Am. J. Hum. Genet. 65:1030–1039, 1999

A Missense Mutation of Cytochrome Oxidase Subunit II Causes Defective Assembly and Myopathy

Shamima Rahman,[1,2] Jan-Willem Taanman,[1] J. Mark Cooper,[1] Isabelle Nelson,[3] Ian Hargreaves,[4] Brigitte Meunier,[5] Michael G Hanna,[3] José J. García,[7] Roderick A. Capaldi,[7] Brian D. Lake,[6] James V. Leonard,[2] and Anthony H. V. Schapira[1,3]

[1]University Department of Clinical Neurosciences, Royal Free and University College Medical School, [2]Metabolic Unit, Institute of Child Health, [3]Department of Clinical Neurology, Institute of Neurology, [4]Department of Biochemistry, National Hospital for Neurology and Neurosurgery, and [5]Department of Biology, University College London, and [6]Department of Histochemistry, Great Ormond Street Hospital for Children, London; and [7]Institute of Molecular Biology, University of Oregon, Eugene

Summary

We report the first missense mutation in the mtDNA gene for subunit II of cytochrome *c* oxidase (COX). The mutation was identified in a 14-year-old boy with a proximal myopathy and lactic acidosis. Muscle histochemistry and mitochondrial respiratory-chain enzymology demonstrated a marked reduction in COX activity. Immunohistochemistry and immunoblot analyses with COX subunit–specific monoclonal antibodies showed a pattern suggestive of a primary mtDNA defect, most likely involving *CO II,* for COX subunit II (COX II). mtDNA-sequence analysis demonstrated a novel heteroplasmic T→A transversion at nucleotide position 7,671 in *CO II.* This mutation changes a methionine to a lysine residue in the middle of the first N-terminal membrane-spanning region of COX II. The immunoblot studies demonstrated a severe reduction in cross-reactivity, not only for COX II but also for the mtDNA-encoded subunit COX III and for nuclear-encoded subunits Vb, VIa, VIb, and VIc. Steady-state levels of the mtDNA-encoded subunit COX I showed a mild reduction, but spectrophotometric analysis revealed a dramatic decrease in COX I–associated heme a_3 levels. These observations suggest that, in the COX protein, a structural association of COX II with COX I is necessary to stabilize the binding of heme a_3 to COX I.

Received May 3, 1999; accepted for publication August 4, 1999; electronically published September 13, 1999.

Address for correspondence and reprints: Professor A. H. V. Schapira, University Department of Clinical Neurosciences, Royal Free and University College Medical School, Rowland Hill Street, London NW3 2PF United Kingdom. E-mail: schapira@rfhsm.ac.uk

0002-9297/1999/6504-0012$02.00

Introduction

Cytochrome *c* oxidase (COX) is the terminal electron acceptor of the mitochondrial respiratory chain and catalyzes the transfer of electrons from reduced cytochrome *c* to molecular oxygen to form water (Capaldi 1990). This enzyme complex comprises 13 subunits, 3 of which (subunits I–III) constitute the catalytic core of the enzyme and are encoded by the mitochondrial genome, a circular double-stranded 16.5-kb DNA molecule present in multiple copies within mitochondria. The remaining 10 subunits are encoded by nuclear genes. The redox centers involved in electron transfer are two heme A moieties (*a* and a_3) and two copper centers (Cu_A and Cu_B). The heme *a* and the heme a_3-Cu_B binuclear centers are associated with COX I, whereas COX II contains the Cu_A center (Tsukihara et al. 1996).

COX deficiency is the most commonly recognized respiratory-chain defect in childhood and is clinically heterogeneous with phenotypes including Leigh syndrome and fatal and benign infantile myopathies (Di Mauro et al. 1983, 1994; Rahman et al. 1996). To date, mtDNA mutations have only been identified in *CO III* and *CO I:* a microdeletion in *CO III* (Keightley et al. 1996) causing cramps and recurrent myoglobinuria, and point mutations in *CO III* in two patients with encephalopathy (see Manfredi et al. 1995; Hanna et al. 1998); and a microdeletion in *CO I* in a patient with motor-neuron disease (Comi et al. 1998). In other cases, combined deficiency of COX and complex I of the respiratory chain may be caused by large-scale rearrangements of the mitochondrial genome, or by point mutations involving mitochondrial tRNA genes (Holt et al. 1989; Ciafaloni 1992). Failure to identify a mutation, even after complete sequence analysis of all COX-subunit genes in a number of patients, has led to the suggestion that many cases of COX mutations may be due to genetic defects in nuclear proteins involved in the assembly of the COX enzyme complex (Adams et al. 1997; Lee et al. 1998). Recently, mutations have been identified in

the *SURF-1* gene on chromosome 9 in patients with Leigh syndrome and COX deficiency (Tiranti et al. 1998; Zhu et al. 1998). The *SURF-1* gene product is thought to be involved in COX assembly or maintenance.

We have studied a boy with isolated COX deficiency and have identified the first heteroplasmic point mutation in the mitochondrially encoded gene for COX II. We provide evidence that this mutation affects the assembly or stability of the COX holoenzyme.

Patient and Methods

Clinical History

All studies were performed with the approval of the ethics committee of the Royal Free Hospital National Health Service Trust. A 14-year-old boy was referred for investigation of a five-year history of muscle weakness and fatigue. There was no family history of neuromuscular disease, the parents were healthy and unrelated, and four younger siblings were all asymptomatic. On examination, the patient was generally thin, but there was no focal muscle wasting. There was mild weakness of shoulder and pelvic-girdle musculature. Tendon reflexes were normal. There was no evidence of ophthalmoplegia or retinopathy. Resting-blood lactate level was elevated (4 mmol/l [reference range 0.9–1.8 mmol/l]) at age 11 years but was normal (0.97 mmol/l) at age 14 years. Mild elevation of cerebrospinal fluid lactate (2.37 mmol/l) was noted at age 14 years. Results of magnetic resonance imaging of the brain, echocardiography, electrocardiogram, blood-creatine kinase, and investigations of renal tubular function were all normal. Histochemical staining of biopsied skeletal muscle, at age 11 years, had revealed reduced COX activity, but biochemical assays of other respiratory-chain complexes were not performed. A further quadriceps-muscle biopsy was obtained at age 14 years for detailed biochemical analysis, after informed patient and parental consent was given.

Muscle Histochemistry and Immunohistochemistry

For histochemical studies, cryostat muscle samples cut into 8-μm sections were stained, to demonstrate the activities of COX and succinate dehydrogenase (SDH), with use of standard methods (Filipe and Lake 1990; Stoward and Pearse 1991). Sections were also stained with the modified Gomori trichrome. A library of subunit-specific mouse monoclonal antibodies was used to identify COX subunits immunohistochemically in 10-μm serial sections. Antibodies were directed against subunits I and II (mitochondrially encoded) and against subunits IV, Va, and VIc (nuclear encoded) (Taanman et al. 1996). Visualization was with the Strept-ABC (Dako) technique, with 3,3′-diaminobenzidine hydrochloride.

Biochemical Studies

Polarographic and spectrophotometric enzyme assays of respiratory-chain complexes were performed in isolated mitochondria, as previously described by Cooper et al. (1992). For spectral analysis of laser-flash photolysis and recombination of carbon monoxide (CO), mitochondria were prepared from 10–20 mg of skeletal muscle (Darley-Usmar et al. 1983) and dissolved into 600 μl of 50 mM Tris/Cl, 10% ficoll, and 0.05% lauroyl maltoside (pH 8); 500 μl were used for optical measurements. The samples were reduced by addition of sodium dithionite and gassed for 2 min with CO to generate the CO-ferroheme a_3 compound. Room-temperature laser-flash photolysis of CO was performed as previously described (Meunier and Rich 1998*b*). Short actinic-light pulses were provided by a frequency-doubled Nd-YAG laser. Optical signals of 430 and 445 were recorded. Ten transients, at each wavelength, were signal averaged and the data were plotted as ΔA at 430 − 445 nm versus time. The observed rate constants (K_{obs}) of CO recombination were obtained by the fit of exponential decays to the traces.

Immunoblot Analysis

A mitochondrial cell fraction was prepared by the method of Darley-Usmar et al. (1983), scaled down for a small sample size of ~20 mg, and cellular proteins, including mitochondrial membrane proteins, were extracted from myoblast and fibroblast cell pellets as described by Taanman et al. (1997). Immunoblot analysis was performed with use monoclonal antibodies against COX subunits (Taanman et al. 1994, 1996), the flavoprotein subunit of SDH (Marusich et al. 1997), the voltage-dependent anion channel (VDAC) (32HL; Calbiochem), core protein 1 of complex III, and the α subunit of F_1-ATP synthase. These last two monoclonal antibodies were generated with bovine enzymes and chosen after first being screened for specificity by reaction with pure beef complex III or with ATP synthase, as well as with bovine mitochondria. Their utility in immunohistochemistry and western blotting with human enzymes was confirmed with mitochondria isolated from human skin fibroblasts.

mtDNA Amplification and Sequence Analysis

Total genomic DNA was extracted from blood, skeletal muscle biopsy, cultured myoblasts, and skin fibroblasts by established methods (Sambrook et al. 1989). Sequence analysis was performed on muscle DNA. All mitochondrial tRNA and COX genes were amplified, by use of oligonucleotide primers with bacteriophage M13 tails. PCR products were sequenced with a *Taq* FS dye primer cycle sequencing kit (PE Applied Biosystems) and

were analyzed on an automated DNA sequencer (373A; PE Applied Biosystems).

RFLP Detection of the Point Mutation

A mismatch PCR method was designed to detect the mutant mtDNA molecule by creation of a restriction site for the enzyme *Tru* 9I (Promega). The sequences of the PCR primers used were as follows: (forward) 5′(7651)-CTT TCA TGA TCA CGC CCT *T*A-(7670)3′ (mismatch nucleotide italicized), and (reverse) 5′(7865)-AGG GAT CGT TGA CCT CGT CT-(7846)3′. The PCR cycling conditions used to amplify the target sequence were as follows: 4 min denaturation at 94°C followed by hot-start addition of *Taq* polymerase, then 30 cycles of 30 s denaturation at 94°C, 30 s annealing at 55°C and 30 s extension at 72°C. A final extension step of 10 min at 72°C was performed after the last cycle. After digestion with *Tru* 9I, the 215-bp PCR product from the mutant allele yielded two fragments, of 197 bp and 18 bp, whereas the wild-type allele remained uncut.

Quantification of Mutant and Wild-Type PCR Products

The relative proportions of wild-type and mutant mtDNA were determined by the addition of fluorescent dUTP (PE Applied Biosystems) just before the final (20th) cycle of the PCR, followed by *Tru* 9I restriction digestion of the PCR product and separation onto a 5% nondenaturing polyacrylamide gel. The fluorescent products were analyzed with GENESCAN (PE Applied Biosystems).

Single-Fiber PCR Analysis

The percentage of mutant mtDNA in individual fibers was correlated with COX activity by use of a single-fiber PCR assay, as previously described (Moraes and Schon 1996). Muscle sections of 30 μm were stained for COX activity and were fixed in 50% ethanol. Individual muscle fibers were selected in accordance to COX staining (either positive or negative) and were microdissected with a borosilicate microcapillary tube under an inverted-light microscope. The removed fiber segments were placed in tubes containing 10 μl of water, centrifuged for 10 min, and then lysed at 65°C for 1 h in a solution containing 200 mM KOH and 50 mM DTT. A neutralizing solution of 5 μl (900 mM Tris-HCl [pH 8.3] and 200 mM HCl) was added, and the samples were then subjected to fluorescent mismatch PCR amplification and restriction digestion, as described above, to determine the proportion of mutant mtDNA in single-muscle fibers.

Studies in Cultured Cells

A primary myoblast culture was established, as described by Yasin et al. (1977), from skeletal muscle biopsied when the patient was 14 years of age. Myoblasts were grown in Dulbecco's modified Eagle's medium containing 25 mM glucose and 4 mM L-glutamine and were supplemented with 20% FCS, 2% detoxified chick embryo extract, 1 mM sodium pyruvate, 200 μM uridine, 50 U/ml penicillin, and 50 μg/ml streptomycin. Cultured skin fibroblasts were grown in the same medium, but without chick embryo extract and with 10% rather than 20% FCS. Cells were cultured in a humidified 37°C incubator containing 8% CO_2 in air. Cultured myoblasts and fibroblasts were stained for COX activity and were immunostained for COX subunit I, as previously described (Taanman et al. 1997). COX and citrate synthase assays were performed in freshly harvested cell pellets, as described by Hartley et al. (1993).

Results

Morphological and Biochemical Studies

Muscle biopsy revealed severe COX deficiency in 97% of fibers (fig. 1) with increased SDH staining, but showed no frank ragged red fibers with the modified Gomori trichrome stain. Muscle morphology and histochemistry remained unchanged in two biopsies taken three years apart, at age 11 years and 14 years, respectively. A defect localized to COX was demonstrated by polarographic and enzyme assays performed on the second biopsy (table 1). Immunostaining of frozen muscle sections demonstrated almost complete absence of COX II, with preserved immunostaining of subunits I, IV, and Va, but with reduced staining of subunit VIc (fig. 1). Immunoblot analysis of a mitochondrial protein fraction, prepared from skeletal muscle, confirmed the immunohistochemical finding of a severe reduction of COX II compared to controls (fig. 2). Further immunoblot analysis with antibodies directed against other COX subunits demonstrated reduced levels of all subunits investigated. There was mild reduction of subunits I, IV, and Va, with more marked reduction of subunits III, Vb, VIa (heart/muscle isoform), VIb, and VIc (fig. 2). In addition, immunoblot analysis revealed increased steady-state levels of other respiratory-chain and oxidative phosphorylation subunits (flavin protein of SDH, core 1 subunit of complex III, and the α subunit of F_1F_0-ATP synthase). Immunoblotting for VDAC was used to demonstrate equal loading of samples (fig. 2).

Spectrophotometric Studies

To measure the COX I–associated heme a_3 levels in the muscle biopsies from controls and the COX-deficient patient, mitochondria were prepared from 10–20 mg of skeletal muscle. The samples were reduced by dithionite and treated with CO, and CO flash-photolysis and recombination signals were monitored (Meunier and Rich 1998*a*). Figure 3 shows the optical signals obtained with

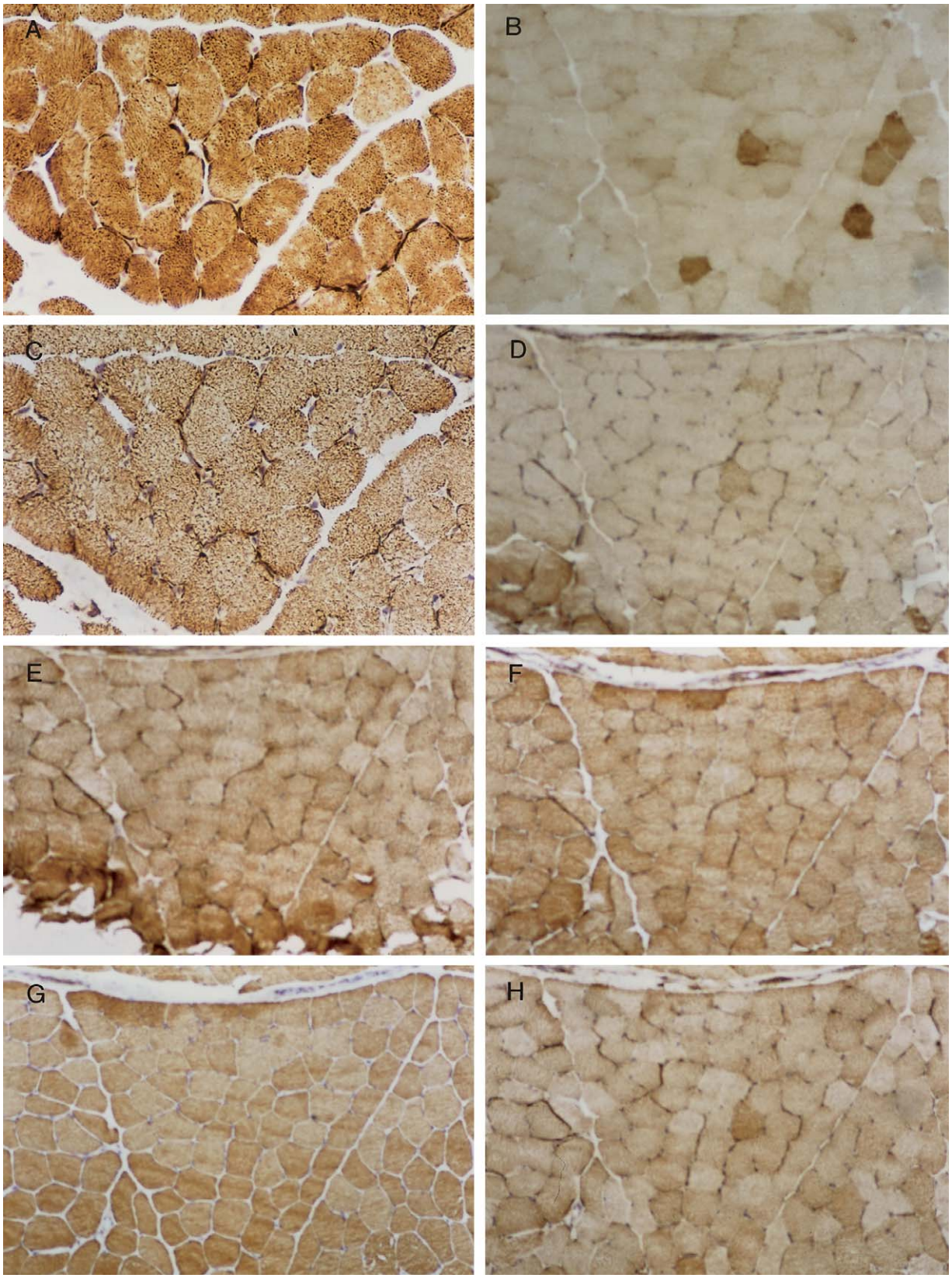

Figure 1 COX-activity staining (panels *A* and *B*) and COX immunohistochemical staining (panels *C–H*) in muscle. A = control; B = patient; C = COX subunit II (control); D = COX subunit II (patient); E = COX subunit I (patient); F = COX subunit IV (patient); G = COX subunit Va (patient); and H = COX subunit VIc (patient). Control sections stained for subunits I, IV, Va, and VIc appeared the same as section C.

Table 1

Polarography

Mitochondrial Oxygen Uptake		
Substrate	*n* Atoms (0/min/mg)	Control Range
Glutamate/malate	26.5	53.3–312.0
Succinate	34.5	68.6–376.1
TMPD-ascorbate[a]	65.4	124–467.7
Respiratory-Chain Enzyme Assays		
COX Complex	nmol/min/mg	Control Range
Complex I	167.1	(27.8–190.0)
Complex II/III	87.3	(18.3–234.3)
Complex IV[b]	2.4	(5.4–46.0)
Citrate synthase	1,745.92	(289.7–1,916.5)

[a] TMPD = N,N,N′,N′-tetramethyl-*p*-phenylenediamine.
[b] Units measured were k/min/mg.

mitochondria from a control and the patient. The traces were biphasic. The fast component (K_{obs} = 500–600 s^{-1}) was the result of CO recombination with contaminating hemoglobin (or myoglobin). The slow component (K_{obs} = 60–65 s^{-1}) arose from CO recombination with heme a_3. The major component (~95%) of the CO recombination signal obtained with the COX-deficient mitochondria was due to hemoglobin. In the control trace, the main signal (~65%) arose from CO reaction with heme a_3 (K_{obs} =~ 60 s^{-1}). The concentration of heme a_3, estimated from the slow-component photolysis signal by use of an extinction coefficient (ε) of 113 $mM^{-1}(cm^{-1})$ at 430–445 nm, was ~0.7 nM in the patient sample and ~11 nM in the control sample. heme a_3 content was measured in a second control sample at a concentration of 6 nM (data not shown). It appeared, therefore, that CO-reactive heme a_3 content was significantly decreased in the mitochondria from the patient.

mtDNA studies

Southern blot and long-range PCR analyses excluded the presence of large-scale rearrangements of mtDNA in total genomic DNA extracted from the patient's muscle (data not shown). In view of the immunohistochemical and immunoblot findings, we performed further analysis of mtDNA (Rahman et al. 1997). Direct-sequence analysis revealed a novel thymine to adenine transversion at nucleotide position 7,671 of mtDNA (T7671A), within the gene encoding COX II (fig. 4), resulting in a missense substitution of a lysine for a methionine at amino acid residue 29 of the polypeptide. Other base changes from the Anderson sequence (Anderson et al. 1981) were either previously reported or were silent polymorphisms, except for a previously unreported change in the D-loop (G468A), which appeared homoplasmic in the sequence chromatogram.

The T7671A mutation was present at 90% in DNA extracted from both skeletal muscle biopsies (done at age 11 years and age 14 years) but at only a low percentage in the patient's blood (4.5%–6%). Single-fiber PCR analysis in muscle samples revealed a significantly higher mean mutant load in COX-negative muscle fibers (81%, n = 13) than in COX-positive fibers (45%, n = 4) (P = .01, Mann-Whitney U test). The mutation was not detected in the mother's blood, nor in 110 normal controls, nor in 15 individuals with mitochondrial disease in whom the underlying mutation was not known.

Cell-Culture Studies

No mutation was detected in cultured myoblasts or fibroblasts at early cell passage, and staining for COX activity and COX I immunoreactivity and spectrophotometric assays of COX activity were normal in these cultures (data not shown). Immunoblot analysis of COX subunits was also normal in cell protein extracts, prepared from myoblast and fibroblast cell pellets (data not shown)

Discussion

We describe a patient with isolated COX deficiency. Biochemical, immunohistochemical, and immunoblot analyses strongly suggested a mutation in the mtDNA gene for COX II. A missense thymine to adenine transversion at nucleotide position 7,671 was identified in *CO II,* and evidence for an etiologic role of this mutation is presented. Sequencing of the remaining mtDNA molecule did not identify any other base changes of likely pathogenic relevance.

Pathogenicity of the T7671A mutation is supported by several lines of evidence. First, the mutation is het-

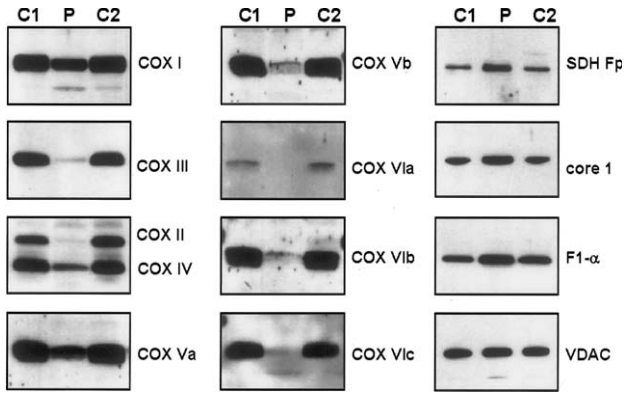

Figure 2 Immunoblot analysis of skeletal muscle mitochondrial fractions, from the patient (P) and two controls (C). Blots were developed with subunit-specific monoclonal antibodies to COX subunits, the flavoprotein subunit of SDH (SDH F_p), core protein 1 of complex III (core 1), the α subunit of F_1-ATP synthase (F_1-α), and VDAC.

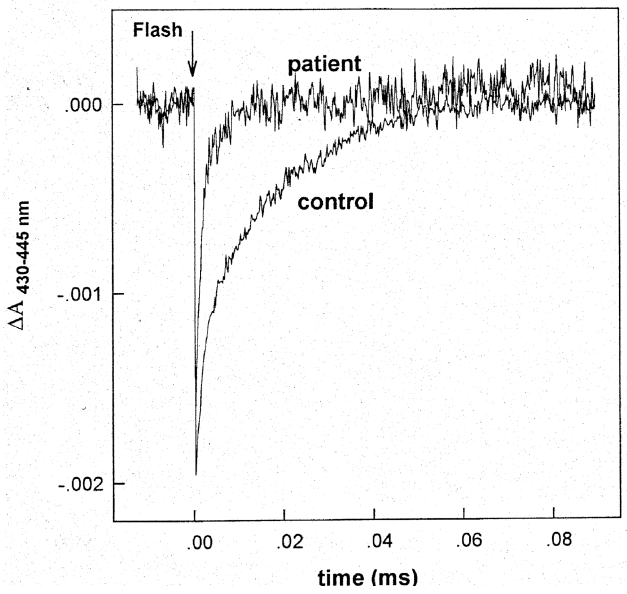

Figure 3 Flash photolysis and recombination spectra of the CO-ferroheme a_3 compound of dissolved muscle mitochondria, from the patient and a control. After reduction by sodium dithionite and treatment with CO, laser-flash photolysis and recombination of CO was monitored at 430 − 445 nm versus time.

eroplasmic and present at high levels of mutant load (90%) in skeletal muscle, the only clinically affected tissue, but at very low levels of mutant load (~6%) in blood. Second, this genotype-phenotype correlation is further supported by the single-fiber PCR studies, which demonstrated significant correlation between mutant-load level and COX activity in individual muscle fibers. Third, the mutation alters an amino acid residue that is relatively conserved in vertebrates (fig. 5). A noncharged methionine residue is replaced by a basically charged lysine, in the middle of the first N-terminal membrane-spanning domain of COX II (Tsukihura et al. 1996). Although the noncharged methionine residue is replaced by other noncharged amino acid residues, in some species, there is no known case in which it is substituted for a charged amino acid residue. Fourth, the mutation was not identified in a large number of ethnically matched control subjects.

The mechanism of pathogenesis of the T7671A mutation may be inferred from analogy with known yeast mutations. Yeast studies have allowed classification of COX mutations into two major groups: "activity" mutations, and "assembly" mutations that affect the assembly and/or stability of the COX holoenzyme (Meunier and Rich 1998*b*). Activity mutations are generally clustered in *CO I* and affect the electron-transfer function or the proton-pumping function of the enzyme. COX II is anchored to the mitochondrial inner membrane with an N-terminal helix-hairpin, whereas its

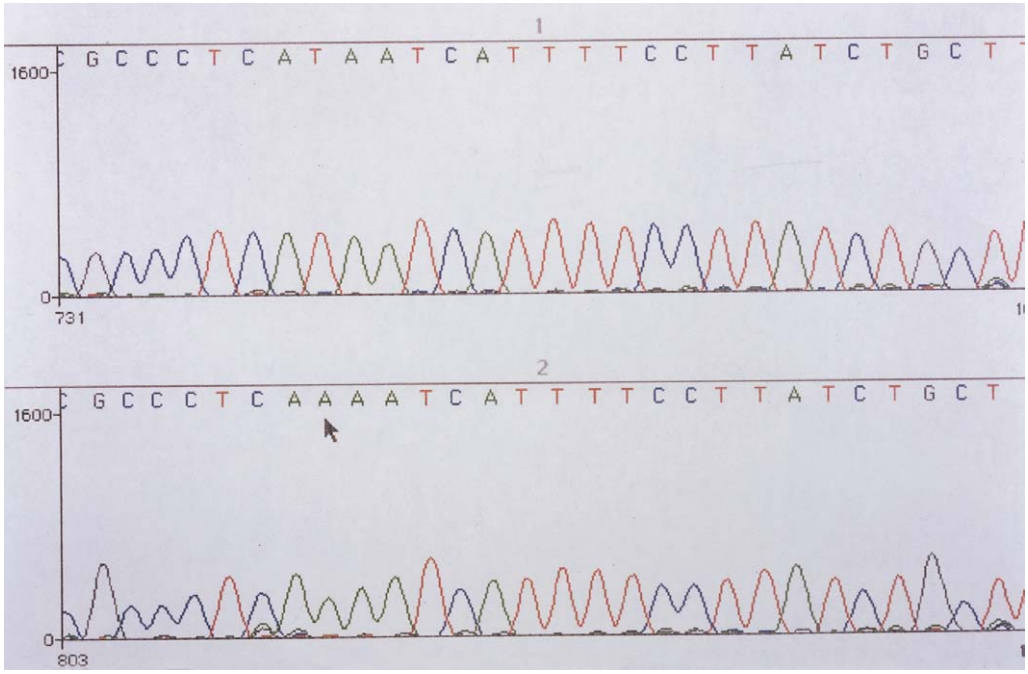

Figure 4 Sequence chromatogram of mitochondrial COX II gene, in control sequence (top) and patient's sequence (bottom). The arrow indicates mutated base (adenine in place of wild-type thymine).

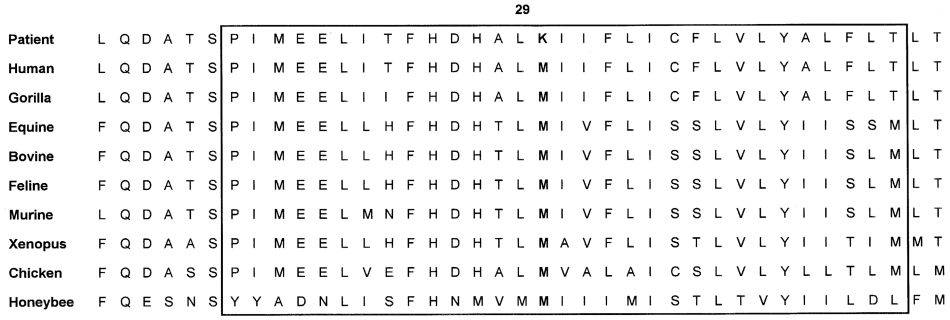

Figure 5 Alignment of amino acid sequence in COX subunit II (mitochondrial genome sequence data obtained from gopher directory at the Molecular Evolution and Organelle Genomics web site). The large box represents the first α-helical region in the bovine structure (Tsukihara et al. 1996). The mutation in our patients involves amino acid residue 29.

large C-terminal hydrophilic domain, which protrudes into the intermembrane space, contains the mixed-valence binuclear Cu_A center and serves as the docking site for cytochrome *c* (Capaldi 1990; Tsukihara et al. 1996). The mutation, in our patient, converts an uncharged amino acid residue in the highly conserved transmembrane segment of the COX II polypeptide into a basic charged residue (fig. 6). The mutation is, therefore, predicted to interfere with the anchoring of COX II within the mitochondrial membrane and to affect the assembly or stability of the COX holoenzyme, rather than the electron-transfer function of the subunit.

Studies of the assembly of COX in cultured human cells have identified two assembly intermediates of the

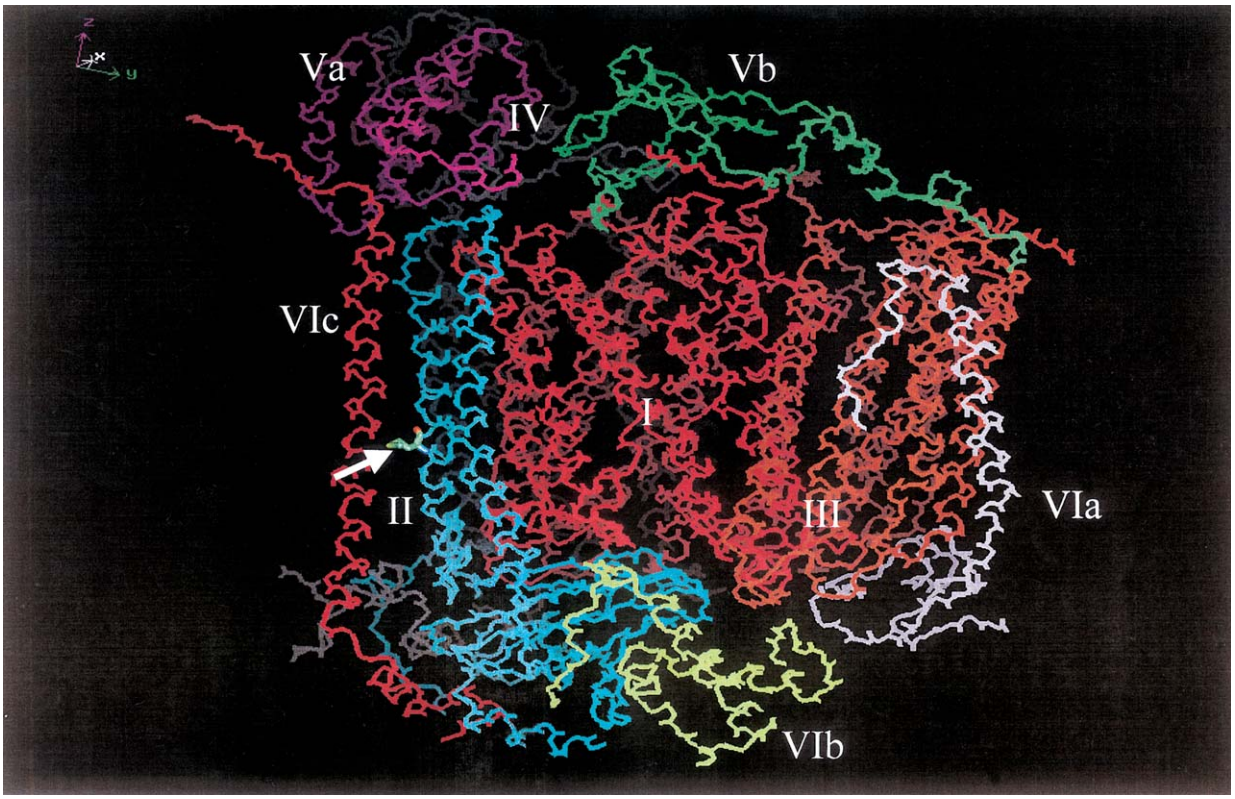

Figure 6 Structure of monomer of bovine cytochrome *c* oxidase. The diagram shows the assembled bovine holoenzyme, with an arrow indicating the site, in subunit II, that is mutated in our patient. The diagram was constructed with use of the data published by Tsukihara et al. (1996) in the Quanta program.

enzyme (Nijtmans et al. 1998). The first contains subunits I and IV. Subunits II and III are subsequently added, together with subunits Va, Vb, VIb, VIc, VIIa or VIIb, VIIc, and VIII. Finally, subunits VIa and VIIa or VIIb are incorporated, to complete the assembly of the holoenzyme. Results of the immunohistochemical and immunoblot analyses performed in our patient indicate that the COX subunit II mutation identified here arrests assembly of the COX holoenzyme after formation of the first assembly intermediate, because the components of this intermediate (subunits I and IV) are relatively preserved (as determined by immunoblotting) compared to the other subunits investigated.

To determine the steady-state levels of subunit I–associated heme a_3, we followed the CO flash-photolysis and recombination signals spectrophotometrically (Meunier and Rich 1998*a*). CO reacts with reduced COX and produces the CO-ferroheme a_3 compound. CO can be photolyzed by a high-intensity short-duration laser flash and will then recombine with the heme on a millisecond time-scale. CO photolysis and rebinding can be monitored optically and provide a sensitive means of quantitation of COX. Because of the high photolysis yield (>95%) and the high ε of the CO-COX compound (ε = 113 $mM^{-1}(cm^{-1})$ at 430–445 nm), it is possible to measure accurately COX content even in samples with low levels of enzyme. Quantitation of COX can be hindered by the presence of other hemoproteins, such as hemoglobin or myoglobin, which also react with CO. The optical signal induced by the binding of CO to these hemoproteins overlaps that of the CO compound of COX. However, the kinetics of CO recombination with hemoglobin and myoglobin are significantly faster ($K_{obs} > 500\ s^{-1}$) than the kinetics of CO recombination with COX (~60 s^{-1}), allowing the deconvolution of the contribution of COX from hemoglobin and myoglobin, especially when their concentration is low.

Immunohistochemical (fig. 1) and immunoblot (fig. 2) analyses showed that the apo–subunit I levels in the patient were only marginally affected. In contrast, subunit I–associated heme a_3 levels were markedly decreased in the patient (fig. 3). Taken together, our data suggest that a structural association of subunit II with subunit I is required to secure binding of the heme a_3 prosthetic group to apo-subunit I.

The surprisingly mild phenotype associated with the mutation in our patient probably reflects the tissue distribution of the mutation. It is likely that skeletal muscle, the only tissue affected clinically, is also the only tissue with a mutant-load level above the threshold required to reduce oxidative phosphorylation capacity. This threshold is generally ~85% for mitochondrial tRNA mutations but may be lower for protein-coding mutations (Hanna et al. 1998). The mutant load in skeletal muscle in our patient has not changed over a period of three years, and this is mirrored by clinical stability over this same period. Stability of mutant load was also observed in our patient with the stop mutation at nucleotide position 9,952 in the mitochondrial *CO III* gene (Hanna et al. 1998), and may be a common feature of mtDNA COX gene mutations. In contrast, mutant load has frequently been observed to increase with time in mtDNA tRNA mutations (Fu et al. 1996; Weber et al. 1997). The explanation for this is not clear. Demonstration of low levels of mutation in peripheral blood cells from our patient may reflect clearance of mutation in this rapidly dividing tissue, and this would be supported by the lower mutant load (4.5%) at 14 years compared to a mutant load level of 6% at 11 years.

The absence of the mutation in maternal blood, with the presence of mutation in both muscle and blood of the patient, suggests that the mutation may have arisen sporadically in early embryogenesis. However, the possibility of the presence of the mutation in the maternal germline cannot be excluded.

The T7671A mutation was not detected in cultured myoblasts, despite the high mutant load in mature skeletal muscle. The lack of mutation in satellite muscle cells has previously been reported with another COX point mutation (Hanna et al. 1998) and also with tRNA point mutations (Fu et al. 1996; Weber et al. 1997). Absence of the mutation from satellite cells has potential therapeutic implications. Previous studies have demonstrated that, in patients with strong segregation of mutation between satellite and mature muscle cells, induction of muscle necrosis—either by bupivicaine local anaesthesia (Clark et al. 1997) or by local trauma (Shoubridge et al. 1997)—was followed by repopulation of the muscle with cells containing only wild-type mtDNA. It remains to be seen if induction of widespread muscle necrosis will be a viable therapeutic option for such patients.

Acknowledgments

S.R. is a Medical Research Council Clinical Training Fellow. This work was also supported by the Research Trust for Metabolic Diseases in Children, the Muscular Dystrophy Association (Great Britain), and the Wellcome Trust. Mr. J. R. Muddle performed the quantitation of COX-negative fibers by image analysis.

Electronic-Database Information

The URL for data in this article is as follows:

Molecular Evolution and Organelle Genomics, http://megasun.bch.umontreal.ca (for COX subunit II mitochondrial genome sequence data)

References

Adams PL, Lightowlers RN, Turnbull DM (1997) Molecular analysis of cytochrome c oxidase deficiency in Leigh's syndrome. Ann Neurol 41:268–270

Anderson S, Bankier AT, Barrell BG, De Bruijn ML, Coulson AR, Drouin J, Eperon IC, et al (1981) Sequence and organization of the human mitochondrial genome. Nature 290: 457–465

Capaldi RA (1990) Structure and function of cytochrome c oxidase. Annu Rev Biochem 59:569–596

Ciafaloni E (1992) MELAS: clinical features, biochemistry and molecular genetics. Ann Neurol 31:391–398

Clark KM, Bindoff LA, Lightowlers RN, Andrews RM, Griffiths PG, Johnson MA, Brierley EJ, et al (1997) Reversal of a mitochondrial DNA defect in human skeletal muscle. Nat Genet 16:222–224

Comi GP, Bordoni A, Salani S, Francheschina L, Sciacco M, Prelle A, Fortunato F, et al (1998). Cytochrome c oxidase subunit I microdeletion in a patient with motor neuron disease. Ann Neurol 43:110–116

Cooper JM, Mann VM, Schapira AHV (1992) Analyses of mitochondrial respiratory chain function and mitochondrial DNA deletion in human skeletal muscle: effect of ageing. J Neurol Sci 113:91–98

Darley-Usmar VM, Kennaway NG, Buist NR, Capaldi RA (1983) Deficiency in ubiquinone cytochrome c reductase in a patient with mitochondrial myopathy and lactic acidosis. Proc Natl Acad Sci USA 80:5103–5106

DiMauro S, Hirano M, Bonilla E, Moraes CT, Schon EA (1994) Cytochrome oxidase deficiency. In: Schapira AHV, DiMauro S (eds) Mitochondrial disorders in neurology. Butterworth-Heinemann, Oxford, pp 91–115

DiMauro S, Nicholson JF, Hays AP, Eastwood AB, Papadimitriou A, Koenigsberger R, DeVivo DC (1983) Benign infantile mitochondrial myopathy due to reversible cytochrome c oxidase deficiency. Ann Neurol 14:226–234

Filipe MI, Lake BD (1990) Histochemistry in pathology, 2d ed. Churchill Livingstone, Edinburgh

Fu K, Hartlen R, Johns T, Genge A, Karpati G, Shoubridge EA (1996) A novel heteroplasmic $tRNA^{Leu(CUN)}$ mtDNA point mutation in a sporadic patient with mitochondrial encephalomyopathy segregates rapidly in skeletal muscle and suggests an approach to therapy. Hum Mol Genet 5:1835–1840

Hanna MG, Nelson IP, Rahman S, Lane RJM, Land JM, Heales S, Cooper MJ, et al (1998) Cytochrome c oxidase deficiency associated with the first stop-codon point mutation in human mtDNA. Am J Hum Genet 63:29–36

Hartley A, Cooper JM, Schapira AHV (1993) Iron induced oxidative stress and mitochondrial dysfunction: relevance to Parkinson's disease. Brain Res 627:349–353

Holt IJ, Harding AE, Cooper JM, Schapira AHV, Toscano A, Clark JB, Morgan-Hughes JA (1989) Mitochondrial myopathies: clinical and biochemical features of 30 patients with major deletions of muscle mitochondrial DNA. Ann Neurol 26:699–708

Keightley JA, Hoffbuhr KC, Buton MD, Salas VM, Johnston WSW, Penn AMW, Buist NRM, et al (1996) A microdeletion in cytochrome c oxidase (COX) subunit III associated with COX deficiency and recurrent myoglobinuria. Nat Genet 12:410–416

Lee N, Morin C, Mitchell G, Robinson BH, Saguenay LSJ (1998) Cytochrome oxidase deficiency: sequence analysis of nuclear encoded COX subunits, chromosomal localization and a sequence anomaly in subunit VIc. Biochim Biophys Acta 1406:1–4

Manfredi G, Schon EA, Moraes CT, Bonilla E, Berry GT, Sladky JT, DiMauro S (1995) A new mutation associated with MELAS is located in a mitochondrial DNA polypeptide-coding gene. Neuromusc Disord 5:391–398

Marusich MF, Robinson BH, Taanman J-W, Kim SJ, Schillace R, Smith JL, Capaldi RA (1997) Expression of mtDNA and nDNA encoded respiratory chain proteins in chemically and genetically-derived rho^0 human fibroblasts: a comparison of subunit proteins in normal fibroblasts treated with ethidium bromide and fibroblasts from a patient with mtDNA depletion syndrome. Biochim Biophys Acta 1362:145–159

Meunier B, Rich PR (1998*a*) Quantitation and characterization of cytochrome c oxidase in complex systems. Analyt Biochem 260:237–243

——— (1998*b*) Second-site reversion analysis is not a reliable method to determine distances in membrane proteins: an assessment using mutations in yeast cytochrome c oxidase subunits I and II. J Mol Biol 283:727–730

Moraes CT, Schon EA (1996) Detection and analysis of mitochondrial DNA and RNA in muscle by in situ hybridization and single-fiber PCR. Meth Enzymol 264:522–540

Nijtmans LGJ, Taanman JW, Muijsers AO, Speijer D, van den Bogert C (1998) Assembly of cytochrome-c oxidase in cultured human cells. Eur J Biochem 254:389–394

Rahman S, Blok RB, Dahl HHM, Danks DM, Kirby DM, Chow CW, Christodoulou J, et al (1996) Leigh syndrome: clinical features and biochemical and DNA abnormalities. Ann Neurol 39:343–351

Rahman S, Lake BD, Taanman J-W, Cooper JM, Hanna MG, Schapira AHV, Leonard JV (1997) Metab Dis (suppl 1) 20: 56

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual, 2d ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp 9.16–9.19

Shoubridge EA, Johns T, Karpati G (1997) Complete restoration of a wild-type mtDNA genotype in regenerating muscle fibers in a patient with a tRNA point mutation and mitochondrial encephalomyopathy. Hum Mol Genet 6: 2239–2242

Stoward PJ, Pearse AGE (1991) Histochemistry, 4th ed. Churchill Livingstone, Edinburgh

Taanman J-W, Bodnar AG, Cooper JM, Morris AAM, Clayton PT, Leonard JV, Schapira AHV (1997) Molecular mechanisms in mitochondrial DNA depletion syndrome. Hum Mol Genet 6:935–942

Taanman J-W, Burton MD, Marusich MF, Kennaway NG, Capaldi RA (1996) Subunit specific monoclonal antibodies show different steady-state levels of various cytochrome *c* oxidase subunits in chronic progressive external ophthalmoplegia. Biochim Biophys Acta 1315:199–207

Taanman J-W, Turina P, Capaldi RA (1994) Regulation of cytochrome c oxidase by interaction of ATP at two binding sites, one on subunit VIa. Biochemistry 33:11833–11841

Tiranti V, Hoertnagel K, Carrozzo R, Galimberti C, Munaro M, Granatiero M, Zelante C, et al (1998) Mutations of SURF-1 in Leigh disease associated with cytochrome c oxidase deficiency. Am J Hum Genet 63:1609–1621

Tsukihara, T, Aoyama H, Yamashita E, Tomizaki T, Yamaguchi H, Shinzawaitoh K, Nakashima R, et al (1996) The whole structure of the 13 subunit oxidized cytochrome c oxidase at 2.8 angstrom. Science 272:1136–1144

Weber K, Wilson JN, Taylor L, Brierley E, Johnson MA, Turnbull DM, Bindoff LA (1997) A new mtDNA mutation showing accumulation with time and restriction to skeletal muscle. Am J Hum Genet 60:373–380

Yasin R, Van Beers G, Nurse KC, Al-Ani S, Landon DN, Thompson EJ (1977) A quantitative technique for growing human adult skeletal muscle in culture starting from mononucleated cells. J Neurol Sci 32:347–360

Zhu ZQ, Yao JB, Johns T, Fu K, De Bie I, Macmillan C, Cuthbert AP, et al (1998) SURF1, encoding a factor involved in the biogenesis of cytochrome c oxidase, is mutated in Leigh syndrome. Nat Genet 20:328–343