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Altered kinetics of cytochrome c oxidase in a patient with severe mitochondrial encephalomyopathy

Leo G.J. Nijtmans a,b, Peter G. Barth a,c, Carsten R. Lincke a,c, Micke J.M. Van Galen a,b, Rob Zwart a, Petr Klement a,h, Pieter A. Bolhuis a, Wim Ruitenbeek d, Ronald J.A. Wanders c, Coby Van den Bergert a,b,*

a Department of Neurology, Academic Medical Center, University of Amsterdam, PO Box 22700, 1100 DE Amsterdam, The Netherlands
b Department of Biochemistry, Academic Medical Center, University of Amsterdam, PO Box 22700, 1100 DE Amsterdam, The Netherlands
c Department of Pediatrics, Academic Medical Center, University of Amsterdam, PO Box 22700, 1100 DE Amsterdam, The Netherlands
d Department of Pediatrics, University of Nijmegen, Nijmegen, The Netherlands

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Abstract

Deficiency of cytochrome c oxidase activity was established in a girl born to consanguineous parents. She showed symptoms of dysmaturity, generalized hypotonia, myoclonic seizures and progressive respiratory failure, leading to death on the seventh day of life. Structural abnormalities of the central nervous system consisted of severe cerebellar hypoplasia and optic nerve atrophy. Biochemical analysis of a muscle biopsy specimen demonstrated deficiency of cytochrome c oxidase activity. Cultured fibroblasts from this patient also showed a selective decrease in the activity of cytochrome c oxidase, excluding a muscle-specific type of deficiency. Further investigations in cultured fibroblasts revealed that synthesis, assembly and stability of both the mitochondrial and the nuclear subunits of the enzyme were entirely normal. The steady-state concentration of cytochrome c oxidase in the fibroblasts of the patient was also normal, suggesting that the kinetic properties of the enzyme were altered. Analysis of the kinetic parameters of cytochrome c oxidase demonstrated an aberrant interaction between cytochrome c oxidase and its substrate, cytochrome c, most likely because of a mutation in one of the nuclear subunits of the enzyme.

Keywords: Cytochrome c oxidase; Enzyme kinetics; Mitochondrial disease; Encephalomyopathy; Fibroblast; (Human)

1. Introduction

Cytochrome c oxidase (complex IV) is the terminal enzyme of the respiratory chain. It catalyses the transfer of electrons from reduced cytochrome c to oxygen and conserves the free energy of this reaction by pumping protons across the mitochondrial inner membrane. The enzyme is composed of thirteen different subunits, of which the three largest ones are encoded by mitochondrial DNA (mtDNA). The other ten subunits are products of nuclear genes that are synthesized in the cytoplasm and transported into the mitochondria where they are processed to the mature form and assembled with the mitochondrial subunits [1]. The subunits encoded by mtDNA constitute the catalytic core of the enzyme: they contain the binding site for cytochrome c and the prosthetic groups, and they are involved in proton pumping [2]. The function(s) of the nuclearly encoded subunits have not yet been elucidated. These subunits might be involved in the process of assembly of a functional enzyme [3], and/or might bind ligands that regulate the catalytic activity of the enzyme [4–7]. A regulatory function is supported by the presence of muscle-specific isoforms of a few nuclear subunits in mammalian cytochrome c oxidase [8,9].

Deficiency of cytochrome c oxidase activity is frequently observed in patients with mitochondrial encephalomyopathies [10,11]. Biochemically, these deficiencies can be classified in four main categories on the basis...
of the origin and function of the gene products that are involved in the biogenesis of the enzyme. In the first group, the deficiency is caused by mtDNA deletions (as is frequently the case in the Kearns-Sayre syndrome and chronic progressive external ophthalmoplegia), by depletion of mtDNA, or by point mutations in mitochondrial tRNA genes [10,12]. These types of mtDNA aberration do not lead to selective loss of cytochrome c oxidase activity: the activity of the other enzyme complexes that contain subunits encoded by mtDNA will also be reduced. In the second group, the disorder is based on mutations of nuclear genes for proteins that are involved in mitochondrial biogenesis, for instance in import or assembly of nuclearly encoded mitochondrial proteins. Such disorders are probably rare, but when they occur cytochrome c oxidase will again not be affected exclusively [13]. The third group consists of deficiencies that are due to mutations in the mitochondrial genes for subunits of cytochrome c oxidase. So far, this type of defect has only been described in a preliminary paper [14]. The fourth group is formed by deficiencies due to mutations in nuclear genes encoding subunits of cytochrome c oxidase, or because of defects in the regulation of the expression of genes for the different isoforms. The latter type of defect has been proposed as an explanation for cases of benign, muscle-specific cytochrome c oxidase deficiency [8,10], but experimental proof is still lacking. Many cases of Leigh’s syndrome are most likely based on defects in nuclear genes for subunits of cytochrome c oxidase that do not show tissue-specific expression [10,11,15], but the mutations in question have yet to be identified.

In general, the concentration of the enzyme is reduced in tissues or cultured cells from patients with a decreased cytochrome c oxidase activity [8,10,16], indicating that assembly or stability of the enzyme are affected. In contrast, here we describe a case of selective deficiency of cytochrome c oxidase where the amount of enzyme was entirely normal. However, the affinity of the enzyme for its substrate, cytochrome c, was significantly decreased. The results clearly suggest that mutations in the nuclear subunits can affect the catalytic activity of cytochrome c oxidase.

2. Materials and methods

2.1. Patient

The clinical data of the patient will be described in detail elsewhere (P.G. Barth et al., unpublished data). In short, the female patient was born after an uneventful pregnancy as the first child to consanguineous (first cousins) parents of Moroccan origin. Severe, persistent hypotonia and myoclonic seizures were observed from the first day of life. Ophthalmoscopy revealed bilateral optic nerve atrophy. Laboratory investigations showed no other abnormalities than an elevated concentration of lactate acid in the cerebrospinal fluid. Magnetic resonance imaging on the fourth day of life showed marked hypoplasia of the cerebellum, in particular of the cerebellar hemispheres. The girl died at the age of seven days from progressive respiratory failure probably due to combined neuromuscular insufficiency and cerebral dysfunction. Muscle and skin biopsy specimens were collected within 1 h after death and immediately processed for further analysis. Examination of the muscle tissue by light microscopy showed no ragged red fibres. Analysis of several mitochondrial enzymes in muscle homogenates revealed a selective decrease in the activity of cytochrome c oxidase.

2.2. Fibroblast cultures

Fibroblasts derived from the patient and from different healthy controls were cultured in Dulbecco’s modified Eagle medium (DME) containing 1 mM pyruvate and supplemented with 10% heat-inactivated fetal calf serum (FCS), at 37°C in a humidified atmosphere of 10% CO₂ in air [16,17]. Cells from exponentially growing cultures were collected as described before [16,18]. The cell pellet was resuspended in phosphate buffered saline (PBS) and the cell concentration was determined using a counting chamber. The cell suspension was divided in small portions, frozen in liquid nitrogen and stored at −80°C.

2.3. Metabolic labeling

After removing the normal culture medium, fibroblasts from exponentially growing cultures were rinsed twice with PBS and cultured in methionine-free DME containing 1 mM pyruvate and supplemented with 10% heat-inactivated FCS in a humidified atmosphere of 10% CO₂ in air. After 1 h, [³⁵S]methionine (specific activity >960 Ci/mmol) was added to a final concentration of 20 μCi per ml. At various time points, cells were collected by centrifugation as described above. In pulse-chase experiments, the [³⁵S]methionine containing medium was replaced by pre-incubated complete DME containing 1 mM pyruvate and 10% FCS after rinsing the cells four times with PBS. In a number of experiments, emetine was used to block the synthesis of nuclear-encoded proteins [19]. Emetine was added 10 min before the addition of labeled methionine, to a final concentration of 100 μg/ml.

2.4. Immunoprecipitation

The volume of samples containing about 5·10⁶ fibroblasts was adjusted to 1 ml with PBS containing 1 mg of bovine serum albumin/ml and 2.5 mM phenylmethyl-sulphonyl fluoride. Lauryl maltoside (final concentration 1.5%) was added to solubilize the cells and to extract membrane proteins. The samples were left on ice for 30 min and then centrifuged for 1 min in a microfuge at
12,000 × g. The supernatants were used for immunoprecipitation by incubation overnight with antibodies coupled to protein A-Sepharose 4B beads as described before [16,18,20]. The immunoprecipitates were eluted by incubating the beads in sample buffer (4% sodium dodecyl sulphate, 20% glycerol in 10 mM Tris-HCl, pH 6.8) at 37°C for 2 h. After centrifugation, the eluates were loaded on to 15% polyacrylamide gels and subjected to electrophoresis as described by Schägger and Von Jagow [21]. In the case of metabolic labeling experiments, the gels were dried under vacuum and labeled proteins were visualized by fluorography [22]. Autoradiographic signals after electrophoresis of 35S-labeled proteins were quantified on gels using a PhosphorImager (Molecular Dynamics, Sunnyvale, USA). Gels containing unlabeled proteins were stained by silver [20].

2.5. Antisera

Subunits I, II, and III of mammalian cytochrome c oxidase are encoded by the mitochondrial genome, the remaining ten smaller subunits are encoded by nuclear genes. According to their eukaryotic mobility these are referred to as subunits IV, Va, Vb, VIa, VIb, Vlc, VIIa, VIIb, VIIc, and VIII, respectively [20]. Human cytochrome c oxidase occurs in two isoforms. One form is present in skeletal muscle and heart and contains muscle-type forms of subunits VIa and VIIa. The other form of cytochrome c oxidase is present in all other tissues, including cultured cells, and contains the so-called liver-type forms of subunits VIa and VIIa [8,9,20,23,24]. A polyclonal antiserum against cytochrome c oxidase from bovine heart was raised in rabbits. The properties of this antiserum have been described in detail before [16,18,20,25,26]. In short, this antiserum precipitates the assembled enzyme complex from cell or tissue homogenates solubilized with lauryl maltoside: after immunoprecipitation and electrophoresis all the subunits are detectable with conventional staining methods. Some of the subunits are under-represented in the immunoprecipitated enzyme, probably because of their partial loss from the enzyme complex by distortions in the interactions between the subunits due to binding of the antisem. The antiserum precipitates unassembled nucleolarly encoded subunits, except subunit VIII. Mitochondrial subunits (and subunit VIII) are, however, only precipitated if these are part of the assembled enzyme complex. The antiserum does not discriminate between both isoforms of cytochrome c oxidase.

2.6. Two-dimensional electrophoresis

Two-dimensional electrophoresis was performed using a modification of the method described by Schägger and Von Jagow (P. Klement et al., unpublished data) [27]. This method allows analysis of the molecular mass and the composition of (mitochondrial) enzyme complexes. In the first dimension separation according to molecular mass of these large complexes occurs under native conditions, and in the second dimension where electrophoresis is performed under denaturating conditions, the individual subunits of the complexes are resolved. Isolation of the complexes prior to electrophoresis by for instance immunoprecipitation is not required, which reduces the possibility of losing one or more subunits during isolation of a complex. Crude mitochondria were used as starting material for two-dimensional electrophoresis, and were obtained by incubating 1 · 10^6 cells/ml PBS for 10 min at 4°C with 20 μg of digitonin [28,29]. The samples were centrifuged for 1 min at 12,000 × g in a microfuge to obtain a mitochondrial pellet. The gels were stained with silver [20].

2.7. Mitochondrial functions in fibroblasts

Mitochondrial ATP synthesis was measured in fibroblasts from exponentially growing cultures, permeabilized with low concentrations of digitonin (1 μg/10^6 cells) [30]. ATP synthesis was initiated by the addition of ADP to a final concentration of 10 mM. Glutamate (10 mM) + malate (2 mM) or pyruvate (10 mM) + malate (2 mM) were used as substrates for oxidative phosphorylation via complexes I, III and IV. Succinate (10 mM) (+2 μg rotenone/ml to inhibit complex I) was used as a substrate for oxidative phosphorylation via complexes II, III and IV. Ascorbate (10 mM) + tetramethylphenylenediamine (0.5 mM) were used as substrates for ATP production via complex IV. In the latter case 2 μg rotenone/ml and 2 μg antimycin/ml were present to inhibit the activities of complex I and III, respectively.

The activity of mitochondrial enzymes and the protein content of the cells were determined in freshly prepared cell lysates, obtained by treatment with lauryl maltoside as described under immunoprecipitation. The activity of cytochrome c oxidase was measured spectrophotometrically [20] at 25°C in 25 mM phosphate buffer (pH 7.4), using 20 μM reduced bovine heart cytochrome c as the substrate, and is expressed as the first-order reaction-rate constant, k. The steady-state kinetics of the enzyme were measured under the same conditions, but the concentration of reduced cytochrome c was varied between 1 and 67 μM. The final concentration of cytochrome c oxidase in these measurements was calculated via quantitative immunoprecipitation as described before [20]. K_m and V_max values were determined for the high-affinity reaction by computer analysis of the data which were expressed as reversed Eadie-Hofstee plots.

The citrate synthase activity was measured according to the method of Srere [31]. The activities of complex II and III were measured at 25°C in 25 mM phosphate buffer (pH 7.4), containing 1 mM MgCl_2. Succinate (10 mM) was used as substrate for measurements of complex II, where the reduction of dichlorophenolindophenol (50 μM) was
followed in the presence of rotenone (2 μg/ml), antimycin (2 μg/ml) and KCN (2 mM). Antimycin (2 μg/ml) sensitive complex III activity was determined in the presence of both rotenone (2 μg/ml) and KCN (2 mM), using decylubiquinol (80 μM) as substrate and oxidized bovine heart cytochrome c (40 μM) as electron acceptor. The enzymatic activities are expressed as nmol/mg protein in cell lysates/min. The protein content of the samples was estimated using a modified Lowry method [32].

2.8. Sequencing of the gene for subunit II

Mitochondrial DNA sequences were amplified by polymerase chain reaction from DNA isolated from fibroblasts of the patient. Primers were selected by means of computer program Primer (S.E. Lincoln, M.J. Daly and E.S. Lander, MIT Center for Genomic Research and Whitehead Institute for Biomedical Research, Cambridge, USA). Primers were synthesized on an Expedite Nucleic Acid Synthesis System™ (Millipore, Bedford, USA). To amplify the mtDNA fragment corresponding to nucleotide number 7578–8303 appropriate forward and reverse primers were synthesized, and to sequence this fragment internal primers (forward and reverse) for the nucleotide numbers 7771–7849 and 8009–8089. The polymerase chain reaction program was: 95°C, 30 s; 55°C, 60 s; and 72°C, 90 s for 30 cycles. The polymerase chain reaction products were purified by electrophoresis and recovered from the gel using Magic™ PCR Preps column (Promega, Madison, USA). The sequencing reactions were carried out according to standard methods, using [35S]dATP and the T7 polymerase sequencing kit (Sequenase, USB, Cleveland, USA).

3. Results

3.1. Analysis of the activities of mitochondrial enzymes in cultured fibroblasts

The activity of cytochrome c oxidase in cultured fibroblasts from the patient, expressed per mg cellular protein, was 25% of the corresponding mean control value. The activities of other mitochondrial enzymes were entirely normal (Table 1). Deficiency of cytochrome c oxidase activity was therefore not limited to muscle of the patient. This ruled out muscle-specificity of the primary defect, such as involvement of the muscle-type specific subunits Va and V1a. Furthermore, this allowed us to study the biosynthesis and other properties of the enzyme from the patient in detail.

3.2. Synthesis and assembly of cytochrome c oxidase

Fig. 1 shows the results of studies on the synthesis, assembly and degradation of subunits of cytochrome c oxidase in fibroblasts from the patient and in control fibroblasts. Experiments with intact cells labeled in the presence of emetine indicated that the expression of mitochondrial genes in fibroblasts from the patient was comparable to that in control fibroblasts, both with respect to the amount of label incorporated into mitochondrial gene prod-

![Fig. 1. Synthesis and stability of mitochondrial and nuclear subunits of cytochrome c oxidase in control fibroblasts and fibroblasts from the patient. Fibroblasts were labeled with [35S]methionine for 1 h or 2 h. After labeling for 2 h, the cells were cultured further in the absence of labeled methionine for 3 h or 21 h. The subunits of cytochrome c oxidase were isolated by immunoprecipitation from cells solubilized with lauryl maltoside. The same number of cells (0.5 • 10⁶) was used for each experiment. COX: cytochrome c oxidase.](image)
Mitochondrial functions in cultured fibroblasts

<table>
<thead>
<tr>
<th>Enzyme activities</th>
<th>Patient</th>
<th>Controls</th>
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<tbody>
<tr>
<td>citrate synthase</td>
<td>79 (2)</td>
<td>78 (51; 58–80)</td>
</tr>
<tr>
<td>complex II</td>
<td>6.2 (2)</td>
<td>7.6 (9; 5.6–7.8)</td>
</tr>
<tr>
<td>complex III</td>
<td>9.6 (2)</td>
<td>10.7 (7; 10.3–13.1)</td>
</tr>
<tr>
<td>complex IV</td>
<td>0.15 (2)</td>
<td>0.58 (51; 0.45–0.62)</td>
</tr>
<tr>
<td>ATP production</td>
<td></td>
<td></td>
</tr>
<tr>
<td>glutamate + malate</td>
<td>1355 (3)</td>
<td>1393 (7; 1104–1402)</td>
</tr>
<tr>
<td>pyruvate + malate</td>
<td>1231 (3)</td>
<td>1239 (6; 1056–1320)</td>
</tr>
<tr>
<td>succinate</td>
<td>977 (3)</td>
<td>997 (6; 672–1116)</td>
</tr>
<tr>
<td>ascorbate + TMPD</td>
<td>700 (2)</td>
<td>703 (3; 688–725)</td>
</tr>
</tbody>
</table>

The activity of mitochondrial enzymes were measured in fibroblasts solubilized with lauryl maltoside. The activities are expressed as μkat per min per mg protein (cytochrome c oxidase) or as nmol per min per mg protein (other enzymes), as described in Section 2. The ATP production was measured in fibroblasts permeabilized with digitonin and is expressed as nmol ATP per h per mg protein. The mean values obtained in different experiments are shown. The assays with fibroblasts from the patient were performed in duplicate, the number of independent experiments is indicated between brackets. The assays with control fibroblasts were also performed in duplicate, the number of independent experiments and the minimal and maximal values obtained for various control fibroblasts are given between brackets. TMPD: tetramethyl phenylenediamine.

The activity of mitochondrial enzymes were measured in fibroblasts solubilized with lauryl maltoside. The activities are expressed as μkat per min per mg protein (cytochrome c oxidase) or as nmol per min per mg protein (other enzymes), as described in Section 2. The ATP production was measured in fibroblasts permeabilized with digitonin and is expressed as nmol ATP per h per mg protein. The mean values obtained in different experiments are shown. The assays with fibroblasts from the patient were performed in duplicate, the number of independent experiments is indicated between brackets. The assays with control fibroblasts were also performed in duplicate, the number of independent experiments and the minimal and maximal values obtained for various control fibroblasts are given between brackets. TMPD: tetramethyl phenylenediamine.

...and with respect to the molecular mass of these products (data not shown). Immunoprecipitation of subunits of cytochrome c oxidase after labeling in the absence of emetine demonstrated that the rate of synthesis of nuclear subunits also did not differ significantly between fibroblasts from the patient and control fibroblasts (Fig. 1, pulse-experiments). The same was the case for the turnover rate of the subunits, determined in pulse-chase experiments (Fig. 1). Since the antiserum that was used only precipitates mitochondrial subunits of cytochrome c oxidase when these are assembled with nuclear subunits (see Section 2), the assembly of mitochondrial and nuclear subunits in cells from the patient was apparently normal: the ratio between the signals of, e.g., subunit II and IV was always comparable to that seen in control cells at the same pulse or pulse-chase time.

3.3. Concentration of cytochrome c oxidase

Fig. 2 shows an example of two-dimensional electrophoresis of mitochondrial enzyme complexes from control fibroblasts and those from the patient. The complexes could be easily identified because of the mobility in the first dimension and the characteristic electrophoretic pattern of the subunits in the second dimension [27]. In addition, we confirmed the position of cytochrome c oxidase by immunoblotting of the first-dimension and second-dimension gels; immunoreactivity was found only in the area where the assembled enzyme was expected because of its molecular mass. A comparison of the concentrations of complexes I, II, III, IV, and V derived from the same number of control or patient’s fibroblasts showed no differences: the concentration of the complexes, including that of complex IV, was normal in fibroblasts from the patient. Careful analysis of the staining intensity of the subunits of cytochrome c oxidase also did not reveal significant differences in concentration of individual subunits between controls and the patient.

3.4. Mobility of nuclear subunits of cytochrome c oxidase

Minor differences in mobility of individual nuclear subunits of cytochrome c oxidase will not be noticed.

![Fig. 2. Composition and concentration of mitochondrial enzyme complexes in control fibroblasts and fibroblasts from the patient. Mitochondria obtained by digitonin treatment of control fibroblasts (C) or fibroblasts from the patient (P) were used as starting material for 2D-electrophoresis. The gels were stained by silver. I, II, III, IV, V: complexes I, II, III, IV and V. The position of these complexes after electrophoresis in the first dimension is indicated at the top. COX: cytochrome c oxidase (complex IV). The position of the individual subunits of cytochrome c oxidase after electrophoresis in the second dimension is indicated on the right.](image)
under the experimental conditions used above. In view of this, longer gels on which a much higher degree of resolution can be obtained [18,20] were used to analyse the mobility of the nuclear subunits. Before electrophoresis, cytochrome c oxidase was isolated from the same number of control and patient’s fibroblasts by immunoprecipitation. The intensive staining of polypeptides derived from the antiserum in the upper part of the gel obscures the mitochondrial subunits [16,20], but the nuclear subunits were clearly visible and differences in mobility were not observed (Fig. 3). In addition, the concentration of the individual subunits as judged by silver staining was comparable between controls and patient, except for that of subunit VIII (Fig. 3, right side).

3.5. Kinetic analysis of cytochrome c oxidase

The steady-state kinetic reactions of cytochrome c oxidase in fibroblasts from the patient and in control fibroblasts are shown in the form of reverse Eadie-Hofstee plots in Fig. 4. Under conditions of low ionic strength, biphasic plots are obtained for cytochrome c oxidase reactions that show a high-affinity and a low-affinity phase [20], but at intermediate ionic strength as used here, the low-affinity phase tends to disappear. Analysis of the data shown in Fig. 4 resulted in a value of $V_{\text{max}}$ for the high-affinity reaction of 14 nmol per min per mg cell protein in control cells, in the patient it proved to be 6 nmol per min per mg protein. Analysis of the amount of cytochrome c oxidase using quantitative immunoprecipitation [20] showed that 1 mg of cellular protein per ml equalled a cytochrome c oxidase concentration of 0.025 $\mu$M, both in control fibroblasts and in fibroblasts from the patient. Therefore, the maximal turnover number per molecule cytochrome c oxidase from control fibroblasts was 9.3 per s, and 4 per s for cytochrome c oxidase from the patient. Calculation of

![Fig. 3. Mobility of nuclear subunits of cytochrome c oxidase in control fibroblasts and fibroblasts from the patient. Cytochrome c oxidase was isolated from $8 \times 10^6$ control fibroblasts (C) or $8 \times 10^6$ fibroblasts from the patient (P) by immunoprecipitation. The gels were stained with silver. R: human heart cytochrome c oxidase, used as reference [20]. The position of the subunits of human cytochrome c oxidase is indicated on the left. In order to show subunit VIII more clearly, the right-hand part of Fig. 3 shows an overexposed picture of the same gel as shown in the left part.](image)

![Fig. 4. Steady-state kinetics of cytochrome c oxidase in control fibroblasts and in fibroblasts from the patient. The activity of cytochrome c oxidase was measured in fibroblasts solubilized with lauryl maltoside at concentrations of reduced bovine heart cytochrome c that ranged from 1 to 67 $\mu$M. The rate of oxidation of cytochrome c is plotted against the oxidation rate at a given substrate concentration (reversed Eadie-Hofstee plots). The combined results of 2 independent experiments are shown. The lines indicate the reaction kinetics for the high-affinity phase. C: control fibroblasts; O: patient fibroblasts.](image)
the \( K_m \) values showed more significant differences: in control cells the \( K_m \) of the enzyme for reduced bovine cytochrome \( c \) was 40 \( \mu \)M whereas the \( K_m \) for reduced cytochrome \( c \) of cytochrome \( c \) oxidase from the patient was increased to 160 \( \mu \)M.

### 3.6. Mitochondrial function

Table 1 shows that there were no differences between cells from the patient and control fibroblasts when their capacity for mitochondrial ATP production was compared. ATP production as the result of oxidation of NADH (derived from either glutamate or pyruvate) via complexes I, III and IV, and ATP production resulting from the oxidation of succinate via complexes II, III and IV was normal in the patient's fibroblasts, indicating that the reduced activity of cytochrome \( c \) oxidase was not limiting in this respect. ATP production measured in the presence of inhibitors of complex I and complex III was also not affected when ascorbate + tetramethylphenylenediamine were used as artificial electron donors for complex IV.

### 3.7. Sequence analysis of the gene for subunit II

The sequence of the gene for subunit II of cytochrome \( c \) oxidase from the patient proved to be identical to the Cambridge sequence [33].

### 4. Discussion

We identified selective deficiency of cytochrome \( c \) oxidase activity in a patient who presented shortly after birth with severe hypotonia and severe cerebellar hypoplasia and who died of progressive respiratory failure on the seventh day of life. The only major abnormality in the results of extensive clinical laboratory investigations was a moderately elevated level of lactate in the cerebrospinal fluid, compatible with mitochondrial dysfunction in brain tissue. The hypoplastic cerebellar hemispheres, documented by cranial magnetic resonance imaging, indicated that normal growth and development of the fetal brain were affected by the primary defect. Cerebellar hypoplasia can be caused by various metabolic and non-metabolic diseases [34], and the findings in this patient suggest that deficiency of cytochrome \( c \) oxidase may be another cause.

Studies in cultured fibroblasts from the patient showed also a selective deficiency of cytochrome \( c \) oxidase activity, but metabolic labeling experiments demonstrated that the rate of synthesis and turnover of mitochondrial and nuclear subunits of cytochrome \( c \) oxidase did not differ between patient and control cells, suggesting that the steady-state concentration of the enzyme complex was normal (Fig. 1). This was confirmed by the results from two-dimensional electrophoresis. The position of the assembled enzyme complex after electrophoresis in the first dimension was the same as that of normally-sized cytochrome \( c \) oxidase and the steady-state concentration of individual subunits of cytochrome \( c \) oxidase, visualized by staining after electrophoresis in the second dimension, was comparable in fibroblasts from the patient and control cells (Fig. 2). The reduced activity of the enzyme in the patient was therefore not due to a reduced amount of complex IV, but to a diminished activity per molecule of the enzyme.

To investigate the diminished activity of cytochrome \( c \) oxidase from the patient in more detail, the kinetics of the reactions of the enzyme were studied. These studies were performed at intermediate ionic strength and the reactions were analyzed for the high-affinity phase, since these conditions are supposed to resemble the physiological situation [20,35,36]. This analysis showed that the activity per molecule of enzyme (or turnover number) was indeed decreased in the patient, and, moreover, that the \( K_m \) for reduced bovine cytochrome \( c \) was 4-fold increased (Fig. 4). In view of the difficulty to obtain sufficient material for classical kinetic assays with purified mitochondrial enzymes from patients, the analyses were carried out with supernatants of cells treated with lauryl maltoside. Using the same approach, the kinetic parameters for human cytochrome \( c \) oxidase from different tissues were compared in a previous study [20]. The values for \( K_m \) and maximal turnover number per molecule enzyme obtained in that study for cytochrome \( c \) oxidase from, e.g., human kidney are well comparable to those found in the present study for control fibroblasts.

The activity of cytochrome \( c \) oxidase from the patient showed no abnormalities when ascorbate and tetramethylphenylenediamine were used as artificial electron donors in experiments with permeabilized fibroblasts (Table 1). The interaction (binding and dissociation) of cytochrome \( c \) and cytochrome \( c \) oxidase is not rate-limiting for the activity under the latter conditions, whereas this is the case when activity measurements are carried out with cytochrome \( c \) as substrate [36]. The reduction of cytochrome \( c \) oxidase activity in the patient is thus most probably related to defects in the interaction with cytochrome \( c \) and not caused by defects that influence the electron transfer or proton-pumping properties of the enzyme. The most obvious explanation for this would be a mutation in subunit II of the enzyme, since this subunit contains the cytochrome \( c \) binding site [37]. However, sequence analysis of the entire gene revealed no mutations, the genes for subunits I and III showed no major rearrangements, and the expression of all three mitochondrial genes for subunits of cytochrome \( c \) oxidase was completely normal.

Alternatively, a mutation in a nuclear gene for one of the subunits of cytochrome \( c \) oxidase might be the cause of the aberrant kinetic parameters of the enzyme in the patient. This possibility would suggest an autosomal recessive mutation. This could be in line with both the consanguinity of the parents of the patient and the birth of a second, healthy child. The postulated mutation in a nuclear
subunit of the enzyme should indirectly influence the binding of cytochrome c to explain our findings. Possible regulation of the activity of cytochrome c oxidase by binding to the nuclear subunits of effectors such as nucleotides or hormones was proposed already a long time ago [38], and regulation of the activity of the mammalian enzyme by binding of ATP to subunit VIa has been demonstrated [39]. Possible differences in modulation by ATP binding can, however, not account for the different kinetics of cytochrome c oxidase from the patient, since the activity was normal when tested in the presence of ATP and ADP (in permeabilized cells), but abnormal in their absence (as measured with the solubilized enzyme). Mutations of subunits VIa and VIIa can also be excluded, since deficiency of cytochrome c oxidase activity was found both in cultured fibroblasts (containing the liver-type forms of these subunits) and in muscle (containing the muscle-type forms) from the patient.

The postulated mutation does not result in obvious differences in molecular mass of one of the subunits: the electrophoretic mobility of the nuclear subunits of cytochrome c oxidase were the same in the patient and control (Fig. 3). Apparently, it also does not influence the assembly of the enzyme, since the steady-state concentration and the molecular mass of the enzyme were normal after native electrophoresis (Fig. 2). Isolation of the enzyme by (quantitative) immunoprecipitation from fibroblasts from the patient did, however, frequently result in under-representation of subunit VIII (Fig. 3). Since subunit VIII can only be precipitated when it is part of the assembled enzyme complex (see Section 2) this suggests a looser association of the subunits in cytochrome c oxidase from the patient than in the controls. In turn, this might be due to the postulated (point) mutation in one of the nuclear subunits. Experiments to determine the nucleotide sequence of the genes encoding the nuclear subunits are presently in progress. The results will not only help to find the molecular basis of the altered kinetics of the enzyme in this patient, but also increase the insight about the function of the nuclear subunits in the regulation of the activity of cytochrome c oxidase.

This is the first detailed description of a cytochrome c oxidase deficiency that is based on abnormal kinetic properties of the enzyme, expressed in cultured fibroblasts and, in contrast to other cases of cytochrome c oxidase deficiencies [11,40–43] not associated with reduced concentrations of the enzyme. A previous study, in which we compared the concentration, using immuno-flow cytometry, and the activity of the enzyme in cultured fibroblasts derived from patients with selective deficiency of cytochrome c oxidase showed a normal concentration, but a decreased activity in two of the nine tested cell lines [16]. Deficiencies in activity that are based on alterations of the kinetic properties of the enzyme might thus not be that rare. The clinical and pathological consequences of this type of cytochrome c oxidase deficiency can only be roughly estimated. Differential tissue-involvement, as was the case in our patient, could reflect the different requirements for cytochrome c oxidase activity, or differences in the in vivo regulation of the activity of the enzyme. In cultured fibroblasts, the deficiency did not lead to a reduced capacity for ATP production, suggesting that the reduced activity of cytochrome c oxidase was not limiting for this process. Since the patient did not show major metabolic disturbances, the diminished cytochrome c oxidase activity might likewise not have affected normal liver function. The defect appeared, however, to influence muscle function and especially the development of the cerebellum.

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References


