any COX assembly intermediates or accumulated free COXI. These data argue that the stabilization of COX subunit I within the inner mitochondrial membrane requires its relatively rapid association with COX subunit IV, an event that probably involves the insertion of at least a portion of the total heme A complement. It is not, however, clear why compromised heme A biosynthesis leads to a greater reduction of COX subunit II relative to COX subunits I and IV (Antonicka et al. 2003). The lack of assembly intermediates in COX10 deficiency was later confirmed in other studies (Coenen et al. 2004; Williams et al. 2004). An exceptionaly interesting finding came up from a study on a mouse fibroblast COX10 knock-out model. These knockout cells present with one of the most extreme COX deficiencies ever published – no COX activity could be measured, the holoenzyme was completely absent and most of the subunits were barely detectable. Moreover, the levels of complex I were severely affected as well, suggesting an unprecedented hypothesis that the presence of complex IV is necessary for complex I assembly. Normal levels of complex I were, however, restored after transfection even with a mutated form of COX10 that allows for assembly of at least residual amount of complex IV (Diaz et al. 2006).

In a genetic analysis of a large family with multiple cases of neonatal ketoacidotic comas and isolated COX deficiency, the mutations in ***SCO1*** gene were identified to be responsible for the disease. *SCO1* encodes a protein located in the inner mitochondrial membrane which is thought to transport Cu ions from COX17 to the Cu_A site in subunit COX II. All affected cases were compound heterozygous - one allele harbored a 2-bp deletion at position 363 resulting in a premature stop codon and highly unstable mRNA,

the C520→T mutation on the other allele changed a highly conserved proline at position 174 to leucine (Valnot et al. 2000). The protein product of the gene harboring P174→L was not able to rescue the *SCO1* yeast mutant (Paret et al. 2000).

Mutations in ***SCO2*** gene, encoding a *SCO1* protein homolog also involved in copper transport to COX, were first identified in three unrelated infants with fatal hypertrophic cardiomyopathy. They all harbored a G→A transition at nucleotide 1541 changing a conserved glutamate 140 to a lysine on one allele, and the C→T nonsense transition at position 1280 or C→T missense transition at position 1797 found on the other allele. In immunohistochemistry performed on the skeletal muscle, the steady-state levels of subunits I and II were severely reduced, subunit IV was just mildly decreased (Papadopoulou et al. 1999). In another study, three other compound heterozygous patients with the 1541 transition were identified, this time in combination with C→T transition at position 1643 resulting in arginine 171 substitution by tryptophane. In fibroblasts from these patients, the COX activity was reduced to 50 % and normal steady-state level of COX subunits was observed (Jaksch et al. 2000). The three patients homozygous for the G1541→A transition had delayed onset of the disease, however, a Leigh syndrome-like phenotype characterized by necrotic lesions of basal ganglia was present in addition to cardiomyopathy. A 10-bp duplication of nucleotides 1302-1311 combined with the common G1541→A missense mutation resulted in fatal cardioencephalomyoathy with prominent spinal cord involvement (Salviati et al. 2002). Interestingly, addition of $CuCl_2$ to the growth medium of fibroblasts, myoblasts and myotubes (Salviati et al. 2002) or copper-histidine to myoblasts (Jaksch et al. 2001) harboring *SCO2* mutations, was able to rescue the COX deficient phenotype.

In addition to their role in delivery of Cu into COX, both Sco1 and 2 are involved in the regulation of copper homeostasis. Mutations in both genes result in a cellular copper deficiency that is both tissue and allele specific. These results suggest a mitochondrial pathway for the regulation of cellular copper content that involves signalling through Sco proteins, perhaps by their thiol redox or metal-binding state (Leary et al. 2007).

Mutations in the ***COX15*** gene, a missense mutation changing arginine 217 into tryptophane on one allele and splicing-site mutation in the intron 3 on the other allele, were identified in patient with early-onset fatal hypertrophic cardiomyopathy. The *COX 15* encodes a protein from the mitochondrial 3-component heme hydroxylase catalyzing the second step of heme *a* biosynthesis. Decreased level of heme *a* and accumulation of the

heme O intermediate were observed in the patient cells. The decreased levels of COX holoenzyme suggested impaired assembly or decreased stability of COX due to the heme absence (Antonicka et al. 2003).

Table 1 – Mutations Causing Isolated Defects of Cytochrome c Oxidase. *The table summarizes all mutated genes causing isolated COX deficiencies that have been identified to date. Nuclear genes are in yellow boxes, genes located in mtDNA are in blue. Detailed information can be found in text.*

GENE	***FUCTION***	***INHERITANCE***	***CLINICAL PHENOTYPES***
COX1	Structural subunit of the enzyme	Maternal	Multisystem mitochondrial disorder, motor neuron degeneration, myopathy with recurrent myoglobinuria
COX2	Structural subunit of the enzyme	Maternal	Encephalopathy, proximal myopathy and lactic acidosis
COX3	Structural subunit of the enzyme	Maternal	MELAS, Leigh-like syndrome, encephalomyopathy
SURF1	COX assembly – progress from S2 to S3 assembly intermediate	Autosomal recessive	Leigh syndrome
SCO1	Copper delivery to the enzyme	Autosomal recessive	Neonatal hepatic failure and encephalopathy
SCO2	Copper delivery to the enzyme	Autosomal recessive	Neonatal cardioencephalomyopathy
COX10	Heme *a* farnesyl-transferase	Autosomal recessive	Neonatal tubulopathy and encephalopathy, Leigh syndrome, cardiomyopathy
COX15	Heme *a* synthesis	Autosomal recessive	Early-onset hypertrophic cardiomyopathy, Leigh syndrome
LRPPRC	Transcriptional regulation of genes encoding mitochondrial proteins	Autosomal recessive	Leigh Syndrome, French Canadian type

The Leigh Syndrome (LS) Associated with Isolated COX Deficiency (LS^{COX}) - represents the largest group of mitochondrial disorders caused by mutation in nuclear genome. LS (i.e. subacute necrotizing encephalomyopathy) is a progressive neurodegenerative disease. The severe symptoms usually have onset in less than a year after birth and are characterized by general psychomotor retardation and bilaterally symmetrical lesions in the basal ganglia region. An increased level of lactate in both blood and cerebrospinal fluid is observed. The disease is fatal in vast majority of cases, the patients usually die before five years of age (Rahman et al. 1996). Early studies using cybrid construction from LS^{COX} and control cells showed that the defect is of nuclear origin (Tiranti et al. 1995). The analysis of transmitochondrial cybrids constructed from LS^{COX} cells and complementation studies on fibroblasts from different patients revealed that most of the patients studied belonged into one complementation group, suggesting that only one mutation locus was responsible for the disease (Munaro et al. 1997). Nuclear coded COX subunits were considered hot candidates for harboring the pathogenic mutation but complete sequential analysis of all 10 of these COX subunits in one of such patients didn't reveal any mutations (Adams et al. 1997). In 1998, mutations in the ***SURF1*** gene were identified in LS^{COX} patients by two groups independently (Tiranti et al. 1998; Zhu et al. 1998). *SURF1* encodes an inner mitochondrial membrane protein homologous to yeast Shy1 protein which was previously shown to be necessary for proper transfer of electrons between respiratory complexes III and IV (Mashkevich et al. 1997). Recent studies on Shy1 revealed that, in cooperation with Mss51p and Cox14p, it helps regulate the translational activation of the COXI transcript according to the rate of assembly of the newly synthesized COXI into the holoenzyme (Barrientos et al. 2004). The absence of mammalian homologs of the Mss51p and the Cox14p, and the differences in translational regulation between yeast and mammals, preluded the extreme challenge of finding the role of mammalian Surf1p, which is described in the following section.

Surf1p Expression and Function

The *SURF1* mRNA levels were found the same in all studied tissues with the exception of brain, where they were lower. The gene is expressed in substoichiometrical amount relative to COX subunits (Yao and Shoubridge 1999). No *SURF1* mRNA (Tiranti et al. 1999) or Surf1 protein (Surf1p) could be detected in patients with mutations resulting in premature stop codon (Tiranti et al. 1999; Yao and Shoubridge 1999). In a later study,

the *SURF1* mRNA was detected at normal level in patients harboring missense point mutations, however, the Surf1 protein wasn't found either (Poyau et al. 2000).

The secondary structure prediction of Surf1p revealed the presence of two transmembrane domains connected by central loop localized in the intermembrane space. To asses which parts of the proteins were necessary for its function, the cDNA constructs encoding several truncated versions of the protein were used for transfection of LS^{COX} fibroblasts. With the exception of the variant lacking only the five C-terminal amino acids, none of these constructs could complement the COX deficiency (Tiranti et al. 1999; Yao and Shoubridge 1999). Despite the high conservation of the Surf1p in evolution (homologs were found in all groups of eukaryotes and even some prokaryotes) (Poyau et al. 1999), the whole length *SURF1* cDNA construct could not complement the pet phenotype of Δshy1 yeast strain (Tiranti et al. 1999), suggesting that a change of its function indeed in the evolution of multicellular organisms.

The potential role of Surf1p was studied at all levels of COX expression. No changes could be found in the amount of both ncDNA or mtDNA encoded COX transcripts or the efficiency of their translation, suggesting that Surf1p functions at later stage of COX expression. Using two-dimensional BN-PAGE/SDS-PAGE electrophoresis, Tiranti et al. observed accumulation of early assembly intermediates of COX in LS^{COX} patients. The assembly was blocked at the level of S2 intermediate suggesting that Surf1p facilitates the incorporation of one or more of subunits COX II, COX III and majority of ncDNA encoded subunits (Tiranti et al. 1999). About 20 % of control amount of the COX holoenzyme was assembled despite the absence of Surf1p suggesting that there was a certain level of redundancy in the assembly process. Impaired COX assembly in LS^{COX} patients was confirmed in another study (Coenen et al. 1999). Also, using 2D electrophoresis, the assembly defect was confirmed in *Δshy1* yeast. In a wild-type strain, Shy1p was found in a multimeric 250 kDa complex together with COXII subunit suggesting their interaction (Nijtmans et al. 2001).

SURF1 Mutations and Changes in COX Structure and Function

More than 30 *SURF1* mutations have been reported since their discovery as the primary cause of LS^{COX}. Most of them are reviewed in (Pequignot et al. 2001), several more mutations were published since then - a splicing site mutation causing the loss of exon 8 (Williams et al. 2001), three new mutations were found in lymphoblastoid cells from Japanese Leigh syndrome patients (Ogawa et al. 2002). A novel C574→T transition

changing conserved arginine 192 into tryptophane was reported from screening of Czech patients (Capkova et al. 2002). Genetic screening of 65 Chinese patients with Leigh syndrome revealed SURF1 mutations in eight of them, with major prevalence of the G604→C transition changing aspartate 202 into histidine (Yang et al. 2006). Another two novel mutations were identified in patients with distinct clinical phenotype – prominent renal symptons and muscle ragger red fibers (Tay et al. 2005). More mutations will most probably be discovered in near future thanks to routine sequencing of the *SURF1* mutations in numerous clinical laboratories all over the world. The enormous variety and also the frequency of *SURF1* mutations are, as far as I am aware, unrivaled by any other nuclear gene coding for OXPHOS associated proteins. The reason might be that in vertebrates, the *SURF1* gene is located within an unusually surfeit (wherefrom it takes its name) cluster of six housekeeping genes of unrelated function. This DNA region virtually lacks any heterochromatin and bears some peculiar features such as bidirectional promotor for *SURF1* and *SURF2* genes (Duhig et al. 1998). I am tempted to hypothesize that such location may predispose the *SURF1* to become a mutational hotspot.

Severely decreased COX activity was reported in all LS^{COX} patients both before and after the *SURF1* mutations discovery. The spectrophotometrically measured activities ranged between 5 - 40 % of control values (Glerum et al. 1987; Lombes et al. 1991; Zimmermann and Kadenbach 1992; Possekel et al. 1996; Tiranti et al. 1998; Zhu et al. 1998; von Kleist-Retzow et al. 1999; Sue et al. 2000) Kinetic analyses revealed unchanged K_M and dramatic decrease of V_{max} of COX (Lombes et al. 1991; Zimmermann and Kadenbach 1992). The COX complexes in LS^{COX} fibroblasts were found to be more sensitive to lauryl maltoside and increased temperature (Possekel et al. 1996).

The COX assembly defect in LS^{COX} cells is further characterized by general decrease of the steady-state levels of COX subunits (Hayasaka et al. 1989; Zimmermann and Kadenbach 1992; von Kleist-Retzow et al. 1999; Yao and Shoubridge 1999; Poyau et al. 2000; Sue et al. 2000). This fact most probably reflects decreased stability and rapid degradation of unassembled subunits. No major differences between the steady-state levels of mtDNA- and ncDNA-encoded COX subunits were reported for LS^{COX} cells.

Abnormal calcium homeostasis in LS^{COX} fibroblasts was reported and possible connection with low mitochondrial membrane potential was established (Wasniewska et al. 2001). In this study, defect in opening of the calcium store operated channels (SOC) in the plasma membrane was observed in LS^{COX} fibroblasts. In controls cells, the SOC opening is impaired after treatment with an uncoupler of mitochondrial membrane, suggesting that

mitochondrial membrane potential is required for proper control of SOC opening. There were no changes in SOC opening in LS^{COX} fibroblasts regardless of the uncoupler treatment. This most probably reflects low steady state level of membrane potential in these cells.

A possible role of Surf1p in calcium homoeostasis was also suggested in a study on a model of mouse *SURF1* knock-out by group of professor Zeviani from Milano, Italy (Dell'agnello et al. 2007). The absence of Surf1p in mice results in milder COX deficiency than in humans. Therefore, the neurological phenotype is not recapitulated in this model. Paradoxically indeed, the *SURF1* knock-out animals displayed significant resistance to kainate-induced neurodegeneration compared to their control littermates. It seems that *SURF1*-lacking neurons do not face such massive calcium efflux that would lead to excitoxicity and cell death. The authors claim that the change in calcium handling is not due to changes in mitochondrial membrane potential (Dell'agnello et al. 2007). Further studies are necessary to clarify this truly unexpected finding.

Nearly ten years after the discovery that LS^{COX} is caused by *SURF1* mutations, its role still remains enigmatic. Solving this question will definitely require divine enlightenment or a great portion of luck.

LRPPRC (leucine-rich pentatricopeptide repeat-containing protein) is the last nuclear gene that was found to cause a selective COX deficiency, namely the French-Canadian type of Leigh syndrome (LSFC). It is relatively common in the Sagueney-Lac St-Jean region of Quebec – 1 in every 2000 births is affected. This subtype of Leigh syndrome is characterized by a less severe neurological picture, but also by additional involvement of liver pathology (Mootha et al. 2003). The story behind the discovery of the LRPPRC gene is very educating in terms of both how we should approach any future hunt for disease genes and also what kind of results should we expect to get. The access to numerous pedigrees allowed for mapping the disease locus to chromosome region 2p16-21 of about 5 centimorgans, which was found to contain 15 coding genes. Using an impressive combination of bioinformatics and mitochondrial proteomics, Mootha et al. resolved that one of the 15 genes, the LRPPRC, is the only to code for a mitochondrial protein. Indeed, when this gene was sequenced in LSFC patients, a homozygous base change C1119→T was found in all cases. The mutation changes the conserved alanine at position 354 into valine and was not present in 175 unrelated controls. The LRPPRC was previously reported to bind polyadenylated mRNA. Furthermore, it bears a weak homology to yeast Pet309, a mitochondrial protein needed for proper splicing and

translational initiation of mtDNA-encoded COX subunits. The authors therefore suggested that human LRPPRC should be involved in the processing of mtDNA transcripts, which is a mechanism that has not been previously implicated in mitochondrial pathophysiology (Mootha et al. 2003).

This suggestion was later supported by a following study, where expression of mtDNA-encoded COX subunits was analyzed. Transcripts for both COXI and COXIII were decreased in fibroblasts of LSFC patients compared to controls. The reduction was due to decreased rate of translation or lower mRNA stability, as evidenced by [^{35}S] methionine labeling of mitochondrial translation products. Interestingly, LRPPRC was also retrieved in nuclear fraction, suggesting a dual location of the protein (Xu et al. 2004).

The nuclear track was, however, eagerly followed in the most recent work concerning the LRPPRC, which was started by a rather unexpected encounter in the laboratory of B. Spiegelmann, renowned for his achievements in the field of transcriptional coactivators. In their attempt to discover binding partners of PGC-1α, a major transcriptional coactivator of genes involved in energy metabolism, LRPPRC was one of the most prominent proteins to be coimmunoprecipitated (Cooper et al. 2006). The LRPPRC was shown to confer the specifity of PGC-1α function towards coactivating genes involved in gluconeogenesis and mitochondrial electron transport. The inability of mutated LRPPRC to regulate transcription in this manner may, at least in part, explain the clinical phenotype of LSFC patients characterized by lactate acidosis, neurodegeneration and liver pathology (Cooper et al. 2006).

At this point I would again like to mention the LRPPRC homology to yeast Pet309, which belongs to the group of proteins regulating the processing and translational activation of mtDNA transcripts by binding to their untranslated regions. In yeast, more than ten proteins are known to regulate translation of COX subunits alone (Fontanesi et al. 2006). During the course of evolution from unicellular organisms such as yeast to mammals, untranslated regions along with introns were lost from the mtDNA sequence. Proteins functionally associated with them have therefore been left “unemployed”. As exemplified by the LRPPRC, these orphaned proteins may have acquired novel functions. In this case, we are perhaps witnessing the evolution at work, as the protein has a dual localization in mitochondria and nucleus, and it is unclear which of these is more crucial for the pathology of LSFC. Likely, a human homolog of another yeast gene coding for a mitochondrial mRNA binding protein will be recognized as a COX disease gene in near

future. As the LRPPRC teaches us, we should expect only weak sequence similarity, multiple cellular location and very likely a novel function.

1.3.4 COX Involvement in Common Disease Phenotypes

Mutations in mtDNA genes have been analyzed by an increasing number of laboratories in order to investigate their potential role as an active marker of tumorigenesis in various types of cancer (Gallardo et al. 2006). While many studies support an active role of mtDNA in tumorigenesis, there are also many caveats that make the implication of mtDNA variants a debatable issue. It is still unclear whether the somatic mtDNA mutations found in tumors are a consequence of the tumorigenic process or a predisposing condition leading towards it. Numerous investigations associating mtDNA variants with cancer may also be based on sequencing results that failed to recognize polymorphisms belonging to mtDNA haplogroups (Salas et al. 2005). In a recent study taking all possible pitfalls into account, however, a COXI mutation G6276>A was identified in several cancer cell lines. These cell lines originated from different cancer types and carried various mtDNA haplotypes. The COXI G6276>A mutation caused a significant decrease in COX activity and mitochondrial respiration. The mutations was found in tumors but also in normal tissues of cancer patients suggesting that it is germline transmitted and plays a role in predisposing to tumorigenesis (Gallardo et al. 2006).

mtDNA variants and mitochondrial dysfunction are in the spotlight due to their potential role in common pathologies such as type2 diabetes or Alzheimer's disease (AD). The putative involvement of mitochondria in pathogenesis of diabetes and metabolic syndrome is attracting increasing attention, breakthroughs can be hopefully expected in near future (reviewed in (Taylor and Turnbull 2005). The relation between mitochondrial dysfunction and Alzheimer's disease is characterized in more detail. Amyloid beta peptide, the Alzheimer's hallmark, has been shown to accumulate inside mitochondria, where it contributes to decline of ATP production and increased ROS production, in part by associating with cytochrome *c* oxidase (reviewed in (Ohta and Ohsawa 2006). AD pathogenesis may be even more intimately intertwined with COX through MIRTD – a protein translated from an alternative transcript of dihydrolipoamide succinyltransferase (DLST) gene. The levels of the alternative transcript are decreased in brains of AD patients and its absence results in specific defect of COX assembly. The precise role of this protein in COX biogenesis is unfortunately unknown so far.

2. AIMS OF THE THESIS

The present thesis was primarily directed to characterize functional consequences of cytochrome *c* oxidase deficiencies caused by defects of enzyme assembly. Thanks to author's involvement in other projects, the scope of the thesis was broadened by findings that demonstrate the role of cytochrome *c* oxidase in more general pathological states such as cancer or diabetes. The aims of the thesis are:

A) To characterize the structural and functional alterations of cytochrome *c* oxidase in mitochondrial encephalomyopathies caused by defects of enzyme assembly due to mutations in *SURF1* and other genes coding for assembly factors.

B) To determine the COX affinity for oxygen in fibroblasts harbouring *SURF1* mutations – an attempt to establish a novel relation between COX oxygen affinity and human disease.

C) To uncover the putative involvement of mitochondria and specifically cytochrome *c* oxidase in disorders of energy metabolism such as obesity, diabetes and metabolic syndrome; and in tumorigenesis of clear cell renal carcinoma (CCRC) – a hereditary type of cancer caused by deficiency of VHL protein crucial for cellular oxygen sensing

3. SUMMARY OF THE RESULTS

The thesis consists of eight publications. Five publications deal with COX deficiencies in mitochondrial diseases. Two publications are concerned with the role of COX in tumorigenesis of CCRC, and the last publication presents our findings on the involvement of COX in diabetes.

The first four publications deal with structural and functional properties of COX in defects on the basis of mutations in nuclear- encoded COX assembly factors, mainly SURF1.

1. **Functional alteration of cytochrome *c* oxidase by *SURF1* mutations in Leigh syndrome,** *Pecina P., Čapková M., Chowdhury S. K., Drahota Z., Dubot A., Vojtíšková A., Hansíková H., Houšťková H., Zeman J., Godinot C., and Houštěk J.; Biochimica and Biophysica Acta, 1639 (1) : 53 – 63, 2003.*

This publication represents our first encounter with fibroblasts from patients harbouring mutations in *SURF1* gene, which encodes a specific assembly factor of COX. The paper thoroughly documents the experimental basis for our hypothesis that, in addition to severely decreased content of COX holoenzyme, the patient fibroblasts accumulate incomplete forms of the enzyme that retain their electron-transport capacity while their proton pumping ability is severely impaired. We proposed a possible pathogenic mechanism where the inability to maintain mitochondrial membrane potential results in severe defect of energy provision. The hypotheses originated in this publication are currently examined in our laboratory.

2. **Decreased affinity for oxygen of cytochrome *c* oxidase in Leigh syndrome caused by *SURF1* mutations ,** *Pecina P., Gnaiger E., Zeman J., Pronicka E., and Houštěk J.; American Journal of Cell Physiology – Cell Physiology, 278 (5) : C1384 – 1388, 2004.*

Our characterization of the functional consequences of the *SURF1* based COX deficiency was continued in this present study elaborated in collaboration with Erich Gnaiger from Innsbruck University. Our work was aiming to answer the question, whether the incomplete COX assemblies present in patient fibroblasts

have altered affinity for oxygen. Indeed, using high-resolution respirometry, we were able to demonstrate decreased COX affinity for oxygen in patient cells. We hypothesize that the depressed oxygen affinity may *in vivo* lead to respiratory limitation, which might represent an additional pathogenic mechanism of Leigh syndrome. Taking into consideration that central nervous system operates near maximum of its oxidative capacity and that some intercapillary regions of brain have extremely low partial pressure of oxygen, the decrease of COX affinity for oxygen may be responsible for specific degeneration of basal ganglia in Leigh syndrome. To the best of our knowledge, this study was the first to implicate an involvement of COX oxygen affinity in human pathology. Personally, I consider the findings communicated in this publication to be the most interesting and important achievements of my thesis.

3. **Genetic defects of cytochrome *c* oxidase assembly,** *Pecina P., Houšťková H., Hansíková H., Zeman J., and Houštěk J.; Physiological Research, 53 Suppl. 1 : S213 – 223, 2004.*

This review summarizes recent developments in elucidation of the molecular basis of mitochondrial diseases due to specific defects of COX with special focus on *SURF1*, including our achievements that have been published in the two papers discussed above. We devised a model of three distinct COX forms present in mitochondria of LS^{COX} cells – i) reduced content of normally-functioning COX holoenzyme, ii) accumulated COX assembly intermediate lacking several nuclear-encoded subunits with upregulated electron-transport activity and impaired proton-translocating activity, iii) inactive early COX assembly intermediates. The publication further presents "epidemiological" data on COX deficiencies in our country - among more than 60 cases of different types of COX deficiency that we have found in last 8 years in patients from the Czech and Slovak Republic, selective COX defects were present in 29 cases, 9 of which were *SURF1* mutations and **7** the *SCO2* mutations. Some of *SURF1* mutations possibly exert Slavonic prevalence, for example the 845-846delCT mutation is present in half of the cases found in Czech Republic and Poland.

4. **Tissue-specific cytochrome *c* oxidase assembly defects due to mutations in *SCO2* and *SURF1*,** *Stibůrek L., Veselá K., Hansíková H., Pecina P., Tesařová M., Černá L., Houštěk J., and Zeman J.; Biochemical Journal, 392 Part3 : 625 – 632, 2005.*

The analysis of post-mortem tissue samples from patients harbouring either *SCO2* or *SURF1* mutations revealed striking differences in the extent of assembly defect between various tissues. Mutations in *SCO2*, for example, result in severe COX deficiency in heart whereas liver COX is completely unaffected. While these variations could not be correlated with the residual content of mutated *SCO2* protein, the results of this study possibly reflect the existence of tissue-specific functional differences of assembly factors, and/or tissue-specific pathways of COX biogenesis.

The following publication is a study dealing with COX deficiency caused by unique mutation in mtDNA affecting transcription and translation of ATP6/COX3 gene

5. **Diminished synthesis of subunit a (ATP6) and altered function of ATP synthase and cytochrome *c* oxidase due to the 2 bp microdeletion of TA at positions 9205 and 9206 ,** *Ješina P., Tesařová M., Fornůsková D., Vojtíšková A., Pecina P., Kaplanová V., Hansíková H., Zeman J., and Houštěk J.; Biochemical Journal, 383 Part3 : 561 – 571, 2004.*

In this study, we aimed to characterize the molecular basis for dysfunction of mitochondrial ATPase due to 2 bp deletion in stop codon of mitochondrial ATP6 gene. Eventhough our efforts were mainly occupied with the ATP synthase, the rare microdeletion impairs cleavage of polycistronic transcript between RNAs for ATP6 and COX3, and therefore synthesis of COX subunit III is also affected. Deficiency of this subunit leads to defect of enzyme assembly, which is accompanied by accumulation of assembly intermediates, similarly as in COX deficiency caused by *SURF1* mutations, or in a patient with a frameshift mutation in COXIII (Tiranti et al. 2000). Considering our previous findings that such incomplete assemblies have

diminished proton pumping ability, the fibroblasts from this patient represent a unique model where both constitution of membrane potential, but also its discharge were affected, due to defects in COX or ATPase, respectively. While the measurements of mitochondrial membrane potential indicate that ATPase defect has a dominant effect on this bioenergetic parameter, both enzyme deficiencies underlie the decrease of mitochondrial energy provision that leads to the severe multisystemic disorder of the patient.

The role of mitochondrial dysfunction in tumorigenesis of clear cell renal carcinoma is described in the two following publications.

6. **A new role for the von Hippel-Lindau tumor suppressor protein: stimulation of mitochondrial oxidative phosphorylation complex biogenesis,** *Hervouet E., Demont J., Pecina P., , Vojtíšková A., Houštěk J., Simmonet H., and Godinot C.; Carcinogenesis, 26 (3) : 531 – 539, 2005.*

This is a first publication resulting from our long-lasting collaboration with the group of Catherine Godinot from Lyon University focused on study of clear cell renal carcinoma (CCRC). This particular type of cancer is caused by inherited mutations of *VHL* gene, which encodes a protein responsible for downregulation of hypoxia inducible factor (HIF-1α) under normoxia. pVHL deficient cells are therefore adapted to "eternal hypoxia", in this sense the CCRC perhaps represents the very essence of Warburg effect. Our results indicate that downregulation of mitochondrial proteins is one of the most crucial steps in CCRC tumorigenesis. The mitochondrial downregulation is most evident in severe decrease of COX content.

7. **Inhibition of cytochrome *c* oxidase subunit 4 precursor processing by the hypoxia mimic cobalt chloride,** *Hervouet E., Pecina P., Demont J., , Vojtíšková A., Simmonet H., Houštěk J., and Godinot C.; Biochemical Biophysical Research Communications, 344 (4) : 1086 – 1093, 2006.*

Our tackling with CCRC was followed by this study, which primarily aimed to uncover the mechanism of mitochondrial downregulation in this cancer type. Tumor cell transfected with wild type VHL were treated with cobalt chloride, frequently used as a hypoxia mimic in cell culture, due to its ability to inhibit enzymes responsible for hydroxylating HIF -1α in order to destine it for

degradation. Cobalt treatment, unfortunately, had quite pleiotropic effect on CCRC cells. However, we considered one of the side effects very intriguing – namely the impairment of COX subunit IV precursor processing. This was due to cobalt inhibition of mitochondrial intermediate peptidase (MIP), which is used only for several mitochondrial proteins. Our unexpected result indicates that COX IV processing requires special care. This event may likely represent the rate-limiting step of S2 intermediate formation during COX assembly.

Our work on CCRC is still running, we are trying to identify the mechanism of mitochondrial enzymes downregulation. The preliminary results indicate that increased production of reactive oxygen species may be responsible for the observed changes in mitochondria of CCRC cells.

The last publication of the thesis presents an evidence for mitochondrial involvement in obesity

8. **Polyunsaturated fatty acids of marine origin upregulate mitochondrial biogenesis and induce beta-oxidation in white fat,** *Flachs P., Horáková O., Brauner P., Rossmeisl M., Pecina P., Franssen-van Hal N., Růžičková J., Šponarová J., Drahota Z., Vlček C., Keijer J., Houštěk J., and Kopecký J.; Diabetologia, 48 (11) : 2365 – 2375, 2005.*

We were invited to participate in this study aiming to characterize the mechanism, how dietary n-3 polyunsaturated fatty acids (PUFA) contribute to white adipose tissue reduction. Mice were fed with high-fat diet, one group received increased proportion of eicosapentaenoic (EPA) and docosahexaenoic (DHA) fatty acids. The EPA + DHA uptake resulted in upregulation of mitochondrial proteins in white adipose tissue that was associated with increased rate of beta-oxidation. The increased expression of mitochondrial enzymes was due to 3-fold upregulation of PGC1 and NRF1, which are master transcriptional activators of mitochondrial genes. COX seemed to be upregulated to similar extent as complex II, and therefore had probably no specific role in the observed phenomenon.

4. CONCLUSIONS

The results of the thesis clearly demonstrate that mutations of *SURF1* gene result in profound alterations of cytochrome *c* oxidase structure and function, which underlie a pronounced defect of mitochondrial energetics. Deficiency of COX seems to be one of the hallmarks of frequent pathologies such as cancer or metabolic syndrome.

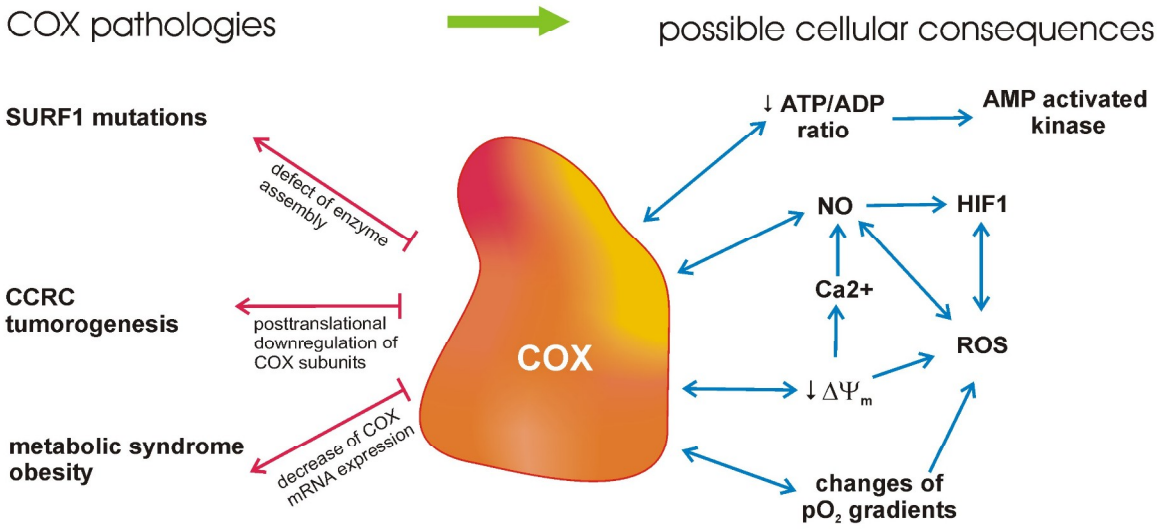
A) Accumulation of incomplete forms of cytochrome *c* oxidase was found in fibroblasts from patients with *SURF1* mutations. These subassemblies retain their electron-transport capacity, while their proton-pumping ability is severely impaired. These functional alterations result not only in serious decrease of energy provision, but underlie even more complex disorder of cellular physiology that ultimately manifests as Leigh Syndrome.

B) The affinity for oxygen of COX assembly intermediates is decreased. This finding implicates that COX deficiency in Leigh syndrome results in even more pronounced impairment of mitochondrial energy provision in tissues with limited oxygen supply.

C) Decrease of cytochrome *c* oxidase content and activity was shown to be involved in metabolic alterations that lead to tumorigenesis of clear cell renal carcinoma caused by inherited mutations of VHL protein. General disorders of energy metabolism such as obesity seem to be associated with cytochrome *c* oxidase decrease. In such cases, however, this phenomenon is not specific and probably reflects downregulation of mitochondrial function in general.

To conclude, I would like to remark that alterations of cytochrome *c* oxidase are most likely involved in vast majority of defects of energy metabolism, which underscores the COX position as one of the key regulatory enzyme of the cell. Extrapolating our findings on cytochrome *c* oxidase function towards a comprehensive model of subsequent changes at the level of cell physiology remains a challenge for future.

Figure 4.1 The Putative Relation of Cytochrome c Oxidase Pathologies to Cell Physiology. *The scheme depicts three different mechanisms of COX deficiency that cause the various pathological conditions as described in the present thesis (left). Crucial aspects of cell physiology that might be influenced by the enzyme dysfuntion are shown on the right.*



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