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Genetic Defects of Cytochrome *c* Oxidase Assembly

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Summary

Cytochrome *c* oxidase (COX), the terminal enzyme of the mitochondrial respiratory chain, is one of the key functional and regulatory sites of the mammalian energy metabolism. Owing to the importance of the enzyme, pathogenetic mutations affecting COX frequently result in severe, often fatal metabolic disorders. No satisfactory therapy is currently available so that the treatment remains largely symptomatic and does not improve the course of the disease. While only few genetic defects of COX are caused by mutations in mitochondrial genome, during the last five years a large number of pathogenetic mutations in nuclear genes have been discovered. All these mutations are located in genes encoding COX-specific assembly proteins including SURF1, SCO1, SCO2, COX10, and COX15. Despite the identification of increasing number of mutations, their precise etiopathogenetic mechanisms, which are necessary for the development of future therapeutic protocols, still remain to be elucidated. This review summarizes recent developments, including our efforts in elucidation of the molecular basis of human mitochondrial diseases due to specific defects of COX with special focus on SURF1 assembly protein.

Key words

Cytochrome *c* oxidase • SURF1 • Leigh syndrome • Mitochondrial disorders

Mitochondrial diseases

Mammalian organisms fully depend on ATP produced by oxidative phosphorylation (OXPHOS), a major metabolic pathway localized in the inner mitochondrial membrane. Changes in the cellular capacity of energy provision rely on biosynthesis of OXPHOS enzyme complexes (Tzagoloff and Myers 1986) – large heterooligomers composed of subunits that are encoded by both the nuclear DNA (nDNA) and mitochondrial DNA (mtDNA) genes.

Human disorders of mitochondrial energy conversion due to dysfunction of OXPHOS enzymes represent large group of mitochondrial diseases, which usually manifest in early childhood, and predominantly affect muscle, brain, heart, and other tissues with high energetic demands (Wallace 1992). Among the most severe belong the disorders of cytochrome *c* oxidase (COX), the terminal enzyme of the respiratory chain, which couples electron transport from cytochrome *c* to oxygen with transport of protons from matrix to cytosol. Mitochondrial diseases are genetically rather complicated as the pathogenetic mutations can reside in mtDNA, as well as in nuclear genes. Up to now, more than 190 different mtDNA point mutations associated with mitochondrial diseases have been described (see

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www.mitomap.org), which affect tRNA or rRNA genes or some of the protein genes for thirteen OXPHOS subunits (Schon *et al.* 1997, Wallace 1992). During the last decade, an increasing number of mitochondrial disorders have been found to result from mutations in nuclear genes that encode either OXPHOS subunits, or specific assembly proteins essential for biosynthesis of these large heterooligomeric complexes (Shoubridge 2001a). Their identification is rather difficult, but up to now, specific defects of nuclear origin have been found in all mitochondrial respiratory chain complexes including COX (Shoubridge 2001b) and ATP synthase (Houštěk *et al.* 1999a).

Structure, function and biosynthesis of COX

The mammalian COX is composed of 13 subunits (Taanman 1997) of which the three largest are encoded by the mtDNA and form the catalytic core of the enzyme. The remaining ten, evolutionary younger, nuclearly encoded subunits are involved in assembly and regulation of the enzyme (Kadenbach *et al.* 1991). The function of mammalian COX can be physiologically modulated and the enzyme represents one of the key regulatory sites of energy metabolism (Kadenbach *et al.* 2000, Ludwig *et al.* 2001).

COX transfers electrons from cytochrome *c* to molecular oxygen, which is reduced to water. The electrons pass from cytochrome *c*, binding at subunit II, through *CuA* and heme *a* cofactors, to the binuclear center buried inside subunit I and composed of heme *a3* and *CuB*, where the incoming four electrons together with four protons from the matrix are sequentially used for oxygen reduction. This exergonic redox reaction is coupled with proton pumping across the inner mitochondrial membrane, but the coupling of the two processes (H^+/e^- stoichiometry) can be modulated. In addition to Mitchell's chemiosmotic theory, a "second mechanism of respiratory control" has been proposed that involves the binding of adenine nucleotides to nuclear-encoded COX subunits. The key event is the phosphorylation of subunit IV. Activity of phosphorylated COX is regulated by ATP/ADP ratio and respiratory rate is precisely controlled according to the ATP utilization. The membrane potential is kept low (100-150 mV) and COX works at high efficiency of proton translocation ($H^+/e^- = 1$). Upon dephosphorylation, however, any ATP/ADP inhibition is abolished. This results in a "slip" of proton pumping ($H^+/e^- = 0.5$), the free energy of the reaction increases leading to a higher rate of respiration and ATP synthesis as well as to an increase of membrane potential and ROS production (Kadenbach *et al.* 2000, Ludwig *et al.* 2001).

Several nuclear-encoded subunits of mammalian COX exist in tissue- and developmentally-specific isoforms (Kadenbach *et al.* 1990). Their role is related to specific energetic demands of different tissues and also to ontogenic development. Tissue specific isoforms of subunits IV, VIa, and VIIa are present in human and mouse (Linder *et al.* 1995). They differ in regulatory properties which are well illustrated by subunit VIa. Heart isoform (VIaH) expressed in heart and skeletal muscle contains a binding site (sensor) for adenine nucleotides and at high ATP/ADP ratios the H^+/e^- stoichiometry of COX is decreased from 1 to 0.5. The enzyme containing the liver isoform (VIaL), which is found in most tissues, translocates protons at a low stoichiometry of 0.5, independently of the ATP/ADP ratio (Kadenbach *et al.* 2000, Lee and Kadenbach 2001). The dissipation of energy by COX containing VIaL is thought to be involved in thermogenesis (Huttemann *et al.* 1999, 2000). In addition, VIa subunit plays a role in the development of cardiac and skeletal muscle. At early embryonic stages, the VIaL isoform is predominant in all tissues, but increased VIaH expression is observed during differentiation of myogenic cells (Parsons *et al.* 1996) and late fetal stages of heart development (Schagger *et al.* 1995).

The COX biosynthesis and assembly is complicated and timely process involving several rate-limiting steps reflecting the sequential incorporation of the subunits from either the cytosol (nuclearly coded subunits) or from the mitochondrial matrix (subunits I, II and III). These rate-limiting steps result in formation of assembly intermediates (S1-S4), which can be isolated by BlueNative electrophoresis and further resolved by SDS-electrophoresis in the second dimension (Nijtmans *et al.* 1998). Several protein factors are required for the process of COX assembly (Robinson 2000). COX 10 (farnesyl transferase) and COX15 are involved in the synthesis of heme A before its insertion into subunit I; SCO1, SCO2, COX 11 and COX 17 control the incorporation of the copper atoms into subunits I and II; and SURF1 protein (Surf1p) facilitates the assembly step from S2 to S3 intermediate, which is the addition of subunit II to a subunit I+IV subcomplex.

Human disorders of cytochrome *c* oxidase

Selective COX defects are seldom maternally inherited and caused by mtDNA mutations (Schon *et al.* 1997). Among those rare cases, mutations in *COIII* gene encoding COX subunit III and A8344G transition in tRNA(Lys) gene are characterized in most details. The *COIII* mutation results in severe COX assembly defect, suggesting that the presence of subunit III is necessary for stabilization of the enzyme (Hoffbuhr *et al.* 2000, Tiranti *et al.* 2000). The "proteosynthetic mutation" A8344G affects mainly complexes I and IV (complexes with the highest number of mtDNA-encoded subunits) and their inhibition varies to different extent. We have found that A8344G mutation-induced severe COX deficiency was associated with decreased V_{max} but not K_m for cytochrome *c* resulting in decreased formation of mitochondrial membrane potential and decreased ATP synthesis (Antonická *et al.* 1999).

The majority of COX defects thus originate from mutations in nuclear genes (Tiranti *et al.* 1998, Zhu *et al.* 1998, Papadopoulou *et al.* 1999, Robinson 2000). They frequently manifest in the first months of life and their prognosis is practically always very serious, often fatal. Up to now, no mutations could be located in the nuclear genes for COX subunits, but during last years, mutations in the genes encoding several COX assembly factors have been described as a frequent cause of mitochondrial diseases and have been assigned with specific clinical symptoms (Table 1). The dysfunction of COX in these cases is mostly caused by structural changes rather than by the changes in amount of the enzyme. Mutations of *SCO1* are responsible for severe cardiomyopathy (Robinson 2000). Mutations in *SCO2* gene usually manifest as fatal infantile hypertrophic cardiomyopathy (Papadopoulou *et al.* 1999), similarly to mutations in *COX15* gene (Antonická *et al.* 2003b). On the other hand, mutations in the *COX10* gene are associated with multiple early-onset clinical phenotypes including tubulopathy and leukodystrophy, Leigh syndrome, anemia, sensorineural deafness, and hypertrophic cardiomyopathy (Antonická *et al.* 2003a). The most common COX assembly disorders are caused by mutations in the *SURF1* gene (Tiranti *et al.* 1998, Zhu *et al.* 1998). They also represent the majority of cases of the COX-associated Leigh syndrome (LS^{COX}), which is a subacute necrotizing encephalopathy characterized by bilaterally symmetrical necrotic lesions in subcortical brain regions with onset early in infancy (DiMauro *et al.* 1986). The frequency of different types of COX defects can be illustrated by the incidence of mitochondrial diseases diagnosed at biochemical and/or genetic level. For example, 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.

On one hand, in many cases mutations in individual COX-assembly genes could be linked with prevailing specific clinical symptoms (degenerative encephalopathy, cardiomyopathy, leukodystrophy etc) and to some extent with changes in COX structure (Nijtmans *et al.* 1998, Coenen *et al.* 1999, Tiranti *et al.* 1999, Yao and Shoubridge 1999, Hanson *et al.* 2001). On the other hand, it is largely unknown how these genetic defects influence different levels of COX function and its *in vivo* regulation in different tissues, or at different developmental stages. Therefore, the main expected energetic and metabolic consequences at the mitochondrial and cellular levels (such as ΔpH and $\Delta\Psi_m$ formation, regulation of H^+/e^- stoichiometry, mitochondrial ATP production, ROS production) remain to be characterized in detail before the molecular etiopathogenetic mechanism of COX dysfunctions is really well understood.

Owing to the generally unfavorable prognosis and absence of therapy for COX defects, the development of prenatal diagnosis is crucial. A rapid and reliable protocol for analyzing COX in amniocytes has been developed that is based on structural and functional analysis of OXPHOS enzymes (Houštěk *et al.* 1999b, Chowdhury *et al.* 2000, Houšťková *et al.* 2000). Indeed, with increasing number of known pathogenetic mutations in COX assembly genes the prenatal diagnosis in the near future should be mainly based on genetic analysis, but at present, the genetic defects in significant number of COX deficiencies remain unknown.

Mutations in *SURF1* gene for Surf1p assembly factor

SURF1 gene of 4.7 kb is located within the compact surfeit locus of six housekeeping genes on the chromosome 9q34. It contains 9 exons and codes for 30 kDa protein of 300 amino acids. The domain structure of this inner mitochondrial membrane protein is well conserved among eukaryotes and also prokaryotes, which underlines its necessity for the function of the respiratory

chain (Poyau *et al.* 1999). Actually, about 40 different pathogenetic mutations in *SURF1* have been described (Table 1). Several of them were found in Czech patients, including some novel mutations – a splicing site mutation causing the loss of exon 8 (Williams *et al.* 2001), and missense 574C>T transition (Čapková *et al.* 2002, Pecina *et al.* 2003a). 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 (Piekutowska-Abramczuk *et al.* 2001, Čapková *et al.* 2002). Most of *SURF1* mutations are nonsense mutations inducing the formation of a premature stop codon; missense and splicing-site mutations are less common. They cause translation of incomplete forms of Surf1p that are rapidly degraded, or instability of *SURF1* mRNA, in both cases preventing the synthesis of the protein.

Table 1: Mitochondrial diseases and dysfunction of specific COX assembly proteins

Assembly protein	Altered function	Number of known mutations	Clinical phenotype	References
SURF1	Advencement of COX assembly from S2 to S3 intermediate	40	Leigh syndrome associated with severe COX deficiency; isolated leukodystrophy	Bruno *et al.* 2002, Čapková *et al.* 2002, Ogawa *et al.* 2002, Pequignot *et al.* 2001, Piekutowska-Abramczuk *et al.* 2001, Sacconi *et al.* 2003, Williams *et al.* 2001
SCO1	Transport of copper atoms to COX subunits I and II	2	Neonatal-onset hepatic failure and encephalopathy	Valnot *et al.* 2000a
SCO2	Transport of copper atoms to COX subunits I and II	7	Fatal infantile hypetrophic cardiomyopathy; Leigh-like syndrome and cardiomyopathy; spinal muscular atrophy	Jaksch *et al.* 2000, Papadopoulou *et al.* 1999, Sacconi *et al.* 2003, Salviati *et al.* 2002
COX10	heme *a* synthesis – farnesylation of protoheme B to heme O	5	Leukodystrophy and tubulopathy; anemia and Leigh syndrome; sensorineural deafness and fatal infantile hypertrophic cardiomyopathy	Antonická *et al.* 2003a, Valnot *et al.* 2000b
COX15	Heme *a* synthesis – hydroxylation of heme O	2	Fatal infantile hypertrophic cardiomyopathy	Antonická *et al.* 2003b

References for SURF1 include reference for a review of SURF1 mutations in 2001 (Pequignot *et al.* 2001), and references for novel mutations discovered thereafter.

It has been proposed that the severe isolated COX defects due to absence of Surf1p result from impaired assembly of the complex, which stops at the S2 intermediate before insertion of subunits II and III into the heterodimer of subunits I and IV (Nijtmans *et al.* 1998, Coenen *et al.* 1999, Tiranti *et al.* 1999). The decreased steady-state level of COX subunits (Yao and Shoubridge 1999), pointing to a lesser amount of the COX holoenzyme, is consistent with severely decreased COX activity measured spectrophotometrically (Zhu *et al.* 1998). However, the exact role of Surf1p in COX assembly and functional manifestation of *SURF1*

mutations are still poorly understood. Despite the fact that the activity of COX in various tissues of the patients is decreased to 1-20 % of the controls, the onset of clinical symptoms is delayed and during the first months of life the patients seem to be clinically normal (for review see Shoubridge 2001b). The assembly of COX is also not completely prevented and significant amounts of normal COX complexes are found in patients completely lacking the Surf1p (Tiranti *et al.* 1999).

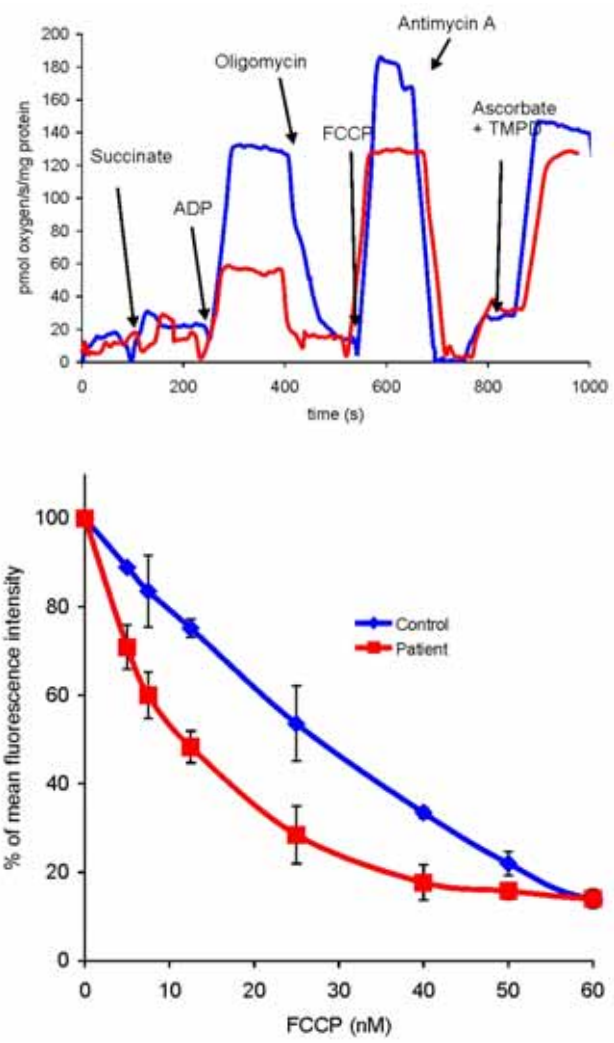

Fig. 1. Functional analysis of LS^{COX} fibroblasts. Upper part: Oxygen consumption in control and LS^{COX} fibroblasts. Measurements were performed with 1.0 mg cell protein/ml and permeabilized by 0.05 mg digitonin/mg protein. Subsequent additions of 10 mM succinate, 0.5 mM ADP, 1 μM oligomycin, 1 μM FCCP, 0.2 μg/ml antimycin A, 5 mM ascorbate, 0.2 mM TMPD are indicated. Oxygen consumption is expressed as negative values of the first time derivative of changes in oxygen concentration (pmol O_2/s/mg protein). **Lower part:** Cytofluorimetric measurement of mitochondrial membrane potential using TMRM probe in fibroblasts permeabilized by 0.05 mg digitonin/mg protein. The potential was titrated with uncoupler FCCP of indicated concentrations. Data are expressed as means ± S.D.

Changes in COX structure and function

It seems that sensitive measurements of COX function at conditions close to *in vivo* situation are essential for better understanding of pathogenetic mechanism of *SURF1* mutations. This can be achieved by analyzing electron transport and H^+ pumping in cultured fibroblasts or muscle fibers with intact mitochondria by high resolution respirometry (Gnaiger 2003, Wenchich *et al.* 2003), and cytofluorimetry with membrane potential-sensitive fluorescent probe TMRM (Floryk and Houštěk 1999). The latter, rather novel approach proved to be useful in studies of mitochondrial membrane potential $\Delta\Psi_m$ in cells with COX defect or ATPase defects (Antonická *et al.* 1999, Houštěk *et al.* 1999b, Dubot *et al.* 2004, Vojtíšková *et al.* 2004). Using these techniques it was demonstrated that the electron transport activity of COX and the whole respiratory chain in fibroblasts from Leigh syndrome patients harboring different *SURF1* mutations (LS^{COX} cells) is considerably less affected in intact cells than indicated by the routine spectrophotometric COX analyses (Fig. 1A). The reason is that COX complexes in LS^{COX} cells are very labile and inactivated upon addition of mild detergent (Zimmermann and Kadenbach 1992, Pecina *et al.* 2003a) that is routinely used in diagnostic assays. On the other hand, the H^+-pumping activity of COX is apparently decreased in *SURF1* mutated cells. Calcium homeostasis studies suggested a decrease of $\Delta\Psi_m$ (Wasniewska *et al.* 2001), which might negatively affect the ATP synthesis, leading to severe pathological phenotype. This was confirmed by direct measurements of steady state levels of $\Delta\Psi_m$ and its sensitivity to uncouplers (Fig. 1B), which demonstrated a decreased ability of LS^{COX} fibroblasts to maintain normal values of $\Delta\Psi_m$ under functional load (Pecina *et al.* 2003a).

At present, the functional changes can also be better related to altered COX structure and assembly. (Tiranti *et al.* 1999) found that *SURF1* mutations interfere with assembly of COX subunits and detected only a small residual portion of fully assembled enzyme complex. It is also well established that cells and tissues from LS^{COX} patients contain decreased amounts of various COX subunits, coded for by mitochondrial and nuclear DNA, but the data differ in various studies (Yao and Shoubridge 1999, Poyau *et al.* 2000, Sue *et al.* 2000, Hanson *et al.* 2001, von Kleist-Retzow *et al.* 2001, Williams *et al.* 2001). Our quantification in mitochondria from cultured fibroblasts using subunit-specific antibodies showed an

approximately 50 % decrease of the content of subunits I and IV, a 70 % decrease of the subunit II and an 80 % decrease of subunit VIc (Pecina *et al.* 2003a), a pattern that was quite similar with other reports (Yao and Shoubridge 1999, von Kleist-Retzow *et al.* 2001). The differential decrease of individual COX subunits and their distribution among complexes solubilized by a mild detergent further indicated that normal copies of fully assembled COX complex account for no more than 15 % of the control (Coenen *et al.* 1999, Tiranti *et al.* 1999, Pecina *et al.* 2003a). The remaining are incomplete COX assembly intermediates of different size and their function is modified according to subunit composition. These incomplete COX assemblies are also unable to form higher supracomplexes (Pecina *et al.* 2003a) that are normally created between COX and respiratory chain complexes I and III (Schagger and Pfeiffer 2000).

Some of the incomplete assemblies are apparently inactive, but since a near-normal function of respiratory chain-linked dehydrogenases is found in oxygraphic experiments, others have to be capable of electron transport activity, but not of proton pumping (see Fig. 2 - model of the respiratory chain function in LS^{COX} patients). As hemes *a* and a_3 are present in COX subunit I, the subcomplexes containing subunits I and II should be capable of electron transfer activity and contribute substantially to oxygen consumption determined in polarographic experiments. As the LS^{COX} cells show pronounced reduction of subunit I and II, this necessarily means that some of the incomplete, but functional COX assemblies must be more active in electron transport but less in H^+ pumping activity than the complete COX complexes. The explanation might be that they lack the nuclear-encoded regulatory subunits. Studies by Kadenbach and coworkers showed that the activity of COX is increased upon removal or dissociation of subunit COX VIb (Weishaupt and Kadenbach 1992) or COX VIa (Kadenbach *et al.* 1991) This is in accordance with the hypothesis that small nucleus-encoded subunits physiologically downregulate the activity of the mammalian enzyme (Kadenbach *et al.* 1991, 2000). It would be thus possible that the absence of some of these subunits, as is generally found in LS^{COX} patients, may in fact paradoxically serve as some kind of a rescue mechanism that keeps the COX electron transport activity *in situ* only mildly decreased. This would provide near-normal electron flux through the respiratory chain and allow proton pumping at complexes I and III ensuring at least decreased level of ATP synthesis.

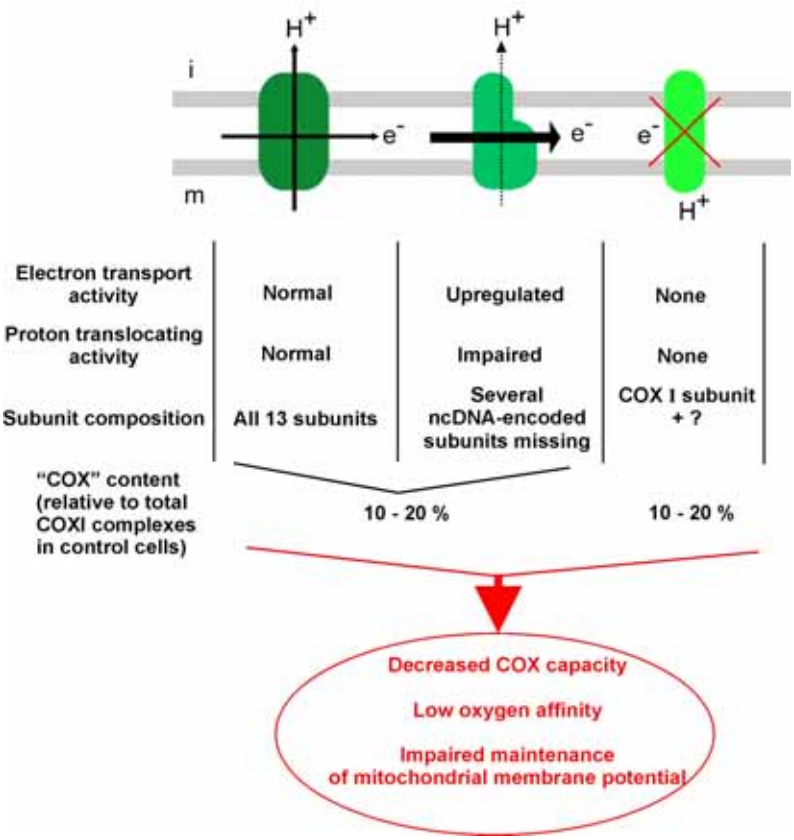

Fig. 2. The model of different COX complexes present in LS^{COX} cells

COX capacity and affinity to oxygen

Individual components of OXPHOS system are physiologically present with excess capacity, which means that their inhibition is tolerated until the threshold value for given component is reached. COX has been shown to possess only slight excess capacity, thus exhibiting substantial control strength over the respiratory rate (Villani and Attardi 1997, Villani *et al.* 1998, Kunz *et al.* 2000). Therefore the regulation of COX activity and H^+/e^- ratios by ATP and reversible phosphorylation could serve as a "second mechanism of respiratory control" (Kadenbach *et al.* 2000, Ludwig *et al.* 2001). COX reserve capacity, similarly as reserve capacity of other OXPHOS complexes, rather differs in mammalian tissues. Interestingly, the COX threshold values for brain, the most affected organ in LS^{COX}, are rather high as compared with other tissues, e. g. skeletal muscle or heart (Rossignol *et al.* 2003). As the COX is the only affected mitochondrial enzyme in patients with *SURF1* mutations, there must be other, still unknown factors that are crucial for pathogenicity of these mutations. One of potentially important factors could be the second substrate of the enzyme – oxygen. The oxygen is supplied to tissues through the respiratory cascade characterized by a drop of the oxygen partial pressure (pO_2) from 20 kPa in the inspired air to low intracellular pO_2 (Richardson *et al.* 1995). Under physiological conditions, mitochondria operate at pO_2 as low as 0.3 kPa in some tissues (reviewed in Gnaiger *et al.* 2000). Nevertheless, mitochondrial and cellular respiration is rarely studied under low oxygen conditions, and the oxygen affinity of COX is frequently assumed to prevent any oxygen limitations of the respiration under normoxia. As all previous studies with LS^{COX} cells were performed at much higher pO_2 than is actually present in cells in vivo, we analyzed the oxygen kinetics of COX and determined the p50 value in LS^{COX} cells by high-resolution respirometry and observed a two- to three-fold decrease of COX oxygen affinity compared with control cells (Pecina *et al.* 2003b). This suggests that at tissue oxygen tension of 0.3 kPa found *in vivo* at normoxic conditions the COX in patients would be only 70 % saturated compared to 90 % saturation in controls. This would cause the impairment of oxidative energy production at normoxic conditions and especially at hypoxia. NO-induced decrease of COX affinity has been shown to lower the expression of "hypoxic genes" (Mateo *et al.* 2003) and analogous situation due to *SURF1* mutations could mean that also glycolytic energy production can also be affected due to lower expression of hypoxic genes. Indeed, the clinical symptoms in patients with Leigh syndrome are known to significantly worsen during respiratory infections (Pronicka and Halikowski 1984, Pronicka *et al.* 2001), especially when the oxygen supply to tissues is decreased (Usen and Webert 2001).

Conclusions

While numerous pathogenetic mutations are now routinely detected in isolated COX deficiencies, the protocols for characterizing the functional impact of these mutations are still in early stages of development. The integrative approach combining multiple bioenergetics analyses performed in whole cells is particularly promising, as the best way to investigate the resulting pathogenetic changes occurring *in situ*. The general severity of the functional changes in COX defects suggests that the development of effective drugs is very unlikely, and that only gene therapy might lead to efficient treatment of these diseases in the future.

Abbreviations

COX, cytochrome *c* oxidase; OXPHOS, oxidative phosphorylation; LS^{COX}, Leigh syndrome caused by COX deficiency.

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