Cytochrome c Oxidase Subassemblies in Fibroblast Cultures from Patients Carrying Mutations in COX10, SCO1, or SURF1*

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Cytochrome c oxidase contains two redox-active copper centers (Cu₄ and Cu₆) and two redox-active heme A moieties. Assembly of the enzyme relies on several assembly factors in addition to the constituent subunits and prosthetic groups. We studied fibroblast cultures from patients carrying mutations in the assembly factors COX10, SCO1, or SURF1. COX10 is involved in heme A biosynthesis. SCO1 is required for formation of the Cu₄ center. The function of SURF1 is unknown. Immunoblot analysis of native gels demonstrated severely decreased levels of holoenzyme in the patient cultures compared with controls. In addition, the blots revealed the presence of five subassemblies: three subassemblies involving the core subunit MTCO1 but apparently no other subunits; a subassembly containing subunits MTCO1, COX4, and COX5A; and a subassembly containing at least subunits MTCO1, MTCO2, MTCO3, COX4, and COX5A. As some of the subassemblies correspond to known assembly intermediates of human cytochrome c oxidase, we think that these subassemblies are probably assembly intermediates that accumulate in patient cells. The MTCO1-COX4-COX5A subassembly was not detected in COX10-deficient cells, which suggests that heme A incorporation into MTCO1 occurs prior to association of MTCO1 with COX4 and COX5A. SCO1-deficient cells contained accumulated levels of the MTCO1-COX4-COX5A subassembly, suggesting that MTCO2 associates with the MTCO1-COX4-COX5A subassembly after the Cu₄ center of MTCO2 is formed. Assembly in SURF1-deficient cells appears to stall at the same stage as in SCO1-deficient cells, pointing to a role for SURF1 in promoting the association of MTCO2 with the MTCO1-COX4-COX5A subassembly.

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The fact that only contains a CuB site (19). This observation together with mutation and co-immunoprecipitation studies in yeast (26, 27) C− suggest that SCO proteins transfer copper to the CuB site. An unrelated Cu−-binding protein, COX11, appears to be involved in the provision of copper to the CuB site (28, 29). It is not clear why two SCO proteins are present in human mitochondria. Mutations in SCO2 have been reported in several unrelated patients (21, 30, 31), but only a single family with SCO1 mutations has been described (32). The patients from this family carried a frameshift mutation on one allele, introducing a premature termination codon, and a missense mutation on the other allele, resulting in a proline to leucine replacement immediately adjacent to the CXXC motif. Cytochrome c oxidase activity was markedly decreased in patient tissues, but there was still some residual activity, suggesting that the amino acid substitution does not completely eliminate SCO1 function.

COX10 is a farnesyltransferase that catalyzes the conversion of heme B (ferric protoporphyrin IX) to heme O (33, 34). Heme O is subsequently converted into heme A in a biosynthetic pathway involving COX15 (35). COX10 and COX15 are considered constituents of the mitochondrial inner membrane. Studies in yeast have indicated that both proteins are necessary for heme biosynthesis (36, 37). Three families with COX10 gene mutations have been documented (38, 39). Patients were either homozygous or compound heterozygous for missense mutations, changing evolutionarily conserved amino acid residues. Their tissues retained some residual cytochrome c oxidase activity, suggesting that the mutated farnesyltransferase was still partially active. Consistent with this view, a mutant human COX10 was able to complement the respiratory-defect of a yeast cox10 null mutant when present on a high copy plasmid but, unlike wild-type human COX10, not when present on a low copy plasmid (38).

The fibroblast cultures which we investigated were derived from four unrelated patients with SURF1 mutations (40, 41), a patient with SCO1 mutations (32), and a patient with a COX10 mutation (38). Quantitative immunoblot analysis of native gels revealed residual levels of fully assembled enzyme in all cultures but also some accumulation of subassemblies. The subunit composition of the subassemblies identified in the patient cells provides insights into the temporal positioning of MTCO1, COX4, and COX5A assembly, heme A insertion, and formation of the CuA center within the assembly pathway of human cytochrome c oxidase.

EXPERIMENTAL PROCEDURES

Cell Cultures—Fibroblasts were grown from explant cultures of skin biopsies derived from four infants carrying SURF1 mutations (P1–4), one infant carrying SCO1 mutations, one infant carrying a COX10 mutation, and four pediatric controls. All patients harboring SURF1 mutations suffered from Leigh syndrome and died at <4 years of age (40, 41). The patient harboring SCO1 mutations presented with liver failure and encephalopathy and died at age 2 months (32). The patient harboring a COX10 mutation presented with tubulopathy and leukodystrophy, and died at 2 years of age (38). The mutations carried by the patients have been reported elsewhere (32, 38, 40, 41). Control skin biopsies were taken from children younger than 3 years undergoing orthopedic surgery. Informed parental consent, in accordance with the guidelines of the participating institutions, was obtained for all biopsies. The A549 human lung carcinoma cell line was obtained from the European Collection of Cell Cultures (Salisbury, UK). The cell line was depleted of its mtDNA by long term exposure to ethidium bromide to yield cell line A549/H (42). Cells were cultured under standard conditions in medium supplemented with 110 mg/ml sodium pyruvate and 50 μM uridine to allow the respiratory chain-deficient cells to grow (42).

Preparation of Mitoplasts and Mitochondria—Mitoplast-enriched pellets were prepared from fibroblasts as described (44), using a solution of 4 mg/ml digitonin. Mitoplasts were washed with phosphate-buffered saline (PBS). Mitochondria were isolated by differential centrifugation of digitonin-treated, homogenized cells (45). Samples were stored at −80°C. Protein concentrations were determined with the BCA™ protein assay kit (Pierce).

Enzyme Activity Assays—Cytochrome c oxidase and citrate synthase activities of mitochondrial preparations were measured spectrophotometrically essentially as described (46, 47). Each reported value is the mean of 4–6 independent measurements. Histochemical staining for cytochrome c oxidase activity in native gels was performed as described previously (48).

Primary Antibodies—We used mouse monoclonal antibodies raised against human MTCO1 or MTCO2 (49), yeast MTCO3 (50), and bovine COX4, COX5A, COX5B, COX6B, or COX6C (51). In addition, we used a rabbit polyclonal antiserum against succinate:ubiquinone oxidoreductase (SDHIA; Molecular Probes) (52), the voltage-dependent anion-selective channel (VDAC; monoclonal 3HIL, Calbiochem), and cytochrome c. The preparation of mitoplasts and mitochondria has been described previously (48).

Immunoblot Analysis—Gel electrophoresis and blotting was carried out with the Mini Protein®3 System (Bio-Rad). For immunoblot analysis of one-dimensional denaturing gels, mitochondrial fractions were dissociated in 50 mM Tris–HCl (pH 6.8), 12% glycerol, 4% SDS, 2% β-mercaptoethanol, and 0.01% bromphenol blue for 30 min at 37°C and resolved on either 7.5, 12.5, or 15% polyacrylamide, 0.1% SDS, 5.5 M urea gels run according to Laemmli (53). For immunoblot analysis of one-dimensional native gels, mitochondrial or mitoplast fractions were solubilized with n-dodecyl-β-maltoside, as described elsewhere (11, 54), and resolved on blue native 8–16% polyacrylamide gels, as devised by Schagger (55). For immunoblot analysis of two-dimensional native/ denaturing gels, n-dodecyl-β-maltoside-solubilized mitochondrial fractions were first separated on blue native 8–16% polyacrylamide gels, followed by separation of single sample lanes on 13.5% polyacrylamide, 0.1% SDS, 5.5 M urea gels. First dimension gel lanes were soaked in 50 mM Tris–HCl (pH 6.8), 1% SDS, 1% β-mercaptoethanol for 15 min, followed by two 10-min soaks in 50 mM Tris–HCl (pH 6.8), 1% SDS. Gel strips were mounted horizontally in the Mini Protein®3 System, and second dimension gels were poured around them, with a 3% polyacrylamide stacking gel surrounding the first dimension gel strip.

Proteins were electrotransferred from the gels onto Immobilon™-P polyvinylidine difluoride membranes (Millipore, Bedford, MA) (56). Blots were air-dried overnight, rinsed three times with PBS, incubated with residual dye, rinsed with PBS, and incubated in PBS, 10% nonfat dry milk for 1 h to saturate protein-binding sites. Subsequently, blots were incubated with primary antibodies in PBS, 0.3% Tween 20 for 2 h. After this 10-min washes in PBS, 0.3% Tween 20, blots were incubated with either rabbit anti-mouse (Dakocytomation, Ely, UK) or goat anti-rabbit (Bio-Rad) IgG horseradish peroxidase conjugate for 1 h. To enhance detection, blots of two-dimensional gels were washed twice in PBS, 0.3% Tween 20, and incubated with a peroxidase-labeled anti-peroxidase mouse monoclonal antibody (Dakocytomation) for 1 h. All blots were washed three times in PBS, 0.3% Tween 20 and twice in PBS. Immunoreactive material was visualized by chemiluminescence (Western Lightning™ Chemiluminescence Reagent Plus, PerkinElmer Life Sciences). Exposures of film to autoradiographic emulsion were such that the intensities of the signals from the samples were within the linear range of the diluted control samples. Signals were quantified using the NIH Scion Image application. All blotting experiments were repeated with independently isolated mitochondrial samples. Duplicate experiments yielded consistent results.

Purification of MTCO1—Human heart cytochrome c oxidase was a kind gift of Dr. A. O. Muijsers (University of Amsterdam). The enzyme was dissociated in 3% SDS, and subunits were resolved under denaturing conditions on a Bio-Gel® P-60 (Bio-Rad) gel filtration column (57). To remove excess of SDS, MTCO1 was precipitated by addition of 3 volumes of ethanol at room temperature. The protein pellet was washed with 80% ethanol, dried, and dissolved in PBS, 0.1% SDS.

1 The abbreviations used are: PBS, phosphate-buffered saline; VDAC, voltage-dependent anion-selective channel; HSP, heat shock protein.
values, serial dilutions of control mitochondria were loaded on the same gels. Development of the immunoblots with antibodies against cytochrome c oxidase subunits revealed that, in all patient samples, COX4 steady-state levels were around 60% of control values, whereas MTCO2, COX5A, and COX5B steady-state levels were around 25% of control values, and MTCO3 steady-state levels were 10–15% of control values (Fig. 1). Interestingly, other subunits showed patient-specific differences. MTCO1 steady-state levels were 35–40% of control values in the SCO1 and COX10-deficient samples, but 65–70% of control values in the SURF1-deficient samples. In contrast, COX6A1 steady-state levels were around 25% of control values in the SCO1- and COX10-deficient samples but below the detection limit of 25% of control values in the SURF1-deficient samples. Likewise, COX6B steady-state levels were around 50% of control values in the SCO1- and COX10-deficient samples, but around 25% of control values in the SURF1-deficient samples. Steady-state levels of COX6C varied between 10 and 25% of control values in SCO1-deficient mitochondrial preparations, and were around 25% of control values in the SURF1- and COX10-deficient preparations.

**Immunoblots of One-dimensional Native Gels**—To investigate possible associations between the remnant subunits in the patient mitochondria, we prepared immunoblots of mitochondrial fractions resolved on native gels. The same mitochondrial fractions as described above were used, and the amount of the sample loaded per lane was identical to that used for the one-dimensional denaturing gels. Blue native gel electrophoresis has been designed specifically to separate mitochondrial membrane complexes under largely native conditions (55). It uses the neutral detergents n-dodecyl-β-maltoside to gently extract membrane proteins from the lipid bilayer and employs Coomassie Blue dye to give proteins a negative charge, allowing the protein moieties to migrate toward the anode irrespective of their isoelectric point.

Probing of the immunoblots with the anti-MTCO1 antibody revealed several distinct bands in the SURF1-deficient sample, which we termed a–f (Fig. 2). Prolonged exposure of the blots showed that many of these bands co-migrated with bands present in the other samples (Fig. 2). Gel staining for cytochrome c oxidase activity demonstrated that the enzyme activity was associated with band a (not shown), which is the most prominent band of the control samples. Sedimentation velocity and equilibrium studies have shown that cytochrome c oxidase exists as a monomer at the n-dodecyl-β-maltoside concentrations used in our experiments (59). Therefore, we assume that band a represents the monomeric holoenzyme complex. The holoenzyme band a was also present in all three patient samples, albeit at much reduced levels. Comparison with the lanes containing serial dilutions of the control sample suggest that the steady-state levels of the holocomplex were just under 10% of control values in the SURF1-deficient samples and just above 10% in the SCO1 and COX10-deficient samples. In addition to holoenzyme band a, the anti-MTCO1 antibody recognized three faster migrating bands, b, d, and e, in the control sample. Band b was barely discernible in the three patient samples, but bands d and e were present at similar or higher levels. Bands c and f, detected with the anti-MTCO1 antibody at relatively high levels in the SURF1-deficient sample, were also noticed in the SCO1-deficient sample but not in the COX10-deficient sample or the control.

Probing of immunoblots with the anti-COX4 antibody revealed three bands in the SURF1-deficient sample (Fig. 2). Alignment of immunoblot strips of SURF1-deficient samples, probed with the anti-MTCO1 or anti-COX4 antibodies, indicated that the three bands detected with anti-COX4 correspond
cytochrome c oxidase subunits to which we have antibodies. The specificity of the cytochrome c oxidase antibodies used in this study have all been rigorously checked with immunoblots of denaturing gels (49, 51). We have, however, noticed that sometimes secondary antibodies that produce specific reactions on blots of denaturing gels give rise to nonspecific signals on blots of native gels. To verify the specificity of the bands found in the previous experiment, we exploited the human cell line A549 \( p^0 \), which lacks mtDNA and is therefore unable to synthesize cytochrome c oxidase. Mitochondrial fractions from A549 \( p^0 \) and its parental cell line A549 were loaded in alternating lanes of a blue native gel, and immunoblot strips were probed with the antibodies against MTCO1, COX4, or COX5A (Fig. 3). The banding pattern of the A549 sample corresponded with the banding pattern of the control fibroblast sample shown in Fig. 2, but no immunoreactive material was present in the A549 \( p^0 \) sample. This demonstrates the specificity of the antibodies when used in immunoblot experiments of one-dimensional blue native gels.

To test whether the cytochrome c oxidase subassemblies found in the SURF1-deficient patient fibroblasts shown in Fig. 2 are characteristic for SURF1-deficient patients in general, we compared fibroblast cultures from four patients (P1–4) carrying different SURF1 mutations (40, 41) with two controls (C1 and C2). Mitoplast-enriched pellets from \(-1.5 \times 10^7\) cells were solubilized with n-dodecyl-\(\beta\)-malto-side and subjected to blue native gel electrophoresis. Immunoblots were probed with the anti-MTCO1 antibody (Fig. 4A). Although the relative intensity of the bands varied somewhat in the four different SURF1-deficient samples, the patterns matched the pattern of the SURF1-deficient, mitochondrial sample from patient P1 shown in Fig. 2. In addition, the banding patterns of the two control samples, C1 and C2, were identical to the pattern of the different control samples shown in Fig. 2. These results indicate that the banding patterns identified here represent reproducible, disease-specific differences in cytochrome c oxidase subassemblies. The differences in intensity of the bands seen in Fig. 4A are most likely due to subtle variations in protein/detergents ratios achieved during solubilization of the samples, caused by variation in cell size and number.

We wondered whether the fastest migrating band detected with the anti-MTCO1 antibody in the SURF1-deficient samples (band \( f \)) represents free, unassembled apoMTCO1. To investigate this, the MTCO1 polypeptide was isolated from purified human cytochrome c oxidase and run alongside a SURF1-deficient and a control sample on a blue native gel. Probing of the immunoblot with the anti-MTCO1 antibody revealed that
The purified MTCO1 polypeptide migrates slightly slower than band f but faster than band e (Fig. 4B).

**Immunoblots of Two-dimensional Native/Denaturing Gels**—
Immunoblots of two-dimensional blue native/SDS-denaturing gels were used to confirm the alignment of the bands identified on blots of one-dimensional blue native gels. Blots of each mitochondrial preparation were simultaneously probed with a mixture of the antibodies against MTCO1, MTCO2, COX4, and COX5A (Fig. 5). Blots were re-probed with antibodies against SDHA and HSP70 to obtain molecular weight marker signals. (Under the conditions used, the anti-SHDA antibodies react with the holosuccinate-ubiquinone oxidoreductase complex of 123 kDa and an SDHA-SDHIB-subcomplex of 97 kDa; not shown.) The blots showed some smearing due to compression of the first dimension signals. Nevertheless, the subassemblies characterized on the blots of the one-dimensional blue native gels were clearly identifiable on the blots of the two-dimensional gels. The four SURF1-deficient samples showed identical patterns, indicating the presence of variation in the abundance of the subassemblies in different SURF1-deficient patients. Although we did detect a faint COX5A spot as part of band c on the blots of the SURF1-deficient samples, this spot was not seen on the blots of the SCO1-deficient sample. The immunoreactivity of the anti-COX5A antibody was relatively poor, and we assume that COX5A subassembly spot was below the detection limit. Most blots of Fig. 5 showed a spot migrating between MTCO1 and MTCO2. This spot was also detected on blots not incubated with primary antibodies (not shown) and, therefore, represents a nonspecific signal.

**DISCUSSION**

As part of our studies aimed at the understanding of the assembly pathway of human cytochrome c oxidase, we compared the consequences of mutations in the assembly factors SCO1, SURF1, and COX10. In line with previous studies (12–14, 32, 38, 40, 41), we found that cytochrome c oxidase activity and subunit steady-state levels were drastically reduced in patient fibroblasts, but some residual activity and subunit levels remained. This implies that although cytochrome c oxidase biosynthesis is clearly affected in the patients, the SCO1 and COX10 missense mutations and SURF1 loss of function mutations still allow some functional enzyme to be formed. Interestingly, immunoblot analysis of denaturing gels indicated that mutations in the three assembly factors exert different effects on the cytochrome c oxidase subunit steady-state levels. Steady-state levels of MTCO1 were markedly higher in SURF1-deficient fibroblasts than in SCO1- and COX10-deficient fibroblasts, whereas steady-state levels of MTCO2, MTCO3, COX4, COX5A, and COX5B were similar in all three patient fibroblast cultures (Fig. 1). SCO1, SURF1, and COX10 are not considered to be directly involved in the synthesis of cytochrome c oxidase subunits (9), and evidence from yeast null mutants indicates that the loss of any of these assembly factors leads to rapid proteolytic turnover of cytochrome c oxidase subunits (17, 36, 60). During the last few years, an evolutionarily conserved, quality control system consisting of ATP-dependent metalloproteases has been identified in mitochondria that selectively removes non-assembled polypeptides and prevents their possible deleterious accumulation in the inner membrane (61). This system is most likely responsible for degradation of cytochrome c oxidase subunits in the fibroblasts of patients. The steady-state level of each subunit probably reflects the intrinsic stability of the subunit and its association with other subunits or assembly factors of the enzyme that protect it from proteolysis. The observed differences in steady-state subunit levels, therefore, suggest the presence of unique patterns of cytochrome c oxidase subassemblies in the different patients.

This prediction was confirmed by the use of blue native gel electrophoresis to examine associations between remnant subunits. Immunoblots of blue native gels revealed high levels of cytochrome c oxidase holoenzyme in control fibroblasts and low levels of the holoenzyme complex in patient fibroblasts. The decrease in holoenzyme levels in the patient samples was similar to the decrease in enzyme activity, suggesting that the residual holoenzyme is fully active and that the cytochrome c oxidase deficiency stems from an inability to assemble or maintain adequate enzyme levels. All samples also contained low levels of a subassembly involving at least MTCO1, MTCO2, MTCO3, COX4, and COX5A and two subassemblies involving MTCO1 but apparently no other subunits of the enzyme. In addition, the SCO1 and four SURF1-deficient fibroblast cultures contained two subassemblies not detected in the COX10-deficient sample and the controls: a subassembly involving MTCO1, COX4, and COX5A and a subassembly involving MTCO1 but apparently no other subunits of the enzyme. In agreement with the relatively high residual steady-state levels of MTCO1 in SURF1-deficient fibroblasts, subassemblies involving MTCO1 were particularly abundant in the SURF1-deficient samples.

To our surprise, the fastest migrating band recognized by the anti-MTCO1 antibody (band f in Figs. 2 and 4) migrated slightly faster than purified MTCO1. Blots of two-dimensional native/denaturing gels confirmed that the MTCO1 spot corresponding to band f was of the correct size and therefore was not a proteolytic breakdown product of the subunit. To facilitate purification of MTCO1 from isolated human cytochrome c oxidase, the protein was denatured. Mature MTCO1 contains a covalent bond between residues His340 and Tyr344 (62). These differences between the purified, mature MTCO1 and nascent apoMTCO1 may result in small differences in migration behavior. Therefore, despite the minor disparity in migration, we assume that band f represents free apoMTCO1.

Subassemblies of cytochrome c oxidase in cell cultures from patients with cytochrome c oxidase deficiency have been reported by others. Hanson et al. (63) resolved mitochondrial preparations of SURF1-deficient, MTCO3-deficient, and genetically undefined patients by centrifugation through discontinuous sucrose gradients and analyzed 10 fractions on denaturing gel immunoblots. Some fractions of the SURF1- and MTCO3-deficient samples contained a mixture of MTCO1, COX4, and COX5A but not MTCO2, suggesting a possible...
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Fig. 5. Verification of subunit composition of cytochrome c oxidase subassemblies in COX10-, SURF1-, and COX10-deficient fibroblasts. Immunoblots of two-dimensional blue native/denaturing gels, loaded with 50 μg of mitochondrial protein from a control fibroblast culture, or the COX10, SCO1, or four (P1–4) SURF1-deficient cultures, were probed with monoclonal antibodies specific for subunits MTCO1, MTCO2, COX4, and COX5A. Immunoreactive material was visualized by chemiluminescence. The positions of subassembly bands a–f and the subunits are indicated. The blots were subsequently probed with antibodies against the succinate:ubiquinone oxidoreductase subunit SDHA or the matrix protein HSP70. The top of the native gel strip and the migration and M of native succinate:ubiquinone oxidoreductase (123k), an SDHA/SDHB subcomplex (97k), and HSP70 (70k) are indicated with arrowheads. The migration direction of the first (native) and the second (denatured) dimension is indicated with arrows.

association of the three subunits. The resolution was, however, insufficient to distinguish distinct subassemblies in the SURF1-deficient samples. As in our current study, Tiranti et al. (11) analyzed SURF1-deficient fibroblasts on immunoblots of blue native gels. The authors identified three subassemblies containing MTCO1 (11), whereas we identify five subassemblies involving MTCO1. Differences in gel resolution and the inclusion of a tertiary anti-peroxidase antibody layer in our study probably explain the lower number MTCO1-containing inclusions involving MTCO1. Differences in gel resolution and the inclusion of a tertiary anti-peroxidase antibody layer in our study probably explain the lower number MTCO1-containing subassemblies identified by Tiranti et al. (11).

In whole-cell pellets of SURF1-deficient fibroblasts, heme ατ of cytochrome c oxidase is barely detectable, suggesting that normal levels of the enzyme are not present in these cells. Therefore, it is unlikely that the subassemblies identified in the patient samples represent parts of a labile cytochrome c oxidase, disrupted during sample handling. We think that the subassemblies identified in the patient samples probably represent protected assembly intermediates that accumulate due to compromised biosynthesis of the enzyme. The assembly of mammalian cytochrome c oxidase occurs in an ordered sequence (64, 65). It is possible that the subassemblies represent off-path complexes that are not relevant to the normal route of assembly. Although we cannot rule out this possibility for all five subassemblies that we detect, three of the subassemblies appear to correspond to the assembly intermediates S1–3 that have been identified in metabolic labeling experiments with a human leukemia cell line (65). Band f detected in our SURF1- and SCO1-deficient samples (Figs. 2 and 4) almost certainly corresponds to S1, the unassembled apo-MTCO1.

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The COX10 mutation carried by our patient leads to a decreased cellular availability of heme A (38). The COX10-deficient fibroblasts contained the same two MTCO1-containing subassemblies as the control and had much reduced levels of the holocytochrome c oxidase. This suggests that heme A insertion into MTCO1 occurs after formation of the two MTCO1-containing subassemblies (bands d and e in Fig. 2) but before (or during) formation of the MTCO1-COX4-COX5A subassembly (band c in Fig. 2). Recently, Antonicka et al. (39, 66) identified two additional patients with COX10 mutations and a patient with cytochrome c oxidase deficiency caused by a mutation in COX15. As in our study, the authors did not detect the MTCO1-COX4-COX5A subassembly in mitochondria of their patients and, based on these observations, also concluded that heme A is required for formation of the MTCO1-COX4-COX5A assembly intermediate S2.

The SCO1 mutation carried by our patient is thought to
reduce the efficiency by which the Cu₄ center in MTCO2 is formed (32). The SCO1-deficient fibroblasts had reduced levels of holo-cytochrome c oxidase and contained several subassemblies, including three MTCO1-containing subassemblies also seen in controls, and the MTCO1-COX4-COX5A subassembly and free apoMTCO1, both of which were also seen in SURFI-deficient cells. The fact that the immunoblots revealed no accumulated levels of an MTCO1- and MTCO2-containing sub-assembly suggests that association of the two core subunits of the enzyme occurs after the Cu₄ center is formed. Apparently, once the Cu₄ center is formed, Cu₄-containing MTCO2 associates with the MTCO1-COX4-COX5A subassembly and further assembly proceeds normally.

The function of SURF1 is not known, but the presence of SURF1 orthologues in terminal oxidase operons of several prokaryotes (67) points toward an elementary role for SURF1 involving the evolutionarily conserved, mitochondrially encoded subunits of the oxidase. The subassembly profile of all four of the SURFI-deficient fibroblast cultures studied here is identical to that of SCO1-deficient fibroblasts, except that a greater accumulation of subassemblies was seen in the SURFI-deficient cells. This suggests that, as in SCO1-deficient fibroblasts, the assembly process in SURFI-deficient fibroblasts is stalled before MTCO2 associates with the MTCO1-COX4-COX5A subassembly. We think that SURF1 may promote the association of MTCO2 with the MTCO1-COX4-COX5X5 subassembly. This view is compatible with the proposal of Barrionets et al. (17) that, in yeast, Shy1p may influence the translation of Cox1p (MTCO1) by facilitating downstream assembly events and increasing Cox1p flux through the assembly process. It is also in agreement with an observation of Nijtmans et al. (68), who detected MTCO2 as part of a 250-kDa SURFI-containing complex, suggesting a transient interaction between SURF1 and MTCO2.

Taken together, our findings suggest several additions to the current model of the human cytochrome c oxidase assembly pathway (65). Nascent apoMTCO1 appears to associate transiently with several subnuslip proteins prior to incorporation of the heme A groups and subsequent association of COX4 and COX5A. In the following steps, Cu₄-containing MTCO2, MTCO3, and most of the remaining nuclearly encoded subunits associate with the MTCO1-COX4-COX5A-SCO1 assembly initiated via a mechanism that requires SURF1. In the final step, according to previous models (65, 69), COX6A and COX7A bind to MTCO3 to give the complete enzyme complex. Ongoing experiments are aimed at detection of MTCO1 prosthetic groups according to previous models (65, 69), COX6A and COX7A bind to MTCO3 to give the complete enzyme complex. Ongoing experiments are aimed at detection of MTCO1 prosthetic groups according to previous models (65, 69), COX6A and COX7A bind to MTCO3 to give the complete enzyme complex. 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