Cytochrome c Oxidase-deficient Patients Have Distinct Subunit Assembly Profiles*

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Cytochrome c oxidase (COX) deficiency is the most common respiratory chain defect in childhood and is clinically heterogeneous. We report a study of six patients with COX deficiencies. Two of the patients had as yet undefined defects, three patients had Surf-1 mutations, and one patient had a 15-base pair deletion in the COX III subunit. We show that quantitative measurements of steady-state levels of subunits by monoclonal antibody reactivity, when used in combination with a discontinuous sucrose gradient method, provide an improved diagnosis of COX deficiencies by distinguishing between kinetic, stability, and assembly defects. The two mutants of undefined etiology had a full complement of subunits with one stable and the other partially unstable to detergent solubilization. Both are likely to carry mutations in nuclear-encoded subunits of the complex. The three Surf-1 mutants and the COX III mutant each had reduced steady-state levels of subunits but variable associations of the residual subunits. This information, as well as aiding in diagnosis, helps in understanding the genotype-phenotype relationships of COX deficiencies and provides insight into the mechanism of assembly of the enzyme complex.

Cytochrome c oxidase (COX) is the terminal enzyme complex of the respiratory chain. In eukaryotes, it is located in the inner mitochondrial membrane where it catalyzes the transfer of electrons from reduced cytochrome c to molecular oxygen (1). This reaction is coupled with the translocation of protons across the inner membrane, and the resulting electrochemical gradient is used to drive ATP synthesis and ion transport (2). Human COX is composed of 13 subunits, the three largest of which are encoded by mitochondrial DNA (3). In addition to these 13 structural subunits, there are many "assembly factors" required for the proper functioning of COX. In yeast, over 30 different genetic complementation groups for COX assembly have been reported (4, 5), and a number of genes involved in the assembly of yeast COX have been identified (6–9). In humans, COX assembly genes have been identified by several methods including functional complementation of yeast mutants (10), homology searches of the expressed sequence tags data base (11), and microcell-mediated chromosome transfer (12, 13).

COX deficiency is the most commonly recognized respiratory chain defect in childhood (14). The disease is clinically heterogeneous with phenotypes including Leigh syndrome, hepatic failure, and myopathies (12, 13, 15, 16). COX deficiency has been associated with mitochondrial DNA mutations in COX I, II, and III (17–20), with large-scale deletions of the mitochondrial genome (21) and with point mutations in mitochondrial tRNA genes (22). There have also been reports of autosomal inheritance of COX deficiency that have involved COX assembly factors including Surf-1 (12, 13), Sco-1 (23), Sco-2 (24, 25), and Cox10 (26). To date, there is no unequivocal evidence of a mutation in the nuclear-encoded structural COX subunits causing human disease. One patient with severe mitochondrial encephalomyopathy was reported to have an altered $K_m$ for reduced cytochrome c yet had no mutations in the mitochondrially encoded COX subunits (27). The defect in this patient was presumed to have arisen from a nuclear gene mutation, but no definitive data were provided.

With so many possible gene defects giving rise to COX deficiency, the screening of patients is complicated, and a protocol that localizes the possible mutations to groups of genes (e.g. mtDNA, nuclear-encoded structural subunit genes, or genes for assembly factors) would be useful. Recently the steady-state levels of cytochrome c oxidase subunits in tissues from 17 patients were examined by Western blotting using monoclonal antibodies made in this laboratory (28). These patients showed a range of subunit profiles. Some had normal levels of all COX subunits and were classified as candidates for mutations in nuclear-encoded structural genes. Other patients, including one Surf-1 mutant, had reduced levels of several of the subunits, which were classified as assembly mutants. More recently, immunohistochemistry has been used to study a set of COX-deficient patients, and similar conclusions were drawn (29). In that study, patients showed either a reduction in all of the subunits, none of the subunits, or just the mitochondrial encoded subunits.

Here, we have analyzed cytochrome c oxidase-deficient patients by subunit composition as before (28), but we added an analysis of patient mitochondria using sucrose gradient centrifugation. Our previous studies have established that this approach separates the complexes of oxidative phosphorylation (OXPHOS) efficiently and, in the case of complex I deficiencies, allows assembly of this complex to be examined (30, 31). As we show, in combination, the data from the two analyses improved the classification of cytochrome c oxidase deficiencies and provided insight into both phenotype-genotype relationships and assembly.

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1 The abbreviations used are: COX, cytochrome c oxidase; PD, population doubling; mtDNA, mitochondrial DNA.


EXPERIMENTAL PROCEDURES

**MR5 Fibroblasts**—MR5 fibroblasts were obtained from the American Type Culture Collection (Manassas, VA). The population doubling (PD) level of the cells was in the range of 35–45 at the time of harvesting. All cells were grown as described before in high glucose Dulbecco's modified Eagle's medium supplemented with 10% bovine calf serum, 50 μg/ml uridine, 110 μg/ml pyruvate, and 10 mM HEPES buffer to maximize growth rates (32). Rho0 MR5 fibroblasts were derived by culturing MRC5 fibroblasts (PD = 28–30) continuously in permissive medium supplemented with 50 ng/ml ethidium bromide for a further 16 population PDs.

**Patient Cell Lines**—Primary fibroblast cultures of Patients 1, 2, 3, and 4 were obtained from needle skin biopsies performed during the course of diagnostic evaluation at the Ospedale Pediatrico “Bambino Gesù,” Roma, Italy. The fibroblast culture of Patient 5 was obtained from the Hospital for Sick Children, Toronto, Ontario, Canada. The 100% mutant cybrid cell line containing a 15-base pair deletion of the COX III subunit (Patient 6) was kindly supplied by Dr. Michael King, Thomas Jefferson University, Philadelphia, PA. Specific details concerning this patient and the generation of the hybrid cell line were described previously (19, 33).

**Preparation of Mitochondria from Cell Lines**—Mitochondria were prepared from ~5 × 10⁶ cells by differential centrifugation as described previously (31).

**Activity Assays**—Cytochrome c oxidase activity was measured basically as described previously (34). Briefly, ~80–100 μg of protein from digitonized fibroblasts or homogenized muscle biopsy were added to a cuvette containing 1 ml final volume of 10 mM potassium phosphate, pH 7.4, 1 mg/ml cytochrome c reduced with sodium dithionite, and 0.025% of lauryl maltoside. The oxidation of reduced cytochrome c at 550 nm was then followed spectrophotometrically (extinction coefficient 19.0 mM⁻¹ cm⁻¹). Each reported value is the average of 2–5 independent measurements.

**Immunoblot Analysis**—Proteins were separated on 10–22% gradient SDS-polyacrylamide gels according to Laemmli (35). Western blotting was done as described previously (32), with the following modifications. The proteins were transferred electrophoretically to an 0.45-mm polyvinylidene difluoride membrane in Towbin buffer. Reactive bands were detected using the ECL Plus detection reagent (Amersham Pharmacia Biotech UK) and were imaged using the image analyzer Storm 860 (Molecular Dynamics, Sunnyvale, CA). Fluorescence was quantified using NIH Image and standardized to the loading control, porin.

**The porin monoclonal antibody** (Calbiochem, La Jolla, CA) was used at a 1:120,000 dilution in 5% milk/Ca²⁺/Mg²⁺-free phosphate-buffered saline. The polyclonal Surf-1 antibody was a kind gift from Dr. Eric Shoubridge, Montreal Neurological Hospital. All other monoclonal antibodies used were prepared at the University of Oregon and used in the following concentrations: complex I-39 kDa (2 μg/ml), complex II-30 kDa (5 μg/ml), complex II-70 kDa (0.01 μg/ml), complex III-core 2 (0.4 μg/ml), complex IV (4 μg/ml), COX II (2 μg/ml), COX I (2 μg/ml), COX I (2 μg/ml), COX II (2 μg/ml), and COX IV (0.5 μg/ml).

**Sucrose Gradient Centrifugation**—Mitochondria (1 mg) were resuspended at a protein concentration of 5 mg/ml in 1 ml MEA, 1 μg/ml peptatin, 1 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, 1% lauryl maltoside, 100 mM Tris/HCl, pH 7.8. The mitochondria were incubated in this solution for 20 min on ice with stirring before insoluble membranes were pelleted by centrifugation (10,000 × g, 10 min, 4°C).

**RESULTS**

**Patient Characterization**—Cell lines from six patients demonstrating different degrees of COX deficiency were studied. The clinical presentations of these patients and specific mutations that were identified are shown in Table I. In muscle biopsy samples from Patients 1 and 2, the COX activity was drastically reduced by 77 and 76%, respectively, compared with control values. On the other hand, the COX activity in fibroblasts was much higher, with Patients 1 and 2 losing only 30 and 20% of enzyme activity, respectively. It is not unusual for there to be tissue specificity of a defect when mtDNA mutations are involved, because mtDNA replication and inheritance are stochastic in somatic cells and can result in different mutational loads in different tissues (36). However, muscle biopsies from Patients 1 and 2 were screened for mutations in the mitochondrially encoded COX subunits I, II, and III, and no mutations were found. The defects in these cell lines are thus predicted to be nuclear-encoded. These two patients were also screened for mutations in the COX assembly factors Surf-1, Sco-1, and Sco-2, but again no mutations were found. The unusual tissue specificity of these two mutants is discussed in more detail later.

In three of the patients (Patients 3, 4, and 5), we identified a SURF-1 mutation, resulting in undetectable levels of the Surf-1 protein (Fig. 1). Two of these are frameshift mutations, and produce a premature translation termination. The third (Patient 5) harbored a deletion between exons 1 and 2. Western blotting of Patient 5 using a different antibody from that used here has shown the presence of some Surf-1 of altered migration. Thus, the mutation could result in an incorrectly spliced version of Surf-1. Patient 4 presented with tubulopathy in addition to Leigh syndrome, which is the most common clinical presentation associated with Surf-1 mutations. Patient 6 had a previously reported 15-base pair deletion in COX III (19, 33).

**Western Blot Analysis of Patient Cell Lines**—Western blot analysis was performed on mitochondria isolated from all six...
patient cell lines along with control MRC5 fibroblasts and MRC5 fibroblasts that had been depleted of mitochondrial DNA (rho0). Individual protein signals were normalized using the porin signal as a control for equal loading. The signals of all proteins were set to 100% for the control MRC5 fibroblasts, and the levels of the individual proteins in other cell lines are reported relative to this value. The quantitative subunit profiles for each cell line appear in Fig. 2. For Patients 1 and 2, all of the COX subunits probed were present at normal levels (Fig. 2). Thus, the subunit profile alone does not provide diagnostic information. The data for Patients 1 and 2 contrast with those of Patients 3–6, each of which had a significant reduction in all of the COX subunits probed. As shown in Fig. 2, the levels of subunits I, II, IV, Va, and Vc were quantitatively similar in Patients 3 and 6, one a Surf-1 mutant and the second a COX III mutant. The levels of subunits in the other two Surf-1 mutants were different from Patient 3. Patient 5 had much more of subunits II and Vc, whereas Patient 4 showed intermediate expression with more of subunits II and Vc than Patient 3 but less than Patient 5. Thus, steady-state levels alone do not distinguish mtDNA from Surf-1 mutations.

Sucrose Gradient Analysis of Patient Cell Lines—Mitochondria from the six patient cell lines, control MRC5 fibroblasts, and rho0 MRC5 fibroblasts were subjected to sucrose gradient centrifugation. In the gradient, complexes are separated at a rate depending largely on their size. The positions of complexes in the gradient can then be determined by Western blotting of the fractions with monoclonal antibodies directed against particular subunits. Ten fractions were collected from each gradient, and Western blots were performed on the fractions from each of the gradients using antibodies to complex I-39 kDa subunit, complex V-α, complex III-core 2, complex II-30 kDa subunit, and COX subunits I, II, IV, and Va. Fig. 3 shows a representative Western blot from a sucrose gradient of mitochondria from Patient 4. For simplicity of presentation, the data from each sucrose gradient are graphed by setting the highest intensity signal for each antibody in each gradient to 100% and expressing the levels of each subunit relative to this value. The quantitative subunit profiles for each cell line appear in Fig. 2. For Patients 1 and 2, all of the COX subunits probed were present at normal levels (Fig. 2), but the sucrose gradient shows a fully assembled complex (Fig. 4C). This patient may have a kinetic defect. Patient 2 had a reduction of COX activity of 20%. In this case, there was evidence of instability of the complex to detergent treatment, as a portion of subunits IV and Va were found dissociated from the core complex (Fig. 4D).

Sucrose gradient evaluation of Patients 3, 4, and 5, each with mutations in the COX assembly factor, Surf-1, confirmed that these are all assembly mutants and showed that there are differences between them in the amount and nature of the partially assembled complex (Fig. 4, E–G). A fraction of subunits I, IV, Va, and all of the residual subunit II present were found at the position of fully assembled COX. Both Patients 3 and 4 retained significant COX activity (see Table I), which is related to the amount of COX II present and thereby the amount of assembled complexes. In both of these patients, there were fractions containing subunits I + II + IV + Va, I + IV + Va, and IV + Va. The third Surf-1 mutant (Patient 5) is different from the other Surf-1 patients in regard to both steady-state levels of subunits and in the assembly profile. This patient, with only 11% residual activity, showed no fully assembled enzyme. However, there were fractions containing I + II, I + Va, and IV + Va.

Patient 6, with a 15-base pair deletion in the COX III subunit, also exhibited a severe assembly defect, with each of the four subunits examined shifted in the gradient to lower molecular weight fractions compared with controls (Fig. 4H). As in all the assembly mutants, subcomplexes containing subunits I + II and IV + Va were detected.

**DISCUSSION**

COX deficiency presents with a plethora of phenotypes, which is not surprising given the complexity of enzyme structure and the multiple factors and many steps required for
assembly of this, the terminal oxidase of the respiratory chain (37). The challenge in patient diagnosis is both to decide whether the condition is due to a COX defect alone and to localize the possible mutations to a single or limited number of genes. A functional COX requires three mitochondrial encoded subunits, 10 nuclear-encoded subunits, some of which are tissue specific, and an as yet unknown number of assembly factors that includes Cox10, Sco-1, Sco-2, and Surf-1 (12, 13, 23–26). Mutations of mtDNA include deletions that cause respiratory chain deficiencies involving multiple respiratory chain complexes, including COX and mutations in tRNA that cause MERRF (myoclonus epilepsy and ragged red fibers) and MELAS (mitochondrial encephalomyopathy with lactic acidosis and stroke-like episodes), which also have defects in COX (36). Alternatively, there can be mutations in any of the three mitochondrial encoded COX genes (for subunits I, II, and III) as represented by Patient 6 in this study. An added complication in understanding the genotype-phenotype relationships of mtDNA mutations is the heteroplasmry, i.e. the presence of the mutation in only a fraction of the thousands of copies of the mitochondrial genome. The result is that cells contain a mixture of normal and defective enzyme complexes giving a proportional activity that can affect the functioning of different tissues differently, i.e. the threshold effect (36).

Mutations of nuclear genes can occur in the structural subunits of the COX complex (subunits IV, Va, Vb, VIa, VIb, VIc, VIIa, VIIb, VIIc, or VIII), two of which (VIa and VIIa) have tissue-specific isoforms in humans (34). If the incorporation of nuclear-encoded subunits into the complex is an early event, defects due to these subunits could affect the assembly, stability, and thus the steady-state levels of enzyme or cause a change in the kinetics of enzyme turnover. Finally, there can be mutations in any of a growing number of so called assembly factors that are required to produce a functional COX. One of these factors is Surf-1, represented in three patients in this study. Thus, the routine sequencing of all the possible genes involved will be laborious and even then not definitive until all the factors required are identified.

In the meantime, analysis of the proteins has allowed some differentiation of likely sites of mutation. The studies of von Kleist-Retzow et al. (28) and Rahman et al. (29) show the variation in steady-state levels of COX subunits in COX deficiencies, some of which are due to mtDNA defects, others undefined, and one a Surf-1 mutant. This information provides some indication of which defects allow enzyme assembly and which do not, based on the ratios of subunits. However, the work was not done quantitatively, and assembly was not examined directly. Another recent study has examined several Surf-1 mutants by native blue gel electrophoresis, which, in principle, gives steady-state levels and assembly information (38). In this method, Tiranti and colleagues (38) were able to show that, in the absence of Surf-1, there was some COX assembly of this, the terminal oxidase of the respiratory chain (37). The challenge in patient diagnosis is both to decide whether the condition is due to a COX defect alone and to localize the possible mutations to a single or limited number of genes. A functional COX requires three mitochondrial encoded subunits, 10 nuclear-encoded subunits, some of which are tissue specific, and an as yet unknown number of assembly factors that includes Cox10, Sco-1, Sco-2, and Surf-1 (12, 13, 23–26). Mutations of mtDNA include deletions that cause respiratory chain deficiencies involving multiple respiratory chain complexes, including COX and mutations in tRNA that cause MERRF (myoclonus epilepsy and ragged red fibers) and MELAS (mitochondrial encephalomyopathy with lactic acidosis and stroke-like episodes), which also have defects in COX (36). Alternatively, there can be mutations in any of the three mitochondrial encoded COX genes (for subunits I, II, and III) as represented by Patient 6 in this study. An added complication in understanding the genotype-phenotype relationships of mtDNA mutations is the heteroplasmry, i.e. the presence of the mutation in only a fraction of the thousands of copies of the mitochondrial genome. The result is that cells contain a mixture of normal and defective enzyme complexes giving a proportional activity that can affect the functioning of different tissues differently, i.e. the threshold effect (36).

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**Fig. 4.** Graphical representation of the position of four COX subunits in sucrose gradients. Western blot signals were quantitated, and for each antibody, the fraction with the highest intensity signal was set to 100%. The relative amount of signal found in the other fractions was then represented as a percentage of this value. Shown are data from: A, control MRC 5 fibroblasts; B, rho0 MRC 5 fibroblasts; C, Patient 1; D, Patient 2; E, Patient 3; F, Patient 4; G, Patient 5; and H, Patient 6. COX I is represented in purple, COX II in pink, Cox IV in yellow, and COX Va in green.
present in fully assembled complexes along with partially assembled complexes. These complexes contained subunit I, the only subunit examined in their study.

Here, we have conducted an analysis of subunit steady states based on gel electrophoresis followed by Western blotting, but in a more quantitative way than done previously. Samples from the same six patients studied were then examined by sucrose gradient centrifugation, which allows assembly of the enzyme complex to be evaluated. By adding the sucrose gradient method, we are able to distinguish variants in features of the COX complex not evident from steady-state levels of subunits alone. A comparison of Patients 1 and 2 exemplifies this point. Both patients have reduced cytochrome c oxidase activity but normal levels of all the subunits. In Patient 1, the enzyme appears fully assembled, but in Patient 2, there is an altered sedimentation in the gradient indicative of reduced stability of the complex. The mtDNA of both patients was sequenced, and no mutation was found. Therefore, both must have a mutation in a nuclear gene. In the case of Patient 1, the most likely scenario is that there is a mutation in one of the nuclear-encoded COX subunits that affects enzyme turnover. One candidate is subunit Vb, mutations of which have been found to alter enzyme kinetics in yeast (39). Other possibilities are subunits IV and VIa, which regulate COX activity in response to ATP binding (40, 41). A mutation in a nuclear-encoded COX subunit is also the likely cause of the altered stability of the enzyme in Patient 2. The mutation could be in subunits IV or Va, which are released from the complex by the detergent treatment and thus less tightly bound. Apart from interacting with themselves, these two subunits interact mainly with subunit I (3), so it is unlikely that mutation in other nuclear-encoded subunits would have the observed effect. An interesting feature of Patients 1 and 2 is that their levels of COX activity were significantly lower in muscle biopsy (23 and 24% respectively) than in fibroblasts in culture. This apparent tissue specificity may be an added indicator of the locus of the mutation, perhaps pointing to subunits VIa or VIIa, which have two isoforms in humans. Other possibilities include mutations in as yet undefined assembly factors/chaperones that have tissue specific isoforms. Additional studies are ongoing with these two patients.

Four of the six patients examined here showed clearly altered assembly of COX. This is already suggested for Patients 3, 4, and 6 by the variable steady-state levels of each subunit. In the case of Patient 5, all subunits studied were present in equivalent amounts but at 50% of wild type. The sucrose gradient analysis differentiates these assembly mutants further and can resolve different mutations within the Surf-1 assembly factor by the patterns of subunit associations. In two of the Surf-1 mutations there is an amount of fully assembled enzyme present, which correlates with the amount of residual activity. All of the subunit II is in this fraction. In addition, there are partial assemblies of subunits. The third Surf-1 mutant, with higher levels of subunits, is very different from the two described above. No fully assembled enzyme is detected. Thus, either no COX was ever properly assembled (although the cell lines have 11% residual COX activity), and/or any complex that does assemble is much more labile and disrupted by centrifugation in the detergent lauryl maltoside. Patient 6, with a subunit III mutation, looks very much like Patients 3 and 4, who have defined Surf-1 mutations based on the steady-state levels of subunits, but is more like Patient 5 in having no fully assembled complex. As with Patient 5, the subunit profile on the sucrose gradient could result from failure of the enzyme to assemble, instability of the assembled enzyme, or both effects. However, it has proved possible to remove subunit III from mammalian cytochrome c oxidase without disrupting the core of the enzyme complex (subunits VIa, VIb, and VIIa are also lost) while retaining electron transfer activity (42–44); this would argue that the mutation in COX III affects assembly directly. It is likely then that COX III has some chaperone-type role in assembly, in addition to any (as yet poorly defined) functional role in the complex.

The sucrose gradient analyses of the six patients reveal at least two partial assemblies of subunits. Subunits IV and Va remain associated in Patients 2–6, in each case running together ahead of the position expected of individual subunits. These two subunits are found in a stable aggregate, even in enzyme missing the three mitochondrially encoded subunits, i.e. in rho0 cells. It is generally agreed that subunit I is inserted into the mitochondrial membrane early in assembly with association of subunit II and binding of hemes and copper atoms facilitated by the assembly factors Surf-1, Sco-1, Sco-2, and Cox10 (23, 26, 38, 45, 46). Nuclear-encoded subunits and COX III are thought to be added later in the assembly process (45). It appears that COX IV and Va form a separate subassembly, possibly with other subunits such as Vb or VIIb (not studied here), and that the core part interacts in one step of assembly with the preformed complex of nuclear-encoded subunits. In summary, we have extended the methodology currently available for studying COX deficiencies by adding a sucrose gradient step to Western blotting studies that define which subunits are present but not their assembly state. The information provided by sucrose gradient centrifugation in conjunction with monochondal antibodies differentiates and classifies COX mutants more finely than previously possible. Further studies of COX deficiencies, as here, will aid in defining complementation groups of defects by protein rather than by DNA analysis. The data obtained as different mutants are examined should also shed more light on the pathway of assembly of this complex.

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