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Polarographic Evaluation of Mitochondrial Enzymes Activity in Isolated Mitochondria and in Permeabilized Human Muscle Cells with Inherited Mitochondrial Defects

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Summary

Inherited disturbances of the mitochondrial energy generating system represent a heterogeneous group of disorders associated with a broad spectrum of metabolic abnormalities and clinical symptoms. We used the polarographic and spectrophotometric method for detection of mitochondrial disorders, because these two techniques provide a different insight into mitochondrial function. In six patients suspected of mitochondrial disease we found defects of complex I (two patients), complex III (one patient), complex IV (two patients) and a combination of defect of complex III and IV (one patient). Citrate synthase activity, used as the reference enzyme, was not changed. A comparison of the two methods showed several differences in evaluation of mitochondrial enzymes activity due to the fact that both methods used different conditions for enzyme activity measurements. In contrast to oxygen consumption measurements, where the function of the whole-integrated respiratory chain is characterized, spectrophotometric measurements characterize activities of isolated complexes in disintegrated membranes. However, it may be concluded from our experiments that both methods provide useful and complementary data about mitochondrial energetic functions. Whereas spectrophotometric data are suitable for evaluation of maximal enzyme activities of mitochondrial enzyme complexes, polarographic data provide better information about enzyme activities in cells with mitochondrial defects under *in situ* conditions.

Key words

Mitochondria • Muscle cells • Respiratory chain complexes • Polarography • Spectrophotometry

Introduction

Mitochondria participate in a number of metabolic reactions, but their principal biological function is energy conversion. ATP produced by the oxidative phosphorylation system localized in the inner mitochondrial membrane is the main source of energy for functional activity of mammalian cells. Dysfunction of respiratory chain (RC) complexes and ATP synthase results in a highly heterogeneous group of diseases with very bad prognosis, which predominantly affect tissues with high energetic demands such as muscles, the brain and heart.

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Most of the studies, which analyze the function of RC complexes and ATP synthase in patients with mitochondrial defects are based on the measurement of enzyme activities by spectrophotometric methods in tissue homogenates, postnuclear supernatants and isolated mitochondria. The results of these methods are highly reproducible and can easily be interpreted. Nevertheless, these methods have several limitations. During homogenization of tissue samples approximately 5-20 % of mitochondria lose the integrity of their outer membrane as indicated by the release of cytochrome *c* during the isolation procedure. Furthermore, the yield is rather low, depending on the type of the tissue (Nedergaard and Cannon 1979). Spectrophotometric methods are certainly useful for the estimation of maximal enzyme activities, but these activities are determined under conditions, which do not correspond to the physiological cytosolic environment of mitochondria (e.g. modified osmolarity, pH, high concentration of substrates, presence of detergents). It is, therefore important, especially for the diagnostics of mitochondrial diseases to determine the activity of mitochondrial enzymes under conditions, which are closer to the physiological situation inside the cell. Mitochondria in intact or permeabilized cells do not represent a population of separated organelles, but they interact with the cytoskeleton network being integrated into functional clusters. It was proposed that mitochondria in these functional clusters are connected with each other and can respond to the minimal concentration changes of metabolic substrates and products in their inner and outer environment (Skulachev 2001). Furthermore, mitochondria are not only in a close proximity with each other, but they are also in a close contact with other cell organelles and structures, such as lysosomes, endoplasmic reticulum, and microtubule or actin filaments (Frey and Mannella 2000, Garesse and Vallejo 2001). Such cooperation might be essential for biogenesis of mitochondria and for correct function of the whole mitochondrial energy-providing system. This all clearly enhances the importance of studying the mitochondrial function *in situ* using whole cells. This can be achieved by polarographic measurements of oxygen consumption in intact and permeabilized cells (Villani and Attardi 2000) or permeabilized tissue fragments (Gnaiger *et al.* 1995) which preserve mitochondrial integrity and intracellular as well as interorganelle structural communications.

The major advantage of the polarographic method is that it provides the possibility of assessing the activity of all complexes of the oxidative phosphorylation system in intact mitochondrial membranes and under the conditions, which are much closer to the *in vivo* situation. There is growing evidence that this type of measurements significantly improves diagnostics and our understanding of the molecular basis of different mitochondrial disorders, especially those connected with an altered assembly and stability of mitochondrial oxidative phosphorylation complexes (Rustin *et al.* 1994, Villani and Attardi 2000, Chowdhury *et al.* 2000).

In our study, we analyzed isolated muscle mitochondria and saponin-permeabilized muscle fiber defects of mitochondrial respiratory chain enzymes by polarography in six patients suspected of mitochondrial defects. Furthermore, we discuss the advantages and disadvantages of spectrophotometric and polarographic methods in the diagnostics of mitochondrial diseases.

Material and Methods

Material

The control group consisted of 26 patients (2-20 years old) who were recommended for muscle biopsy with the clinical suspicion of mitochondrial neuromuscular diseases, which were not confirmed.

Mitochondrial defects were found in a group of 6 patients. These patients (1-28 years old) had clinical symptoms of mitochondrial disorders including muscle weakness, progressive hypotonia, myopathy, encephalopathy, cardiomyopathy, psychomotor retardation and lactic acidosis. Muscle biopsies (cca 120-150 mg of muscle tissue) were obtained from the musculus tibialis anterior after informed consent of the patients or from their parents.

Isolation of muscle mitochondria

Muscle mitochondria were isolated according to Makinen and Lee (1968). Tissue samples (about 100 mg) were homogenized at 4 ^{o}C in a KCl medium (100 mM KCl, 50 mM Tris, 2 mM EDTA, aprotinine 10 mg/ml, pH 7.5). The homogenate was centrifuged 10 min at 600x*g*, the supernatant was filtered through a 100 μm nylon mesh and centrifuged 10 min at 10 000x*g*. The mitochondrial pellet was suspended in a KCl medium and centrifuged 10 min at 10 000x*g*. The final mitochondrial pellet was suspended in a KCl medium to a protein concentration of approximately 20 mg/ml.

Spectrophotometric measurements

The activities of respiratory chain complexes in isolated muscle mitochondria were measured spectrophotometrically: NADH-coenzyme Q_{10} oxidoreductase (NQR, complex I) according to Fischer *et al.*

(1986), succinate-coenzyme Q_{10} oxidoreductase (SQR, complex II) according to Fischer *et al.* (1985). Coenzyme Q_{10}-cytochrome c oxidoreductase (QCCR, complex III) was assayed according to Rustin *et al.* (1994) and cytochrome c oxidase (COX, complex IV) according to Wharton and Tzagaloff (1967). Citrate synthase (CS) was measured according to Rustin *et al.* (1994) and proteins were determined by the method of Lowry *et al.* (1951).

Preparation of muscle fibers

Muscle fibers were separated mechanically according to Kunz *et al.* (1993) in a medium containing 10 mM Ca-EGTA (0.1 μM free Ca^{2+}), 20 mM imidazole, 20 mM taurine, 50 mM K-MES, 0.5 mM DTT, 6.56 mM $MgCl_2$, 5.77 mM ATP, 15 mM phosphocreatine, pH 7.1. Bundles of fibers between 15 and 20 mg wet weight were placed into 2 ml of an ice-cold medium. Thereafter, 50 μg/ml saponin (Sigma S-2149) was added and the suspension was gently mixed for 30 min at 4 °C. The fiber bundles were washed to remove saponin and stored on ice before being used for respiration measurements.

Polarographic measurements

The respiration of isolated mitochondria was measured at 30 °C using OROBOROS Oxygraph (Anton Paar, Innsbruck, Austria) in the medium containing 0.5 mM EDTA, 3 mM $MgCl_2$, 20 mM taurine, 10 mM KH_2PO_4, 20 mM HEPES, 200 mM sucrose, BSA 1 g/l, pH 7.1. For saponin-skinned muscle fibers, a medium containing 0.5 mM Na_2EDTA, 5 mM $MgCl_2$, 10 mM KH_2PO_4, 110 mM mannitol, 60 mM KCl, 60 mM Tris-HCl, pH 7.4 was used. Polarographic measurements were performed as multiple substrate-inhibitor analyses (Kunz *et al.* 1993, Sperl *et al.* 1994) in the presence of 1 mM ADP. The following substrates and inhibitors were used for isolated muscle mitochondria: 10 mM pyruvate + 2 mM malate, 10 mM glutamate + 2 mM malate, 5 μM cytochrome *c*, 1.25 μM rotenone, 10 mM succinate, 2.5 μM antimycin A, 0.4 mM ascorbate + 400 μM TMPD (N,N,N',N'-tetramethyl-p-phenylenediamine dihydrochloride). Specific inhibitors for complex I (rotenone) and complex III (antimycin A) together with substrates allowed us to characterize respiratory chain complexes I, II, III and IV within one measurement. TMPD was used as an artificial electron mediator which accelerates the transfer of electrons from ascorbate to membrane-bound cytochrome c (Lee *et al.* 1967). For saponin-skinned muscle fibers, we used the same substrate concentrations except for: 10 mM pyruvate + 5 mM malate, 10 mM glutamate + 5 mM malate, 2 mM ascorbate + 500 μM TMPD and 2.5 μM FCCP (carbonyl cyanide p-(trifluoromethoxy) phenylhydrazone).

Table 1. Spectrophotometric analysis of the respiratory chain complexes in isolated muscle mitochondria of six patients with mitochondrial disorders.

	Patient 1	**Patient 2**	**Patient 3**	**Patient 4**	**Patient 5**	**Patient 6**	**Controls (n=26)**
NQR	128	175	212	**68**	**95**	282	225±125
SQR	20	50	92	84	128	74	60±40
QCCR	**87**	184	**172**	387	473	324	265±85
COX	**295**	**140**	1177	896	861	**334**	1100±450
CS	442	602	751	1195	1390	1003	835±400

NQR - NADH-coenzyme Q_{10} oxidoreductase, SQR - succinate-coenzyme Q_{10} oxidoreductase, QCCR - coenzyme Q_{10} cytochrome c oxidoreductase, COX - cytochrome c oxidase, CS - citrate synthase (activities were expressed as nmol/min/mg protein).

DNA analyses

Total genomic DNA was isolated from muscle biopsies by phenol extraction. Mitochondrial DNA (mtDNA) point mutations A3243G, A8344G and T8993G were analyzed by PCR-RFLP. For each mutation, an appropriate segment of mtDNA was amplified by PCR, labeled with [α-^{32}P]dCTP and digested by restriction endonuclease: Apa I for A3243G, BglI for A8344G, AvaI for T8993G. The levels of heteroplasmy (an amount of mutated mtDNA molecules) were determined densitometrically. Deletions of mtDNA were analyzed by Southern blot. The probe (flanking the

region of mtDNA from 16130 np to 500 np) corresponded to the non-coding D-loop region of the mtDNA and was labeled by [α-^{32}P]dCTP (Houštěk *et al.* 1995, Stratilová *et al.* 1998a,b, Berger *et al.* 2001, Čapková *et al.* 2002) .

Ethics

The Committee of Medical Ethics approved the present study. Informed consent was obtained from the patients or their parents.

Results

The results of spectrophotometric analysis of respiratory chain complexes in isolated muscle mitochondria are shown in Table 1. The individual values of enzyme activities in six patients with mitochondrial disorders are compared with those of the control group. Markedly decreased cytochrome *c* oxidase activity was found in three patients (patients 1, 2 and 6). In two patients, a decrease of complex I was found (patients 4 and 5) and in two patients (patients 1 and 3) a decrease of complex III was ascertained. In patient 1, a combined deficiency of complex III and IV was present. In all patients, the activity of control enzyme citrate synthase was within the reference range.

In two patients, molecular analysis revealed a mutation in mtDNA. In patient 2 with clinical symptoms of the Kearns-Sayre syndrome (muscle weakness, chronic progressive external ophtalmoplegia, heart block) a large scale mtDNA deletion (7.4 kB) was observed. In patient 3 with clinical symptoms of MELAS syndrome (mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes) a heteroplasmic mtDNA mutation A3243G was found (the level of heteroplasmy was 68 %). Molecular analysis in other patients revealed neither mtDNA mutations A3243G, A8344G and T8993G nor any large-scale mtDNA deletion or mtDNA depletion.

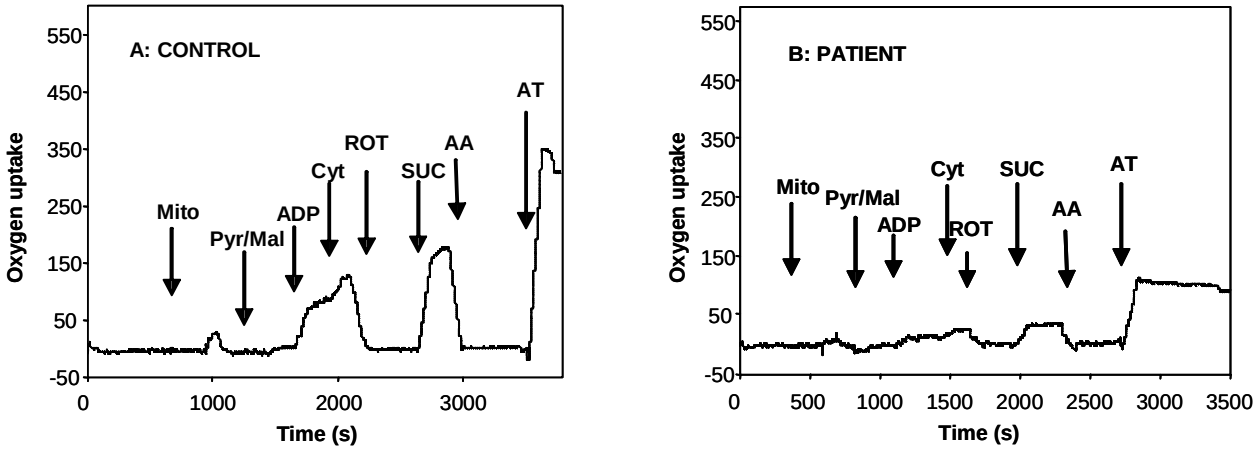

Fig. 1. *Determination of oxygen consumption (pmol oxygen/s/mg protein) using multiple substrate-inhibitor analysis in isolated muscle mitochondria from (A) control and (B) patient 1. Additions: Mito: 90 μg protein of isolated muscle mitochondria; Pyr/Mal: 10 mM pyruvate plus 2 mM malate; ADP: 1 mM ADP; Cyt: 5 μM cytochrome c; ROT: 1.25 μM rotenone; SUC: 10 mM succinate; AA: 2.5 μM antimycin A; AT: 0.4 mM ascorbate plus 400 μM TMPD.*

Mitochondrial enzyme activities were further analyzed by oxygen consumption measurements using isolated mitochondria (patients 1 to 3) and saponin-skinned muscle fibers (patients 4 to 6). Figure 1. shows the typical oxygraphic curves of isolated muscle mitochondria from control subjects and patient 1 which allowed evaluation of the activity of most RC complexes.

Our measurements showed a partial disruption of the outer mitochondrial membrane, because the oxygen consumption by isolated mitochondria was activated by exogenous cytochrome *c*. The oxygen consumption without substitution of cytochrome c was by about 20 % lower in comparison with the respiratory rate in the presence of cytochrome *c* (Table 2). Values of oxygen consumption in isolated mitochondria of patients 1 to 3 are summarized in Table 2. We found a decreased respiration with NADH- and FADH-dependent substrates

and with ascorbate and TMPD in patients 1 and 2. No changes were found in patient 3.

Figure 2 shows typical curves of polarographic measurements with muscle fibers prepared from control and patient biopsies (patient 4). Similarly as with isolated mitochondria, utilization of NADH- and flavoprotein-dependent substrates, and activity of cytochrome c oxidase was evaluated. Under these conditions, respiration was not activated by cytochrome c, indicating that the outer mitochondrial membrane was intact. The values of oxygen consumption from our measurements on permeabilized muscle fibers from patients 4-6 are summarized in Table 3.. These data clearly identified the complex I deficiency in patient 4, but there were no significant changes in oxygen consumption in patient 5. In patient 6 with an evident COX deficiency, the mitochondrial respiration in permeabilized muscle fibers was lower after addition of all substrates.

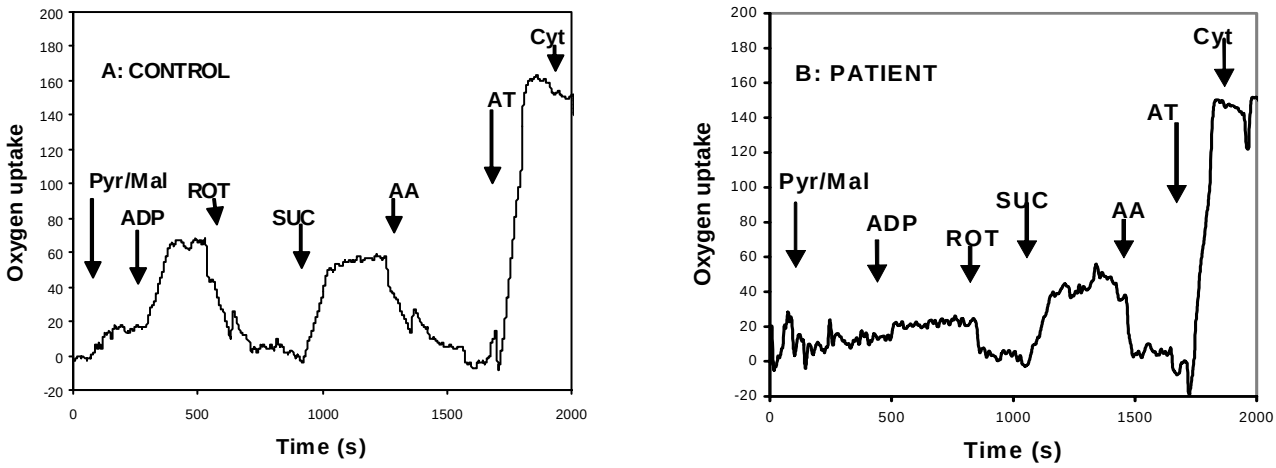

Fig. 2. *Determination of oxygen consumption (pmol oxygen/s/mg wet weight) using multiple substrate-inhibitor analysis in permeabilized muscle fibers from (A) control and (B) patient 4. Additions: Pyr/Mal: 10 mM pyruvate plus 5 mM malate; ADP: 1 mM ADP; ROT: 1.25 μM rotenone; SUC: 10 mM succinate; AA: 2.5 μM antimycin A; AT: 2 mM ascorbate plus 500 μM TMPD; Cyt: 5 μM cytochrome c.*

Discussion

Mitochondrial diseases are characterized by a broad spectrum of clinical symptoms, various age of onset and complicated genetics with Mendelian or maternal inheritance, less frequent are sporadic mitochondrial disorders. Genetic counseling and prenatal diagnostics of mitochondrial disturbances are of particular importance because they are often manifested as a fatal metabolic disorder for which no causal therapy is available.

On the biochemical level, several different approaches are available for the determination of mitochondrial respiratory chain dysfunctions in patients with mitochondrial disorders. The activities of respiratory chain complexes may be analyzed spectrophotometrically as dehydrogenases and oxidoreductases or they may be examined polarographically as the oxygen consumption rate after addition of various substrates.

Using spectrophotometric analysis, we selected six patients with apparent defects of respiratory chain complexes. Three patients had decreased cytochrome c oxidase activity, two patients lower activity of complex I and two patients reduced activity of complex III. One of the patients had a combined defect of complex III and IV.

When we compared the results of spectrophotometric measurements with the polarographic data, we found a defect of cytochrome c oxidase by both methods in patient 1 and 2 and normal cytochrom *c* oxidase activity in patient 3. Contrary to the spectrophotometric measurements, polarographic analysis showed a lower rate of NADH- and flavoprotein-dependent substrate utilization in patient 1 and 2 with decreased cytochrome *c* oxidase activity. This discrepancy indicates that the capacity of these dehydrogenases cannot be fully utilized when the activity of complex III and/or IV is highly depressed.

Polarographic measurements on muscle fibers correlate with those obtained with spectrophotomeric

measurements on isolated mitochondria. A defect of cytochrome *c* oxidase was confirmed in patient 6, but no changes of cytochrome *c* oxidase were found in patients 4 and 5. Similarly as in the polarographic measurements on isolated mitochondria, the defect of cytochrome *c* oxidase activity was accompanied by lower utilization of NADH- and flavoprotein-dependent substrates. In patient 4 with a pronounced decrease of complex I activity (Table 1), the lower utilization of NADH-dependent substrates was also found. However, in patient 5 with a moderate decrease of complex I activity no changes in the oxidation of pyruvate + malate were observed (Table 3). Thus, both oxygraphic analysis in isolated mitochondria and in permeabilized muscle fibers provided similar data in the diagnostics of mitochondrial defects as spectrophotometric measurements.

Table 2. Polarographic analysis of isolated muscle mitochondria from three patients with spectrophotometrically documented disturbances of respiratory chain complexes

Substrates	Patient 1	Patient 2	Patient 3	Controls (n=8)
PM	**80**	**170**	444	527±195
PM+C	**157**	**204**	533	690±265
GM	**94**	**203**	307	576±204
GM+C	**175**	**233**	531	780±320
S	**221**	**240**	634	836±474
AT	**442**	**597**	2011	2090±1110

PM – 10 mM pyruvate, 2 mM malate, 1 mM ADP; PM+C – 10 mM pyruvate, 2 mM malate, 1 mM ADP plus 5 μM cytochrome c; GM – 10 mM glutamate, 2 mM malate, 1 mM ADP; GM+C – 10 mM glutamate, 2 mM malate, 1 mM ADP plus 5 μM cytochrome c; S – 10 mM succinate and 1 mM ADP; AT – 1 mM ADP, 0.4 mM ascorbate plus 400 μM TMPD (oxygen consumption was expressed as pmol O_2/min/mg protein).

The differences in the results between spectrophotometric and polarographic measurements may be explained by the threshold values found in mitochondrial complexes (Villani and Attardi 1997). The calculation of threshold values in animal models had shown a large functional excess of the RC complexes in mitochondrial respiration (the complex I and III threshold values were over 50 % in all rat tissues), except for complex IV (Rossignol *et al.* 1999). This may not only explain the discrepancy between spectrophotometry and polarography, but also truly mirror the "*in vivo*" state of respiratory chain and oxidative phosphorylation system functions.

Table 3. Oxygen consumption in permeabilized muscle fibers from three patients with spectrophotometrically documented disturbances of respiratory chain complexes

Substrates	Patient 4	Patient 5	Patient 6	Controls (n=9)
PM	**6.7**	32	**7.6**	21±5
GM	**7.1**	17,8	**5.6**	16±6
S	14.1	14	**6**	13.5±4.5
AT	51	87	**40**	63±20

PM – 10 mM pyruvate, 5 mM malate and 1 mM ADP; GM – 10 mM glutamate, 5 mM malate and 1 mM ADP; S – 10 mM succinate and 1 mM ADP; AT – 1mM ADP; AT – 2 mM ascorbate plus 500 μM TMPD (oxygen consumption was expressed as pmol O_2/min/mg wet weight of muscle fibers).

The main difference in oxygen consumption studies between isolated muscle mitochondria and permeabilized muscle fibers concerns the disturbed structural integrity of myocytes and partial disintegration of the outer mitochondrial membrane as demonstrated by the activatory effect of added cytochrome c (see Table 2 and Fig. 1B). No similar problems occurred with saponin-skinned muscle fibers, where the activity of mitochondrial enzymes is measured under the conditions which are closer to those in intact cells. The technique of the saponin-skinned muscle fibers thus allows to study the mitochondrial respiration "*in situ*" with the preserved contact of mitochondria with other cell organelles. It was clearly demonstrated that e.g. the activation of actomyosin ATPase by caffeine and Ca^{2+} stimulates mitochondrial respiration of saponin-skinned muscle fibers (Khuchua *et al.* 1994).

The correlation of spectrophotometric and polarographic methods is also very important when the ratio between COX and other RC complexes is

determined. Spectrophotometric measurements showed the COX/SQR ratio to be 18.3, which indicates a high reserve capacity of COX (Table 1). However, polarographic measurements on isolated mitochondria or muscle fibers under conditions, which reflect rather the situation in intact cells, show that this reserve capacity is in fact much lower (2.5- or 4.8-fold, respectively, Tables 2 and 3). This difference is due to the activation of COX activity by detergent used in the spectrophotometric method. Low excess of COX, observed in muscle fibers with intact mitochondria, are thus in agreement with the data observed by measurements of oxygen consumption in different types of cultured cells. These experiments only demonstrated a low excess of cytochrome *c* oxidase capacity, when compared with the capacities of various respiratory chain complexes (Villani and Attardi 1997). Polarographic measurements, indicating that cytochrome *c* oxidase is not in high excess under physiological conditions, are also in agreement with the established role of cytochrome *c* oxidase in the control of respiratory chain function (Gnaiger *et al.* 1998, Villani and Attardi 2000).

The results of our study showed that both methods, the spectrophotometry and polarography provide different but complementary information about the function of respiratory chain complexes and therefore both techniques are important in the diagnostics of mitochondrial disturbances of energy generating system. Such comparative measurements are very important especially in the diagnostics of diseases connected with various mutations of COX genes, because the molecular approaches to diagnostics of mitochondrial disorders in most patients are usually available only after precise assessment of the biochemical defect.

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List of abbreviations

NQR, NADH-coenzyme Q_{10} oxidoreductase (complex I); SQR, succinate-coenzyme Q_{10} oxidoreductase (complex II); QCCR, coenzyme Q_{10}-cytochrom *c* oxidoreductase (complex III); COX, cytochrome *c* oxidase (complex IV); CS, citrate synthase; OXPHOS, oxidative phosphorylation; mtDNA, mitochondrial DNA; RC, respiratory chain; PCR-RFLP, polymerase chain reaction-restriction fragment length polymorphism, TMPD, N,N,N',N'-tetramethyl-p-phenylenediamine dihydrochloride; FCCP, carbonyl cyanide p-(trifluoromethoxy) phenylhydrazone; BSA, bovine serum albumine; DTT, dithiothreitol; EDTA, ethylene diamineteraacetic acid; EGTA, ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid; HEPES, N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid); K-MES, 2-(N-morpholino) ethanesulfonic acid potassium salt; Tris, (hydroxymethyl) aminomethane acetate salt.

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Reprint requests

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