Human diseases with defects in oxidative phosphorylation

1. Decreased amounts of assembled oxidative phosphorylation complexes in mitochondrial encephalomyopathies

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The amount of oxidative phosphorylation enzymes in mitochondrial encephalomyopathy patients has been studied by two-dimensional electrophoresis (blue native PAGE/Tricine-SDS-PAGE). Only 20 mg muscle was required to identify and analyse complexes I, III, IV, and V after Coomassie staining. In most cases reduced amounts of the involved complex(es) correlated well with decreased enzyme activities. The reliability of the method was reflected by the constant mutual ratio of the complexes found in all controls. Deviations from normal ratios were found to be more sensitive indicators for a defect than the absolute quantities, which varied considerably within the control group both in the enzymic and in the electrophoretic analysis. The effect of the mitochondrial tRNA Lys (UUR) mutation in mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes on the amount of oxidative phosphorylation complexes was demonstrated for the first time directly on the protein level. In patients without known DNA mutations, specific defects of single complexes were identified. The new technique is a sensitive method for the identification of oxidative phosphorylation defects, complementary to enzymic measurements.

Keywords. Mitochondrial encephalomyopathies; respiratory chain deficiencies; blue native electrophoresis; mtDNA.

Mitochondrial myopathies are a heterogeneous group of disorders affecting predominantly skeletal muscle and the central nervous system. A defect in the mitochondrial energy metabolism is thought to be the primary cause of disease [1, 2]. Each mitochondrion contains its own mtDNA that codes for 13 genes of oxidative phosphorylation (OXPHOS) proteins plus two structural rRNAs and 22 tRNAs necessary for expression [3]. Recently, several mutations of mtDNA have been described in association with mitochondrial encephalomyopathies or other neurological disorders [4]. Examples of these are large-scale deletions of mtDNA in chronic progressive external ophthalmoplegia (CPEO) [5], point mutations in the mitochondrial tRNA Lys (UUR) or the tRNA Lys (UUR) gene in mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes (MELAS) [6] and in myoclonic epilepsy and ragged-red fibers (MERRF) [7], or in the gene coding for the ATPase 6 subunit in Leigh’s syndrome [8]. These mutations are usually found only in a proportion of the mtDNA molecules (heteroplasmy), which may vary between different tissues and which determines the phenotypic expression of the mutation in a given tissue.

Biochemical analysis of muscle tissue varied from severe isolated or combined deficiencies of complex I and complex IV to no apparent defects [9-11]. In a large proportion of patients with respiratory-chain enzyme deficiencies, no mtDNA mutations have been found [12, 13]. These defects could have arisen from mutations in the nDNA, although such mutations have not yet been identified.

In the present study we used a quantitative two-dimensional electrophoretic procedure (blue native electrophoresis/Tricine-SDS-PAGE) to analyse the amount of assembled OXPHOS complexes in mitochondrial encephalomyopathy patients with mtDNA mutations and/or enzyme defects in the respiratory chain.

MATERIALS AND METHODS

Chemicals. Dodecyl β-D-maltoside was from Boehringer Mannheim, 6-aminoheptanonic acid from Fluka, acrylamide and bisacrylamide (the commercial twice-crystallized products), and Serva blue G (Coomassie blue G 250) from Serva. All other chemicals were from Sigma.

Electrophoretic techniques and sample preparation. The sample preparation for blue native PAGE started from 20 mg muscle that was homogenized with 250 μl 440 mM sucrose, 1 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride, 20 mM Na/Mops pH 7.2, by using a tightly fitting glass/Teflon homogenizer. After a 20-min centrifugation at 20000 g, the supernatant
was discarded and the sediment was suspended in 40 μl 1 M aminohexanoic acid, 50 mM Bistris/HCl pH 7.0, by twirling with a tiny spatula. The membrane proteins were solubilized by adding 15 μl 10% (mass/vol.) dodecylmaltoside. After a 15-min centrifugation at 100 000 g, the supernatant was collected, 7.5 μl 5% (mass/vol.) Serva blue G in 1 M aminohexanoic acid was added, and the total volume was applied to gel wells (10 mm × 1.6 mm) in blue native PAGE, that was performed as described [14]. Second dimension Tricine-SDS-PAGE starting from individual lanes from blue native PAGE was performed as described previously [15].

**Staining and quantification.** All two-dimensional (2D) gels were stained with Coomassie blue G 250 [16]. Densitometric quantification was performed with a personal densitometer (a laser scanning densitometer from Molecular Dynamics, Krefeld), and a Quick Scan Jr filter-densitometer (Desaga, Heidelberg) using a 595-nm filter. The personal densitometer allowed one to use rectangles around individual bands and to quantify the absolute staining intensity of bands. The filter-densitometer was used for one-dimensional scanning through the lanes of the characteristic polypeptide patterns. A 5-mm-broad slit was used in order to include the most intensely stained part of the bands. Both quantification techniques essentially gave the same results.

Several successive steps were applied for quantification of 2D gels. First, the staining intensity of selected protein subunits of complexes was measured. The selected bands, marked by arrows in Fig. 1A, were: the 75-kDa, the unresolved 51-kDa and 49-kDa, and the 39-kDa subunit of complex I [17], subunits α, β, γ, b, d, and α of complex V [18], core proteins I and II together, cytochrome b, iron sulfur protein, 13.4-kDa and 9.5-kDa proteins of complex III [19], subunits II (apparent molecular mass, mapp about 20 kDa), IV (mapp 16 kDa), Va,b (one band, mapp 11 kDa), VIa,b,c (two bands, mapp around 9 kDa) of complex IV [20]. Then the average staining intensity of each individual band from the control group was set to 1.00 and the staining intensity of individual bands from patient samples was compared to the corresponding average staining intensity from the controls. The mean value of these percentages was taken as the percentage of holo-complex in the patient sample. One of the quantified complexes (complex V in the present work, complex III in the studies of Alzheimer disease in the following paper in this volume) was then used as an internal reference in order to obtain the mutual ratios of complexes in each individual gel.

**Patients.** Seven patients were selected for this study based upon low respiratory-chain enzyme activities and/or mtDNA mutations observed in skeletal muscle biopsies. A brief overview of some clinical and laboratory data is presented in Table 1.

The two patients with the MELAS syndrome (P3 and P4) carried the heteroplasmic A3243G mutation in 50% and 60% of their mtDNA, respectively, in their skeletal muscle. Riboflavin therapy in patient P3 has been previously described [21]. The two CPEO patients (P6 and P7) carried a large heteroplasmic deletion in 50% and 75%, respectively, of their muscle mtDNA. This deletion included the cytochrome-c oxidase III gene (COX III). All other patients and controls were free of known mtDNA mutations associated with mitochondrial myopathies or respiratory-chain defects [4].

Control specimens for enzymic measurements were considered those in which a mitochondrial defect had been excluded by normal substrate oxidation rates of intact mitochondria and individual enzyme activities, as described previously [22]. The control group for 2D electrophoretic analysis consisted of ten patients with normal individual OXPHOS enzyme activities.

**Morphology and biochemistry.** All specimens of quadriceps muscle were removed by surgical biopsy, whereas the fresh tissue was processed for histochemical and biochemical studies. From patients P1 and P2 autopsy specimens, that had been removed within 1 h of death, were also studied. The oxidation rates of radiolabeled substrates (pyruvate, malate, and succinate), and the activities of enzyme complexes of the respiratory chain were measured in 600 g supernatants of muscle homogenates [22, 23]. Cytochrome c oxidase (complex IV) was determined according to Cooperstein and Lazarow [24] and citrate synthase according to Strie [25]. The rotenone-sensitive NADH:ubiquinone-1 oxidoreductase (complex I), and NADH:O2 oxidoreductase (complex I-III-IV) were measured according to Fischer et al. [22]. Ubiquinone-1 was a gift from Hoffman-La Roche. Complex III was measured using decylubiquinol and cytochrome c as substrates according to Zheng et al. [26]. All catalytic activities of OXPHOS complexes were first normalized for the citrate synthase activity to account for differences in recovery of mitochondria (values not shown). These ratios then were expressed as the percentage of the mean value of the controls.

**Western blotting.** Mitochondrial proteins, isolated by differential centrifugation of homogenized skeletal muscle specimens, were fractionated (2 μg protein/lane) on 10% or 16.5% slab gels in a Mini-Prtn II apparatus (BioRad) according to Schägger and Von Jagow [16]. Antibodies were raised in rabbits by injection of the purified complex into lymph nodes according to Sigel et al. [28].

The antibodies against the bovine complex I holo-enzyme, its 24-kDa subunit and the ND1 subunit of Neurospora crassa [29] were kind gifts from Dr U. Harnisch (Department of Biochemistry, University of Düsseldorf), Dr. J. Walker (MRC, Cambridge) and Dr. S. Werner (Department of Physiological Chemistry, University of Munich), respectively.

All antibodies were used at a dilution of 1:1000, except for the antibodies against ND4 and ND6, which were used at a dilution of 1:100. The ECL kit (Amersham) was used for detection of bound antibodies.

**RESULTS**

**Muscle morphology.** Quadriceps muscle from six patients (P2—P7) was histologically examined. From patient P1 no histological report was available. No ragged red fibers were observed in patients P2 (isolated complex I defect) and P5 (isolated complex IV defect). The two patients with MELAS showed a higher frequency of these fibers (10.7% in P3 and 8.5% in P4), as compared to the two patients with CPEO (0.5% in P6 and 0.6% in P7).

Cytochrome-c oxidase activity was normal in most fibers of patient P2, whereas patient P5 had a complete lack of this activity. The activity in the ragged red fibers of the two patients with CPEO (P6 and P7) contrasted with that of the two patients with MELAS (P3 and P4). In the CPEO patients the majority of ragged red fibers contained no cytochrome-c oxidase, whereas this was not the case in the MELAS patients.
Table 1. Clinical, laboratory and morphological features of patients studied. Ages are given in years unless otherwise specified (d = days, inf = infantile). For 'Progression', † = indicates death. Other abbreviations: na, not available; ?, not applicable because of young age; CT/MRI, computerized tomography/magnetic resonance imaging.

<table>
<thead>
<tr>
<th>Feature</th>
<th>P1</th>
<th>P2</th>
<th>P3</th>
<th>P4</th>
<th>P5</th>
<th>P6</th>
<th>P7</th>
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<td>M</td>
<td>F</td>
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<td>M</td>
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<td>M</td>
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<td>MELAS</td>
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<td>CPEO</td>
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<td>22</td>
<td>inf</td>
<td>12</td>
<td>5</td>
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<td>Failure to thrive</td>
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Table 2. Quantification of OXPHOS complexes after two-dimensional electrophoresis compared to enzyme activities in fresh 600 g supernatants of patients muscle. Controls were set to 1.00; their values are given as a range ±SD (no.). Stain absolute, total amount of an individual complex in 20 mg muscle deduced from protein stain in 2D gels; stain/V, ratio of the absolute stain of an individual complex and complex V from the same gel, the quantification being described in Methods; act./CS, ratio of catalytic activities of complexes and citrate synthase. All values are normalized to the average values of the controls. Underlined values are below the mean of the control group minus twice its standard deviation (P<0.05); nd, not determined.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Complex I stain/V</th>
<th>Complex I act./CS</th>
<th>Complex III stain/V</th>
<th>Complex III act./CS</th>
<th>Complex IV stain/V</th>
<th>Complex IV act./CS</th>
<th>Complex V absolute</th>
<th>Complex I-III-IV activity/CS</th>
</tr>
</thead>
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<tr>
<td>Controls</td>
<td>0.75—1.42</td>
<td>± 0.22 (10)</td>
<td>0.81—1.37</td>
<td>± 0.16 (10)</td>
<td>0.60—1.39</td>
<td>± 0.24 (24)</td>
<td>0.90—1.29</td>
<td>± 0.14 (10)</td>
</tr>
<tr>
<td>P1 (complex I defect)</td>
<td>&lt;0.23</td>
<td>0.13</td>
<td>1.23</td>
<td>nd</td>
<td>1.15</td>
<td>0.88</td>
<td>0.66</td>
<td>0.16</td>
</tr>
<tr>
<td>P2 (complex I defect)</td>
<td>&lt;0.24</td>
<td>0.33</td>
<td>0.96</td>
<td>0.53</td>
<td>0.97</td>
<td>0.72</td>
<td>0.70</td>
<td>0.16</td>
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<tr>
<td>P3 (MELAS)</td>
<td>0.36</td>
<td>0.27</td>
<td>0.58</td>
<td>0.38</td>
<td>0.47</td>
<td>0.58</td>
<td>1.49</td>
<td>0.22</td>
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<tr>
<td>P4 (MELAS)</td>
<td>&lt;0.12</td>
<td>0.27</td>
<td>0.52</td>
<td>0.55</td>
<td>0.26</td>
<td>0.24</td>
<td>1.85</td>
<td>0.16</td>
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<tr>
<td>P5 (complex IV defect)</td>
<td>0.94</td>
<td>1.60</td>
<td>0.86</td>
<td>nd</td>
<td>0.20</td>
<td>0.23</td>
<td>0.50</td>
<td>0.36</td>
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<tr>
<td>P6 (CPEO)</td>
<td>0.89</td>
<td>0.84</td>
<td>1.03</td>
<td>0.57</td>
<td>1.22</td>
<td>1.18</td>
<td>0.36</td>
<td>1.42</td>
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<tr>
<td>P7 (CPEO)</td>
<td>1.09</td>
<td>0.96</td>
<td>1.33</td>
<td>0.57</td>
<td>0.82</td>
<td>0.72</td>
<td>0.71</td>
<td>0.53</td>
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Biochemistry. Substrate oxidation rates in 600 g supernatants of fresh muscle homogenates were decreased in all patients, except for the two CPEO patients (P6 and P7, results not shown). Also the NADH:O2 oxidoreductase activity (complex I-III-IV, Table 2) was severely diminished in the same patients. An isolated complex I deficiency could be clearly attributed to patient P1, as its activity in muscle was only 13% of the mean of controls (P<0.05). This observation was confirmed in muscle, liver and brain tissue obtained through autopsy after the child had died at the age of 27 days.

In patient P2 a decreased complex I activity was measured, although this was not statistically significant because of the broad range of control values. Since complex III activity was also below the normal range, there was some ambiguity about possible complex I and/or complex III defects.

In the MELAS patients, low activities of complexes I, III, and IV were observed but only the complex III activity of patient P3 and the complex IV activity of patient P4 were significantly reduced.

In patient P5 a severe complex IV deficiency was found (23% of mean control value). The muscle of the two CPEO patients (P6 and P7) did not reveal any markedly decreased enzyme activities, which was in accordance with the observed normal substrate oxidation rates.

Two-dimensional electrophoresis. The electrophoretic and quantification techniques were optimized and validated by using bovine heart mitochondria and human autopsy specimens. The extraction of OXPHOS proteins from mitochondria and tissue homogenates and the protein recovery after 2D electrophoresis were almost quantitative. The Coomassie staining intensity of complexes correlated linearly with the protein load over a much broader range than used for analysis of human biopsy specimens (experiments not shown).
These minimal amounts of complexes, additionally, make it possible to determine the absolute quantification of the complexes and their interactions with the control group. This allows for the quantification of the complexes and their interactions with the control group, which can be used to deduce the absolute quantification of the complexes and their interactions with the control group.
the varying absolute amounts of complexes. The standard deviation, e.g. of the complex IV/V ratios in the control group (1 ± 0.14) was much lower than that of the absolute complex IV amounts (1 ± 0.34).

In muscle of patients P1 and P2 isolated complex I defects were identified by the low absolute amounts (not shown) as well as by the low complex IV/V ratios (Table 2). The results of the electrophoretic and enzymic analysis were in parallel but, due to the smaller standard deviations of the electrophoretic analysis, the complex IV/V ratios were clearly below the P<0.05 limit in both cases.

The two MELAS patients showed varying degrees of defects. Considering absolute amounts, only a significant reduction of complex I in patient P4 was observed (not shown). Complex V, in contrast, was normal or moderately increased, as compared to the controls, resulting in reduced ratios of all individual complexes relative to complex V. Ratios of complexes and enzymic activities paralleled, but only the electrophoretic analysis revealed a significant reduction of all mutual ratios.

In patient P5 the absolute quantities of the complexes were generally below the normal range, but only the quantity of complex IV was significantly reduced. The ratios of the complexes were normal, except for complex IV (0.20). Thus, a generally low content of OXPHOS complexes seemed to be accompanied by a severe complex IV defect.

The two CPEO patients (P6 and P7) showed normal ratios of the complexes. Only in patient P6 were the absolute staining intensities of all complexes below the normal range of controls without reaching the level of significance (not shown).

The subunit compositions of all visible complexes were found to be normal, and no partially assembled complex could be identified in any of the patients.

**Western blotting.** We tested whether the reduced amounts of assembled complexes were caused by a missing subunit, i.e. whether individual subunits were present in abnormal ratios. Mitochondrial proteins were separated by one-dimensional Tricine-SDS/PAGE. After the transfer to nitrocellulose paper, the blots were incubated with different antibodies.

Reactions of mitochondrial proteins from patients P1—P4 with antisera against holo-complex I, against single mitochondrially coded subunits (ND1, ND4 and ND6), and a nuclear-coded subunit (24kDa), were all diminished, whereas the reactions with an antibody against the ATPase β subunit were normal (results not shown). Thus it seemed that the major portion of unassembled subunits was completely degraded.

Antibodies against human heart complex IV that gave a strong reaction with all of the larger subunits, including the mitochondrially coded subunits I, II and III, were used to screen for missing or proteolytically fragmented complex IV subunits. An antibody against bovine heart adenine nucleotide translocase was used as a reference. Patients P4 and P5, who had strongly decreased levels of assembled complex IV in the 2D electrophoresis, also showed reduced levels of individual cytochrome-c oxidase subunits. Reactions with proteolytic fragments were not observed, indicating again that unassembled protein subunits were proteolytically degraded (results not shown).

**DISCUSSION**

Morphological, molecular genetic, biochemical, electrophoretic and Western blotting techniques were applied to characterize three groups of OXPHOS defects: CPEO patients with deletions in the mtDNA, MELAS patients with a point mutation in the tRNA Leu(UUR) gene, and patients with fatal infantile forms without known mtDNA mutations, who suffered from isolated defects either of complex I or of complex IV.

Our special interest was the study of the amounts of assembled OXPHOS enzymes and the mutual ratios of these complexes using a new type of 2D electrophoresis (blue native PAGE/Tricine-SDS-PAGE).

The enzymic and 2D electrophoretic approaches, summarized in Table 2, gave similar results; however, the standard deviations of controls in the electrophoretic analysis were markedly lower than in the enzymic analysis, allowing the results to be interpreted with greater statistical confidence. Both techniques should always be used in parallel because in rare cases, which are under investigation, normal amounts of correctly assembled complexes were observed at very low catalytic activities.

Quantification of a 2D gel from a sample with lower content of OXPHOS proteins (around 50% in patient P5) allowed a single defect of complex IV to be clearly identified by a selectively decreased complex IV/V ratio. Since the detection limit of absolute amounts of complex IV in the Coomassie-stained gels was reached only at 10% of the mean of the controls (approximately 20% in the case of complex I defects), missing or hardly detectable polypeptide patterns clearly indicate a defect, but enzyme activity measurements are required to quantify extremely reduced complexes more exactly. More sensitive silver-staining procedures were not found to be useful for reliable quantification. This may be overcome by the use of suitable antibodies for quantitative immunodetection.

The electrophoretic technique using mutual ratios of complexes has the advantage of quantifying all complexes in one gel, thus minimizing errors in comparing different samples. Since the ratios of complex concentrations were found to be fairly constant in the control group and independent of the protein load, divergent ratios in patients were found to be a superior measure to detect OXPHOS defects. Isolated defects of OXPHOS complexes, as in patients P1, P2, and P5, could be clearly discriminated from multiple defects, as in MELAS patients P3 and P4. Furthermore, complex V, which is difficult to quantify by enzymic analysis, was easily quantified in 2D gels. Complex V defects were not observed in the small number of patients studied, but were found in Alzheimer patients (see following paper in this volume).

In the case of the two MELAS patients (P3 and P4) a reduced rate of mitochondrial protein synthesis [30, 31] would account for a low amount of mitochondrial translation products and a decreased amount of those complexes that comprise at least one mitochondrially coded protein subunit (all, except complex II). The enzymic analysis showed that the activities of complexes I, III, and IV were differentially affected (Table 2). The electrophoretic analysis identified altered mutual ratios of complexes even more clearly and demonstrated a hitherto unidentified effect on complex III. Complex I was more affected than complex IV and the ratio of complex III/V was also found to be reduced. This order in the extent of the deficiencies seemed to parallel the number of subunits encoded in mtDNA of each complex: seven, three and one, respectively. However, complex V also contains two subunits, ATPase 6 and 8, encoded in mtDNA, but was found to be normal or moderately increased (Table 2). It is not known whether this apparent discrepancy is based on differences at the transcription, translation, or protein turnover levels.

Three patients with fatal infantile mitochondrial encephalomyopathy (P1, P2 and P5) had no deletion, depletion, MELAS or MERRF mutation, and their muscles lacked ragged red fibers, which has also been observed in diseases caused by nonsense mutations in mtDNA, like Leber hereditary optic neuropathy and...
Leigh’s syndrome [4]. The localization of the defects (specific complex I or complex IV deficiencies) was achieved by using 2D electrophoresis and enzyme activity measurements. No disease-related missense mutations have been found in the ND genes of patient P1 (De Vries et al., unpublished results). Therefore the complex I deficiency in this patient is most probably caused by a mutation in one of the nuclear genes of complex I or in a gene specifically controlling the assembly of complex I. These hypotheses are under present investigation. No partially assembled complexes could be identified on the basis of their subunit composition (Fig. 1) in any of the 2D gels from patients and controls, not even by using more sensitive silver-staining techniques.

An OXPHOS defect in CPEO patient P6 seemed to be apparent from the absolute amounts of complexes in 2D electrophoresis (32–44% of the mean of controls) that seemed to be in good agreement with the observed 50% deleted mtDNA. However, this correlation was not found in patient P7 with an even higher percentage of deleted mtDNA (75%), but almost normal absolute amounts of complexes. The discrepancy between a high percentage of deleted mtDNA and a hardly detectable defect can be explained by an uneven distribution of deleted mtDNA in the muscle fibers [32, 33]. The few ragged red fibers which predominantly lacked cytochrome-c oxidase had increased amounts of mtDNA, and have been shown to be almost completely homoplasmic for the deletion [34], while most other fibers had normal amounts of heteroplasmic mtDNA. This would result overall in a high percentage of deletion, but the ratio of deleted/wild-type mtDNA would stay below the threshold of phenotypic expression in most fibers. In contrast to the CPEO patients, the MELAS patients P3 and P4 showed multiple defects at only 50–60% mutated DNA, which is more even distributed [32, 33].

Western blotting was performed in order to detect potential variations in subunit quantities and stoichiometries, i.e. to analyze both free and correctly assembled subunits. In fact, reduced reactions of antibodies correlated with reduced mutual ratios of complexes in the electrophoretic and enzymic analysis, but no significant deviations from the normal ratio of individual subunits in complexes were detected. This observation confirms earlier results from other groups, e.g. [13], indicating that free subunits, not assembled into complexes, are proteolytically degraded, and shows that only the assembled complexes are analyzed by Western blotting, similar to 2D electrophoresis and enzymic analysis.

We conclude that 2D electrophoresis (blue native PAGE/Tricine-SDS-PAGE) is a complementary, sensitive and valuable new method for the identification of OXPHOS defects in mitochondrial encephalomyopathies. Work is in progress to use specific antibodies and cell cultures to allow identification of partially assembled complexes and free subunits, which could give clues to the underlying gene defect in patients.

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