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Biochemical, genetic and immunoblot analyses of 17 patients with an isolated cytochrome *c* oxidase deficiency

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Abstract

Mitochondrial respiratory chain defects involving cytochrome *c* oxidase (COX) are found in a clinically heterogeneous group of diseases, yet the molecular basis of these disorders have been determined in only a limited number of cases. Here, we report the clinical, biochemical and molecular findings in 17 patients who all had isolated COX deficiency and expressed the defect in cultured skin fibroblasts. Immunoblot analysis of mitochondrial fractions with nine subunit specific monoclonal antibodies revealed that in most patients, including in a patient with a novel mutation in the SURF1 gene, steady-state levels of all investigated COX subunits were decreased. Distinct subunit expression patterns were found, however, in different patients. The severity of the enzymatic defect matched the decrease in immunoreactive material in these patients, suggesting that the remnant enzyme activity reflects the amount of remaining holo-enzyme. Four patients presented with a clear defect of COX activity but had near normal levels of COX subunits. An increased affinity for cytochrome *c* was observed in one of these patients. Our findings indicate a genetic heterogeneity of COX deficiencies and are suggestive of a prominent involvement of nuclear genes acting on the assembly and maintenance of cytochrome *c* oxidase.

Keywords: Cytochrome *c* oxidase; Respiratory chain; Mitochondrial protein; Enzyme deficiency; Leigh syndrome; (Human)

1. Introduction

Mitochondria play a central role in cellular energy provision by the process of oxidative phosphorylation. In this metabolic pathway, synthesis of ATP is driven by a transmembrane proton gradient across the mitochondrial inner membrane. The proton gradient is sustained by the respiratory chain which couples transfer of electrons from NADH and $FADH_2$ to molecular oxygen with proton pumping across the membrane [1]. Three of the four enzyme complexes of the electron transfer chain contain subunits encoded on mitochondrial DNA (mtDNA) and subunits encoded on nuclear DNA [2]. The mitochondrial genome is transmitted maternally but diseases associated with respiratory chain dysfunction may show any mode of inheritance [3].

Cytochrome *c* oxidase (COX; EC 1.9.3.1) is a complex metalloprotein embedded in the mitochondrial inner membrane and constitutes the terminal

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component (complex IV) of the respiratory chain. COX deficiency is emerging as one of the most common defects in mitochondrial diseases. In mammals, COX is composed of three polypeptides (I, II and III) encoded by mtDNA and ten polypeptides (IV, Va, Vb, VIa, VIb, VIc, VIIa, VIIb, VIIc, VIII) encoded by nuclear DNA [4]. The mitochondrially encoded subunits are homologous to the three polypeptides found in bacterial terminal oxidases and form the catalytic core of the enzyme [5]. The function of the nuclear encoded subunits remains enigmatic. Studies in yeast have shown that most nuclear-encoded subunits are required for assembly of the holo-enzyme [6]. Kadenbach was the first to postulate that some of these subunits are involved in allosteric modification of the catalytic activity [7]. Support for this idea is provided by the presence of tissue specific isoforms of some of the subunits which could optimize the enzymatic activity to the metabolic demands of different tissues. In humans, subunits VIa and VIIa exist as muscle/heart (VIa-H and VIIa-H) and ubiquitously expressed (VIa-L and VIIa-L) isoforms [6,8].

The dual genetic origin and isoform complexity of the enzyme may explain in part the remarkable clinical and biochemical heterogeneity of COX deficiencies, with different tissues being affected and with a variable onset of symptoms [9]. Partial defects of COX activity, frequently accompanied with other respiratory chain abnormalities, are often associated with specific point mutations in tRNA genes or deletions in a subpopulation of the mtDNA (i.e., the mutation is heteroplasmic). For instance, patients with the mitochondrial disorders MELAS (mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes) and MERRF (myoclonic epilepsy with ragged-red fibers) commonly carry a heteroplasmic A3243G transition in their mitochondrial $tRNA^{Leu(UUR)}$ gene or a heteroplasmic A8344G transition in their mitochondrial $tRNA^{Lys}$ gene, respectively, and show a COX deficiency combined with a defect of other respiratory chain enzymes [10].

COX deficiency in childhood shows a wide clinical spectrum and includes infants suffering from Leigh syndrome, a progressive neurodegenerative disorder characterized by subacute necrotizing encephalopathy. Leigh syndrome can result from several inborn errors of energy metabolism and the very first mutation in a nuclear gene encoding a respiratory chain component, the flavoprotein subunit of the succinate dehydrogenase, was reported in two siblings with this syndrome [11]. A maternally inherited form of Leigh syndrome is associated with a heteroplasmic mutation in the mitochondrially encoded subunit 6 of F_1F_0-ATPsynthase. While a low mutant load may result in neurogenic muscle weakness, ataxia and retinitis pigmentosa (NARP), a high mutant load can produce the clinical phenotype of Leigh syndrome [12].

Although much progress has been made in identifying mitochondrial genetic defects associated with COX deficiency, deleterious mutations in only one nuclear gene have been identified to date [13,14]. Diagnosis for COX deficiency has traditionally relied on analysis of the enzyme activity but this gives little information about the primary genetic basis of any abnormality or its prognosis. We have utilized a battery of nine subunit specific monoclonal antibodies which permit a more detailed analysis of the mutant enzyme. Immunocytochemical analysis of 17 patients with isolated COX deficiency showed a large variation in subunit expression patterns and suggest the involvement of many nuclear genes in COX deficiency.

2. Materials and methods

2.1. Case histories

The clinical presentations of patients included in this study are given in Table 1. All patients were diagnosed as COX-deficient before the age of 7 years.

2.2. Preparation of mitochondria-enriched fractions from skin fibroblasts

Human skin fibroblasts were cultured under standard conditions. However, in order to allow the respiratory chain defective cells to synthesize nucleic acids and grow, culture medium was supplemented with 400 μM uridine and 5 mM pyruvate [15]. Mitochondrial fractions were obtained from digitonin-treated cells as described by Bourgeron et al. [16].

2.3. Biochemical analysis

Respiratory chain enzyme activities were measured spectrophotometrically as previously described [17] using freeze–thaw permeabilized fibroblasts. Enzyme activities were calculated both as residual absolute activities and relative to each other, allowing better recognition of partial enzyme defects [18,19]. Kinetic parameters of COX reactions were determined by fitting the rates obtained at varying concentrations of reduced cytochrome *c* to a Michaelis–Menten equation modified to provide estimates of the Hill number as well as V_{max} and apparent K_m [20]. The protein concentration of solubilized mitochondrial fractions was determined with the help of the bicinchoninic acid kit (Pierce, Rockford, IL).

2.4. DNA analysis

Total DNA was extracted from various tissues (muscle, liver, skin fibroblasts, lymphocytes) and amplified by the polymerase chain reaction (PCR) using primers specific for mitochondrial COX genes as described [21]. Investigations aimed at detection of large scale rearrangements of mtDNA and the MELAS A3243G, MERRF A8344G and NARP T8993C mtDNA point mutations were performed according to standard procedures [22]. Total RNA extracted from cultured skin fibroblasts was reverse transcribed using the GeneAmp RNA PCR kit (Applied Biosystems). COX VIb cDNA was amplified using oligonucleotide A5′ 1–21 and A3′ 404–384 according to the sequence previously published [23]. PCR primers for amplification of the SURF1 gene were chosen as follows (position respective to the A of the start codon ATG): A5′ nucleotides −48–27, A3′ 273–253; B5′ 1474–1495, B3′ 1853–1833; C5′ 2494–2515, C3′ 2763–2743; D5′ 3673–3694, D3′ 4063–4043; E5′ 4294–4314, E3′ 4591–4571. Amplification conditions included 30 cycles of 30 s, 94°C; 30 s, 55°C; 30 s, 72°C.

For sequence analysis, amplification products were purified in 2% low-melting-point agarose gels and recovered by heating for 5 min at 65°C. Direct sequencing was performed using 3.2 pmol of the amplification primer, 10 ng of DNA and 8.5 μl of sequencing reaction mixture (Prism Ready Reaction Sequencing kit, Perkin Elmer Cetus) on an automatic fluorimetric DNA sequencer (Applied Biosystems).

2.5. Immunoblot analysis

Proteins (2.5, 5 or 10 μg) were dissociated for 30 min at 37°C in the presence of 4% sodium dodecyl sulfate, 2% 2-mercaptoethanol, and resolved on either 12.5% or 15% polyacrylamide, 5.5 M urea mini-gels. Proteins were subsequently electroblotted onto Immobilon-P poly(vinylidene difluoride) membranes (Millipore, Bedford, MA) as described [24].

Blots were developed with monoclonal antibodies against cytochrome *c* oxidase subunits [24]. After incubation with goat anti-mouse IgG horseradish peroxidase conjugate, immunoreactive material was visualized by chemiluminescence (Renaissance, DuPont NEN). Exposures of films to the blots were chosen such that the signals were always within the linear range. In order to correct for possible unequal loading of the samples, the signals of the COX subunits were compared to the signals obtained with monoclonal antibodies against two other proteins: the mitochondrial inner membrane flavoprotein subunit of succinate dehydrogenase [25] and the mitochondrial outer membrane voltage-dependent anion-selective channel (VDAC; monoclonal 31HL, Calbiochem). Previous experiments have shown that the concentration of these polypeptides is not affected by the absence of the COX complex [25,26].

3. Results

3.1. Biochemical analysis

Respiratory chain enzyme activities were determined spectrophotometrically in freeze–thaw permeabilized, cultured skin fibroblasts from 17 patients and compared to control values (Table 2). Eight patients showed significantly reduced COX activity. In the other nine patients, the absolute residual COX activity was low but within the normal range. The ratio between COX activity and other respiratory chain activities was, however, significantly reduced in these patients, indicating a partial COX deficiency. All other respiratory chain complex activities, as well as citrate synthase, fumarase and lactic dehydrogen-

Table 1
Clinical presentation of 17 patients with isolated COX deficiency

Patient	Consang./Multiplex	Symptoms/syndromes	COX deficiency identified in
1	M	IUGR, growth failure, hepatic failure, deafness, cataract, facial dysmorphism	Muscle Lymphocytes Fibroblasts
2	C	Myoglobinuria	Fibroblasts
3	M	Encepalopathy, liver failure, hypertrophic cardiomyopathy, gut involvement	Fibroblasts
4	C, M	Truncal hypotonia, retinitis pigmentosa, tubulopathy, pyramidal syndrome, growth failure	Muscle Lymphocytes Fibroblasts
5		Leigh syndrome, truncal hypotonia, encephalopathy, pyramidal syndrome	Muscle Fibroblasts
6		Leigh syndrome, growth failure, facial dystonia	Muscle Fibroblasts
7		Truncal hypotonia, cholestatic hepatic failure, growth failure	Liver Lymphocytes Fibroblasts
8		Growth failure, mental retardation, villous atrophy, truncal hypotonia, lactic acidemia	Muscle Fibroblasts
9	C, M	Leukodystrophy, peripheral neuropathy, lactic acidosis	Muscle Fibroblasts
10		Leigh syndrome, cerebellar syndrome, spastic paraplegia, strabismus, nystagmus	Muscle Lymphocytes Fibroblasts
11	C	IUGR, growth failure, recurrent vomiting and diarrhea, lactic acidemia	Muscle Fibroblasts
12	C, M	Cerebellar syndrome, peripheral neuropathy, growth failure, strabismus, retinitis pigmentosa, muscle atrophy	Fibroblasts
13		Truncal hyptonia, growth failure	Muscle Lymphocytes Fibroblasts
14		Myoglobinuria	Fibroblasts
15	C, M	Strabismus, muscle atrophy, lactic acidemia	Lymphocytes Fibroblasts
16	C, M	Leukodystrophy, truncal hypotonia, growth failure, retinitis pigmentosa, tubulopathy	Muscle Lymphocytes Fibroblasts
17	M	Growth failure, hypertrophic cardiomyopathy, nystagmus, tubulopathy, hepatomegaly	Muscle Fibroblasts

C, consanguineous family; M, Multiplex family; IUGR, intrauterine growth retardation.

ase, were normal. In 13 patients, the specific defect of COX found in fibroblasts was also observed in other tissues (circulating lymphocytes, liver and/or skeletal muscle; Table 1).

3.2. DNA analysis

In order to dissect the molecular basis of the COX deficiencies, all patients were initially screened for large rearrangements of mtDNA and mtDNA point mutations commonly associated with MELAS, MERRF and NARP. As this analysis did not reveal aberrant mtDNA, the genes of the three mitochondrially encoded COX subunits (I, II and III) as well as the flanking tRNA genes were sequenced for 10 of the 17 patients (nos. 1, 2, 3, 4, 7, 9, 10, 13, 14, 16). This analysis did not identify any pathogenic mutations.

The recent reports of mutations in the SURF1 gene associated with COX-deficient Leigh syndrome [13,14] prompted us to screen for SURF1 mutations in our patients presenting with Leigh syndrome. While no mutations were found in patients 5 and 10, a homozygous 1-base pair deletion within exon 6 of the SURF1 gene was identified in patient 6. This base change causes a frameshift mutation resulting in a premature stop-codon at amino acid residue 187 of the polypeptide.

3.3. Immunoblot analysis

In order to find additional clues to guide our genetic investigations, we performed immunoblot analysis of mitochondria-enriched fractions from fibroblasts of patients and controls. Fig. 1 shows the results for five of the patients and two controls, and is representative of the various subunit patterns observed in the 17 patients. Patient 1 presented with a profound COX deficiency (Table 2) and showed severely compromised steady-state levels of all COX subunits except subunits IV and Va. Patients 2, 3 and 5 presented with a less severe COX deficiency (Table 2) and exhibited a less pronounced decrease of most COX subunits (Fig. 1). In contrast, patient 4 presented with a severe COX deficiency (Table 2) but this patient showed near normal levels of COX subunits on Western blots (Fig. 1). Antibodies to the flavoprotein protein subunit of the succinate dehydrogenase and to the voltage-dependent anion channel (VDAC) gave similar signals in controls and patient extracts, confirming that protein loading was equal in all lanes.

Table 3 gives a summary of the immunoblot data obtained with the cultured skin fibroblasts of all 17 patients. We did not observe a change in the electrophoretic mobility of any COX subunit. In 13 out of the 17 cases, the steady-state levels of several subunits were found to be decreased with subunits II, III and VIa-L generally being the most severely affected and subunits IV, Va and Vb being the least affected (Table 3). This subunit expression pattern was observed in particular in fibroblasts from patient 6 who was found to carry a mutation in the SURF1 gene. Comparison of the quantitative changes in COX subunits and the severity of the enzymatic deficiency in this group of 13 patients revealed a correlation between these parameters (Fig. 2).

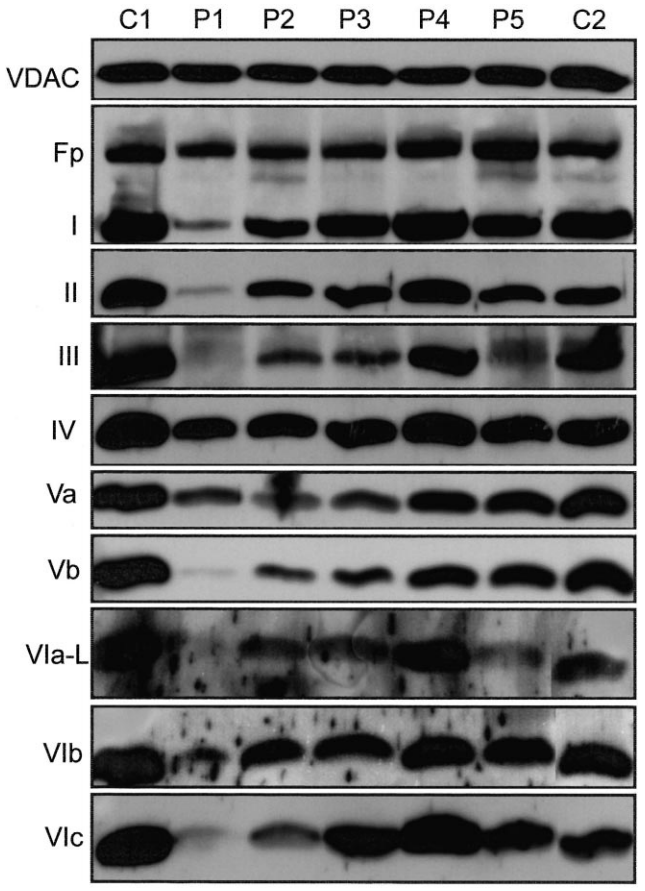

Fig. 1. Immunoblots with mitochondrial proteins isolated from patient (P) and control (C) skin fibroblasts. The upper blot was developed with a monoclonal antibody against VDAC; the second blot was developed with monoclonal antibodies against the flavoprotein (Fp) of succinate dehydrogenase and subunit I of COX; the lower blots were developed with specific antibodies against the various COX subunits as indicated.

Despite the COX deficiency (Table 2), patients 4, 7, 10 and 14 showed near normal enzyme subunit steady-state levels (Table 3). These observations suggest that this group of patients harbors a mutation affecting the catalytic activity of the enzyme that does not affect enzyme stability or assembly. More detailed kinetic studies revealed a decreased K_m for cytochrome *c* (0.38 μM; control $n=5$: 3.5 μm (± 1.0)) in patient 4, while the K_m in the other three patients was normal. Subunits II and VIb are putatively involved in cytochrome *c* binding [4] but no mutations were found in the COX II gene or in the COX VIb cDNA of patient 4.

Table 2
Spectrophotometric analysis of respiratory chain in cultured skin fibroblasts from patients

Patient	Enzyme activity (nmol/min/mg protein)				Activity ratios	
	SQDR	QCCR	COX	SCCR	COX/SCCR	QCCR/SCCR
1	27	40	**4**	20	**0.2**	2.0
2	nd	63	**36**	25	**1.4**	2.5
3	21	107	62	41	**1.5**	2.6
4	nd	80	**12**	32	**0.5**	2.5
5	21	105	54	38	**1.3**	2.8
6	25	86	**30**	28	**0.9**	3.1
7	nd	27	61	51	**1.2**	2.5
8	25	118	**21**	38	**0.5**	3.1
9	21	119	**25**	38	**0.6**	3.1
10	nd	nd	55	28	**1.95**	–
11	21	93	52	36	**1.4**	2.6
12	6	107	54	40	**1.3**	2.7
13	nd	141	**27**	60	**0.4**	2.4
14	30	169	83	52	**1.6**	3.2
15	26	79	61	38	**1.6**	2.1
16	nd	115	**28**	19	**1.4**	2.8
17	21	84	57	26	**2.2**	3.2
Controls ($n=56$)	12–42	33–187	47–182	16–68	3.0 ± 0.4	2.9 ± 0.3

Enzymes were measured as described in Section 2. SQDR, succinate quinone DCPIP reductase (complex II); QCCR, quinol cytochrome *c* reductase (complex III); COX, cytochrome *c* oxidase (complex IV); SCCR, succinate cytochrome *c* reductase (complex II+III); nd, not done. Abnormal results are indicated in bold characters.
Values for controls: range for control values are given for absolute activities; ratios in controls are given as mean ± 1 S.D.

4. Discussion

In this report, we present the results of a comprehensive biochemical, genetic and immunoblot analysis of cultured fibroblasts from patients with isolated COX deficiency. In previous studies, analysis was restricted to one or a few patients and a limited number of COX subunits [27–32]. We have examined 17 cases and have investigated the steady-state levels of nine of the 13 COX subunits with subunit specific monoclonal antibodies.

Heteroplasmic mutations in mitochondrially en-

Table 3
Immunoblot analysis of mitochondrial proteins from skin fibroblasts from 17 patients and 2 controls

	C1	C2	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10	P11	P12	P13	P14	P15	P16	P17
SDH-Fp	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
VDAC	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
COX I	++	++	−−	+−	+	++	+	−	++	+	−−	+	+−	+−	−−	++	+−	+−	++
COX II	++	++	−−	+	+	++	+	−	++	−	−−	++	−	−	−−	++	+−	+−	+−
COX III	++	++	−−	−	−	++	−−	−−	++	−−	−−−	++	+−	+−	−−−	+	−	−	−
COX IV	++	++	+−	+−	+	++	+	+−	++	+−	−−	++	+−	−	−	++	+−	+−	++
COX Va	++	++	+−	−	+	+	+	+	+	+	+−	++	+	+−	+−	++	+	++	++
COX Vb	++	++	−−	+−	+−	+	+	++	++	++	−−	++	+−	+−	−−	++	+	+	+
COX VIa	++	++	−−−	−	−	++	−	−−−	+	−−	−−−	++	+−	−	−−−	+	+−	+	+−
COX VIb	++	++	−−	−	−	+	−	−	++	+	−−	++	+	+−	−−	+	+	+−	+
COX VIc	++	++	−−	−	+	++	+−	−−	++	−	−−	++	+	+−	−−	++	+−	−	+−

Conditions as in Fig. 1. Symbols correspond to percent of densitometric values as compared to controls: ++, >80% (normal); +, 61–80% (somewhat below normal); +−, 31–60% (below normal); −, 11–30% (considerably below normal); −−, 1–10% (detectable only after prolonged exposure); −−−, 0% (not detectable).

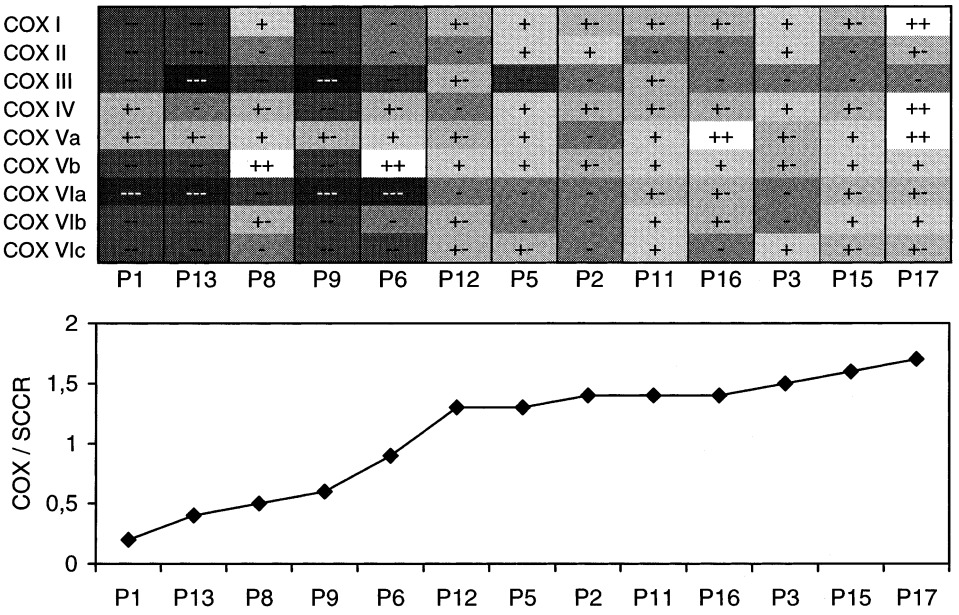

Fig. 2. Relationship between the overall COX subunit steady-state levels and the severity of the enzyme defect. Symbols as in Table 3. Intensities of gray correspond to decreasing concentrations of COX subunits. Enzyme deficiency is shown as COX/SCCR ratio.

coded COX genes have been reported in a small number of cases with isolated COX deficiency [33–36]. We determined the DNA sequence of the three mitochondrially encoded COX genes in ten of our patients but failed to detect any potentially pathogenic base changes. In a previous study, no mtDNA mutations were found in 18 cases with isolated COX deficiency, suggesting that mutations in the genes for subunit I, II and III are not common in patients with COX deficiency [21]. The rarity of mtDNA mutations in these cases is further supported by the reported high rate of parental consanguinity in children with cytochrome *c* oxidase deficiency [3]. Seven of our 17 patients came from consanguineous families (Table 1).

Mutations in the nuclear-encoded COX genes have not been identified [32,37] but, recently, mutations in the SURF1 gene were found to be associated with COX-deficient Leigh syndrome [13,14]. The SURF1 gene was previously shown to be part of a highly conserved gene cluster [38]. The yeast homologue encodes a mitochondrial inner membrane protein required for electron transfer between complexes of the respiratory chain and COX assembly or stability [39]. Sequencing of the SURF1 gene in our three patients with Leigh syndrome revealed in one patient of Italian origin a novel mutation which, like most of the other reported mutations, is predicted to lead to a truncated protein product. On the other hand, the absence of a mutation in the SURF1 gene in two of our patients confirms the heterogeneity of the molecular basis of COX deficiency in Leigh syndrome.

Immunoblot analysis of fibroblast mitochondria revealed a partial deficiency of COX subunits in 13 of the 17 patients. The deficiency affected both mitochondrially and nuclear-encoded subunits (Table 3). COX activity corresponded with the overall subunit steady-state levels in this group of patients (Fig. 2), suggesting that the defect in these patients affects predominantly the concentration of fully formed enzyme complex and not its intrinsic catalytic activity. Studies in yeast have revealed the existence of an ATP-dependent proteolytic pathway responsible for the clearing of unassembled and improperly folded polypeptides in the various subcompartments of mitochondria [40]. This proteolytic system is responsible for the rapid degradation of COX subunits in yeast strains unable to complete assembly of the holo-enzyme due to lack of one of the subunits or of assembly-assisting proteins [41,42]. The proteolytic system appears to be conserved in mammals [43,44]. Low steady-state levels of nuclear-encoded COX subunits have been found in mouse and human cell lines that do not express the mtDNA-encoded sub-

units [25,45–48], and it has been demonstrated by pulse–chase labeling experiments that these low steady-state levels are the result of an increased turnover rate [47]. Furthermore, Hayasaka and colleagues [28] have shown that the reduced COX content in fibroblasts cultures from two patients with COX-deficient Leigh syndrome, was the result of an elevated rate of degradation. Thus, the decreased subunit steady-state levels observed in our patients are most likely the result of proteolytic degradation of unassembled or misfolded subunits.

In some cases, subunit VIa-L was undetectable (Table 3) but it is unlikely that a complete lack of this subunit is the primary cause of the phenotype observed in these patients because studies in yeast have demonstrated that this highly conserved polypeptide is not necessary for activity or assembly of the complex [49]. In most patients, the steady-state levels of subunits IV, Va and Vb were less affected than the levels of the other subunits. Subunit IV appears to have an intrinsic stability [46] and is present at 40% of control values in human cell cultures depleted of mtDNA (rho° cells) [26]. Subunits Va and Vb do not span the inner membrane and are located on the matrix side of the enzyme complex where subunit Va interacts with subunit IV [4]. These subunits may already be folded prior to assembly, with subunits IV and Va forming a subcomplex, making them less prone to proteolytic degradation.

Although we have excluded mitochondrial mutations in ten of the 17 patients, missense mutations resulting in a decreased assembly or stability of the holo-enzyme could be present in the mtDNA of the remaining seven patients. Furthermore, the patients could harbor mutations in nuclear genes involved in the addition of the heme A or copper prosthetic groups, or at other stages of the assembly process unique to COX. Such genes have been shown to be essential for COX assembly in yeast and mutations in these genes lead to reduced steady-state levels of the COX subunits [50–55]. The residual subunit patterns of these mutant yeast strains are different and depend on the mutated gene. In our patients, we observe dissimilar subunit patterns, suggesting different genetic defects. Indeed, sequencing of the SURF1 gene in patients 5, 6 and 10, which all have COX deficiency and Leigh syndrome but clearly distinguishable subunit patterns (Table 3), demonstrated a mutation in patient 6 but not in patients 5 and 10. The SURF1 gene mutation appears to have a drastic affect on COX subunit levels (Table 3, patient 6) and the characterized subunit fingerprint may be useful for diagnostic purposes. Our knowledge of the assembly pathway of human COX is limited to two partly characterized assembly intermediates [56] and we are currently unable to relate the other subunit patterns to specific molecular genetic defects. Detailed studies in yeast and mammalian cells may allow us to link the subunit 'signatures' to specific genetic defects in future.

In four of our 17 patients (patients 4, 7, 10 and 14), immunoblot analysis indicated near normal steady-state levels of all COX subunits despite clearly reduced COX activity (Tables 2 and 3). Enzyme kinetic studies revealed an increased affinity for the substrate cytochrome *c* in patient 4. The reduced COX activity in patient 4 is thus probably related to a defect in the interaction of the enzyme with cytochrome *c*. Abnormal kinetic behavior of COX has been observed in other patients [30,57,58] but an increased affinity for cytochrome *c* has never been reported. Based on the crystal structure, Tsukihara and colleagues [4] have proposed a cytochrome *c* binding site which is comprised of the three mitochondrially encoded subunits I, II and III and the nuclear-encoded subunits VIa and VIb. Sequencing of the mitochondrial COX genes excluded mutations in subunits I, II and III. A mutation in subunit VIa-L is highly unlikely because subunit VIa is expressed as a different isoform in skeletal muscle [8], while the biochemical defect was present in skeletal muscle as well as lymphocytes and fibroblasts (Table 1). Sequencing of subunit VIb cDNA did not reveal a mutation and we are currently considering the sequencing of other COX subunits with domains located in the vicinity of the putative cytochrome *c* binding site.

Kinetic analysis of the enzyme in the other three patients with normal concentrations of COX subunits did not reveal an alteration in the apparent affinity for cytochrome *c*. The decrease in enzyme activity is, therefore, probably related to defects in the electron transfer or proton pumping properties of the enzyme. The mitochondrially encoded subunits play a pivotal role in these activities [5,6] but sequencing of the mitochondrial genes did not reveal

any potentially pathogenic mutations. The nuclear-encoded subunits are thought to play a regulatory role in the catalytic functions of the enzyme [7] and we are now contemplating sequencing of cDNAs for the ten nuclear-encoded subunits.

Recent genetic studies of patients with COX-deficient Leigh syndrome have shown mutations in the SURF1 gene for patients falling into one complementation group [13,14,59]. Our study of 17 patients with isolated COX deficiency suggests a large genetic heterogeneity. DNA sequencing showed that only one of our three patients with COX-deficient Leigh syndrome carried a mutation in the SURF1 gene. We expect that the detailed description of COX subunit expression patterns will guide our future efforts to determine the genetic lesion in the other patients with COX deficiency.

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