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Review

Evaluation of procedures for assaying oxidative phosphorylation enzyme activities in mitochondrial myopathy muscle biopsies

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Contents

Summary

The mitochondrial myopathies (MM) are a heterogeneous group of neuromuscular diseases associated with abnormal mitochondria and defects in mitochondrial oxidative phosphorylation (OXPHOS). Analysis of a broad spectrum of MM patients has revealed that patients with similar clinical symptoms frequently do not have the same muscle OXPHOS defect. To determine whether some of this variation was due to methodological differences between studies, we have made a detailed survey of OXPHOS enzyme analysis procedures. The coupled OXPHOS assays for Complexes I + III and II + III were found to be variable due to competing reactions and complicated interactions between complexes. These problems were resolved by utilizing specific Complex I and III assays. The muscle mitochondria isolated from surgery patients under general anesthesia and prepared by proteinase digestion were observed to give low and highly variable OXPHOS activities. Mitochondria isolated from muscle biopsies

Abbreviations: MM, mitochondrial myopathies; OXPHOS, oxidative phosphorylation; MERRF, myoclonic epilepsy and ragged red fibers; MELAS, mitochondrial encephalomyopathy, lactic acidosis and stroke-like episodes; KSS, Kearns-Sayre syndrome; DB, 2,3-dimethoxy-5-methyl-6-*n*-decyl-1,4-benzoquinone.

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performed under local anesthesia and finely sliced prior to homogenization gave higher and more consistent OXPHOS activities. Assays for Complexes I, III and V required mitochondrial sonication to express maximal activity, but Complex IV was prone to inactivation by excessive mechanical disruption. Mitochondria isolated from frozen muscle or from patients with an OXPHOS disease are more fragile than those isolated from fresh tissue and normal individuals. Hence, Complex IV activity can be preferentially lost from frozen and sonicated myopathy patient samples. These results suggest that variation in muscle OXPHOS analysis techniques may account for some of the discrepancies between clinical manifestations and OXPHOS defects and suggest that no single protocol is sufficient to adequately define the OXPHOS defect in MM patients.

Introduction

Mitochondrial myopathy (MM) generally involves abnormal mitochondria and defects in oxidative phosphorylation (OXPHOS) and is associated with a variety of neuromuscular diseases including myoclonic epilepsy and ragged red fibers (MERRF); mitochondrial encephalomyophathy, lactic acidosis and stroke-like episodes (MELAS); and the Kearns-Sayre syndrome (KSS). A complete evaluation of these diseases requires comprehensive clinical, biochemical and molecular genetic analysis.

The OXPHOS system consists of five enzyme complexes located in the mitochondrial inner membrane whose biogenesis is controlled by both nuclear and mitochondrial genomes. Complexes I, II, III and IV comprise the electron transport chain by which NADH and succinate are oxidized and an electrochemical potential is generated across the mitochondrial inner membrane. Complex V utilizes this potential energy to condense ADP and P_i into ATP (Fig. 1) [1,2]. Biochemical analyses of MM patients have revealed that enzyme defects frequently do not correlate with disease manifestations [3–5] or with identified molecular defects of the mitochondrial DNA (mtDNA) [6–8]. For example, MELAS has been associated with Complex I or IV deficiencies [9,10], MERRF associated with Complex IV, Complex III or combined Complex I and IV deficiencies [11–13], and KSS associated with Complex I, Complex IV, or combined Complex I, IV and V deficiencies [14–16].

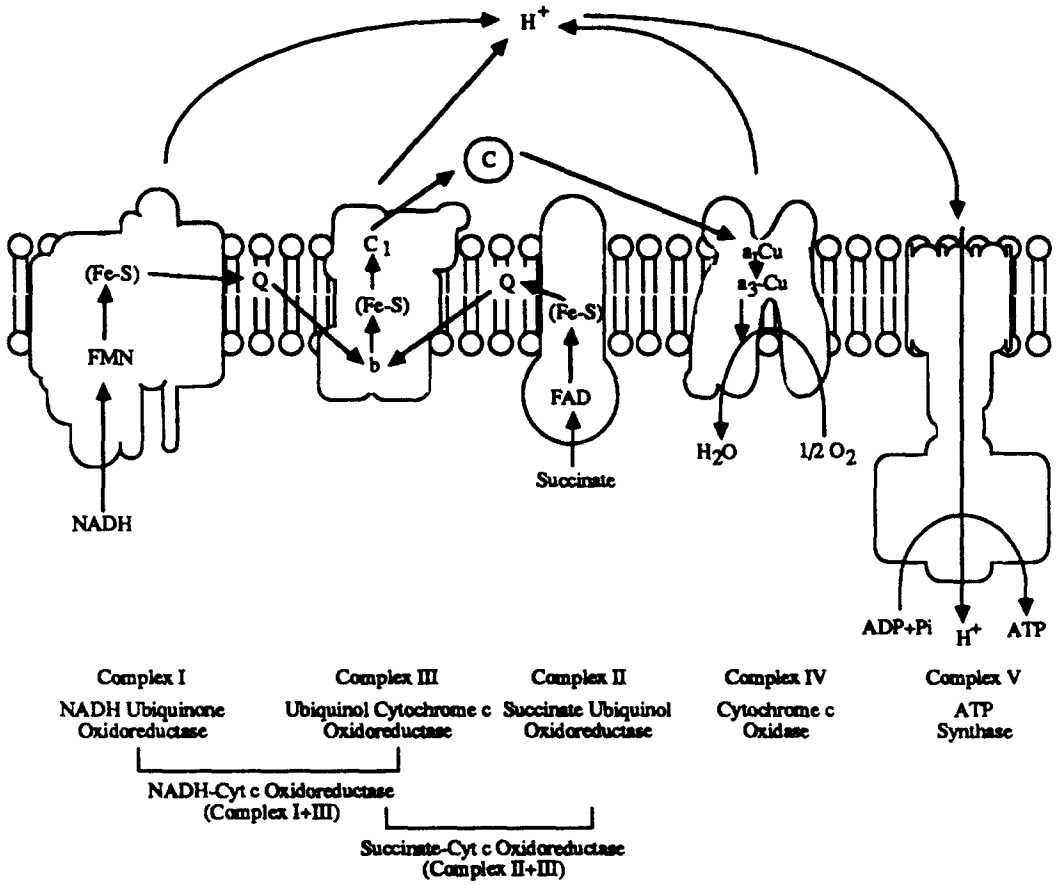

Fig. 1. Electron transport chain and oxidative phosphorylation. FMN, flavin mononucleotide; FAD, flavin adenine dinucleotide; NADH, nicotinamide adenine dinucleotide, reduced; FeS, iron-sulfur center; C, cytochrome *c*; C_1, cytochrome c_1; b, cytochrome *b*; *a*-Cu, cytochrome *a*-Cu reaction center; a_3-Cu, cytochrome a_3-Cu reaction center.

In an attempt to understand the disparity among these biochemical results, we have made a detailed analysis of methods for measuring OXPHOS defects in patients and controls. We have found that coupled assays for Complex I and III give highly variable results, that different methods for mitochondrial isolation and disruption alter the apparent specific activities of OXPHOS Complexes and that Complex IV is more labile in OXPHOS disease mitochondria than those from normal individuals, resulting in its easy destruction.

II. Material and Methods

Muscle specimens

To investigate the basis of OXPHOS enzyme variability, we analyzed two categories of human control specimens. In category I, eight skeletal muscle biopsy samples from the quadriceps, vastus lateralis, or deltoid muscles were obtained under local anesthesia (2% lidocaine) (age range: 25–55 years). In category II, 48 samples of excess muscle tissue from sternocleidomastoid, gluteus maximus, external oblique, rectus abdominus, vastus lateralis, deltoid or pectoralis muscle were collected under general anesthesia (age range: 12–78 years). Samples were collected under protocols approved by the Emory Human Subjects Review Board and consistent with informed consent.

Mitochondrial isolation

Mitochondria were isolated in two ways. For all categroy I and 17 category II samples, 1–4 g of muscle were placed immediately in ice-cold H-buffer (210 mM mannitol, 70 mM sucrose, 1 mM EGTA, 5 mM Hepes (pH 7.2) and 0.5% BSA) [17] and then processed by a Thomas tissue slicer. The sliced tissue was suspended in 40 ml H-buffer. For 31 category II samples, 0.6–2.5 g of muscle were placed in approx. 2 ml of H-buffer, minced with scissors and digested with Nagase (2 mg/g of muscle) for 10 minutes [18]. The minced muscle was washed twice with H-buffer to remove excess Nagase and suspended in 40 ml of H-buffer. Sliced or minced tissues were then homogenized using a motor-driven Thomas homogenizer with a Teflon pestle (2–4 up-down strokes). The homogenate was centrifuged twice at 1475 $\times g$ for 5 min at 4°C to remove cells and debris (Sorvall SS-34 rotor). The mitochondria were then collected from the supernatant by centrifugation at 7710 $\times$ g for 15 min, and the pellet was resuspended in H-buffer and stored in vapor phase liquid nitrogen for 1 or 2 weeks for enzyme assays. Protein concentrations were determined by a modified Lowry procedure [19].

Enzyme assay

Concentrated mitochondria frozen in H-buffer were thawed, diluted to 1.5 mg/ml and disrupted by either three cycles of freeze-thawing or by six seconds of sonication. Sonication was performed using a Heat System Ultrasonic Processor (Model W-385) set at a power output of approx. 38 W. This power is continuously delivered to the sample (0.3–0.5 ml) in three 2 s bursts which are separated by a 5 s cool down period to minimize sample heating. All enzyme assays were performed using an Aminco DW-2000 dual beam spectrophotometer.

The Complex I (NADH-DB assay) specific activity was measured by following the reduction of the coenzyme Q_2 analog, *n*-decyl-coenzyme Q (DB; custom synthesized by Dr. N.M. Witzke) [20] at 272/247 nm ($\varepsilon = 8\ mM^{-1} \cdot cm^{-1}$) (personal communication from Dr. D.S. Beattie). Between 30 and 45 μg/ml of freshly isolated and sonicated (6 s) mitochondria were preincubated in a 1 ml reaction buffer including 50 mM Tris-HCl (pH 7.4), 250 mM sucrose, 1 mM EDTA and 2.5 mM KCN, for 10 min at 25°C. The reaction was started by adding NADH. For kinetics studies at 50 μM DB, NADH concentrations varied from 0.2 to 50 μM. At 50 μM NADH, DB concentration varied from 0.1 to 100 μM. The initial rate was recorded for 1 min. Complex I activity was inhibited by 7.5 μM rotenone.

The Complex III (DBH_2-cytochrome *c* assay) specific activity was measured by monitoring the reduction of cytochrome *c* at 550/540 nm ($\varepsilon = 19\ mM^{-1} \cdot cm^{-1}$). Between 10 and 15 μg/ml of freshly isolated mitochondria were preincubated in a 1 ml reaction buffer (same buffer as complex I) and the reaction started by adding reduced DB (DBH_2). For kinetic studies at 50 μM of DBH_2, cytochrome *c* concentrations varied from 1.6 to 150 μM cytochrome *c*, the DBH_2 concentration varied from 0.4 μM to 100 μM. The nonenzymatic activity was measured at 50 μM DBH_2 and varying concentrations of cytochrome *c*. The initial rate was monitored for 30 s. Complex III activity was inhibited by the addition of 10 μg/ml antimycin A.

DBH_2 was prepared by adding a few crystals of KBH_4 to 10 mM DB dissolved in absolute alcohol. The reaction was initiated with a few μl of 0.1 M HCl. When the yellow color was bleached, the solution was decanted from the excess KBH_4 and 2–3 μl of 1 M HCl was added to retard auto-oxidation. The reduced DB was made fresh every week and stored at −20°C. Autooxidation was minimal under this condition.

Assays for Complex I + III (rotenone-sensitive NADH-cytochrome *c* oxidoreductase), Complex II + III (antimycin A-sensitive succinate-cytochrome *c* oxidoreductase), Complex IV (cytochrome *c* oxidase) and Complex V (F_1-ATPase) were performed as previously described [13,21]. The average concentration of ferrocytochrome was 0.62 ± 0.05 mM ($\varepsilon_{550} = 2.7\ mm^{-1} \cdot cm^{-1}$) [22].

Statistical analysis of enzyme activity controls were performed to calculate 5–95% confidence intervals [23].

III. Results

III-A. Kinetic studies of NADH-DB and DBH_2-cytochrome c assays

Specific assays were developed for Complexes I and III which avoid the effects of interactions between complexes and non-specific side-reactions. Freshly isolated and sonicated mitochondria from category I specimens were used for kinetic analysis.

NADH-DB assay. Fig. 2 shows the kinetics of the Complex I reduction of DB with NADH. At 50 μM DB, the NADH concentration was rate-limiting up to 12.5 μM (Fig. 2A). The K_m estimated by the Lineweaver-Burk plot was 0.82 μM and the V_{max} 184 nmol/min per mg. At 50 μM NADH, DB concentrations were rate-limiting up to about 12.5 μM (Fig. 2B). The calculated K_m and V_{max} were about 0.75 μM and 196 nmol/min per mg, respectively. Under saturated conditions (50 μM NADH and 50 μM DB), the reaction was linear over a 8-fold range of protein concentrations (5.6 μg/ml to 45 μg/ml) (Fig. 2C). Hence, for routine analysis of human category I and II samples, protein concentrations between 30 and 45 μg/ml were chosen. With 45 μg/ml of mitochondrial protein, the average variability between repeats of a sample was only 6%. Rotenone at 7.5 μM inhibited 95% of the activity. Hence, this assay is highly specific and reproducible.

DBH_2-cytochrome c assay. Fig. 3 presents the kinetics of the Complex III reduction of cytochrome *c* with reduced DBH_2. At 50 μM DBH_2, cytochrome *c* concentrations were rate-limiting up to 40 μM (Fig. 3A). The estimated K_m was 4.7 μM and V_{max} 1771 nmol/min per mg. At 50 μM cytochrome *c*, DBH_2 was rate-limiting up to about 12.5 μM (Fig. 3B). The computed K_m and V_{max} were 3.1 μM and 2651 nmol/mg per min, respectively. The reaction rate was linear over a 8-fold range of mitochondrial protein concentrations from 1.88 to 15 μg/ml (Fig. 3C). Therefore, mitochondrial protein concentrations between 7.5 and 15 μg/ml were chosen for routine analysis of mitochondria Complex III activity in controls and affected individuals.

To determine the specificity of the Complex III assay, we added 10 μg/ml of antimycin A to the reaction and measured antimycin non-inhibitable activity. We observed that at 50 μM DB, 6% of total activity was insensitive to antimycin, but this was due to nonenzymatic activity. Hence, this assay is highly specific for Complex III. At saturated conditions with a mitochondrial protein concentration of 15 μg/ml, the variability between duplicates was approximately 3%.

III-B. Higher and less variable OXPHOS enzyme activities obtained with mitochondria from category I specimens isolated by the tissue slicer technique

Two techniques for mitochondrial isolation were evaluated using category II specimens: the scissor-Nagase technique ($II_{scissor\text{-}Nagase}$) and the tissue slicer technique (II_{slicer}) (Table I). The mean OXPHOS enzyme activities for all assays were lower for category $II_{scissor\text{-}Nagase}$ isolated mitochondria than for category II_{slicer} mitochondria, with the differences for Complex I,

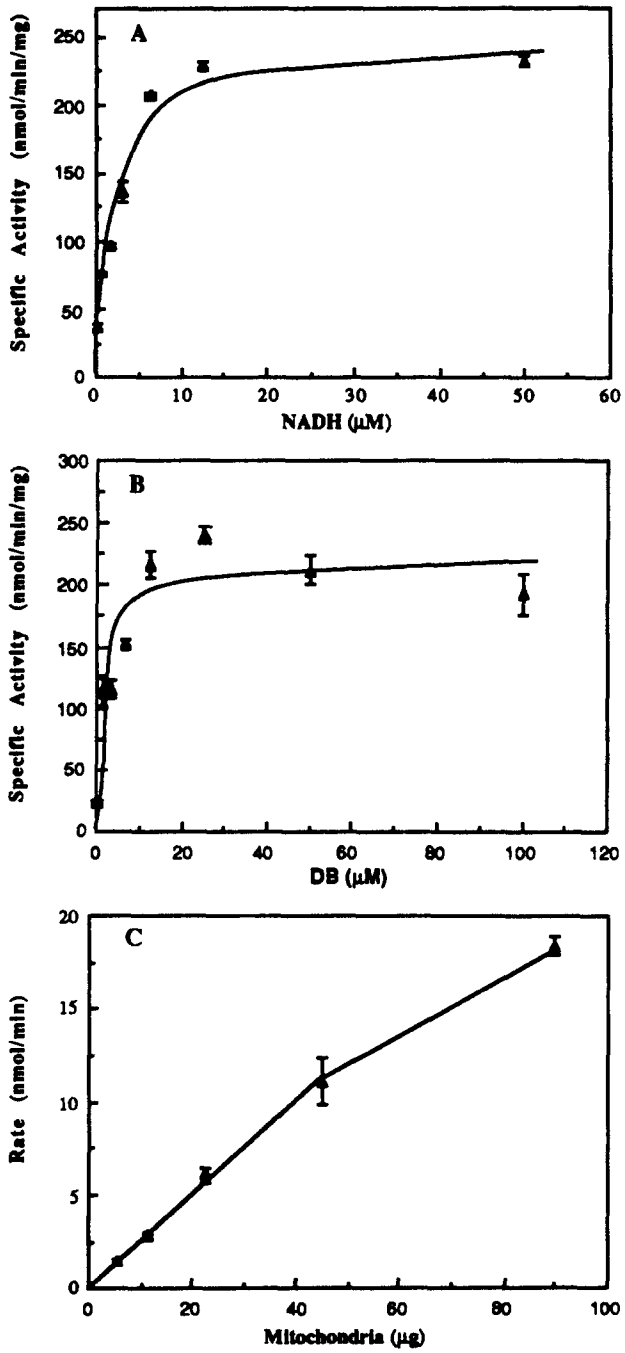

Fig. 2. Kinetics of NADH-DB assay on human muscle mitochondria. (A) Saturation curve for NADH at DB concentration of 50 μM. (B) Saturation curve for DB at NADH concentration of 50 μM. Mitochondria isolated from Category I samples and using mitochondria protein concentration of between 15 and 30 μg/ml. (C) Relationship between the initial rate of DB reduction and mitochondria protein concentration at substrate concentrations of 50 μM NADH and 50 μM DB. All data were reported as the average of two or three determinations.

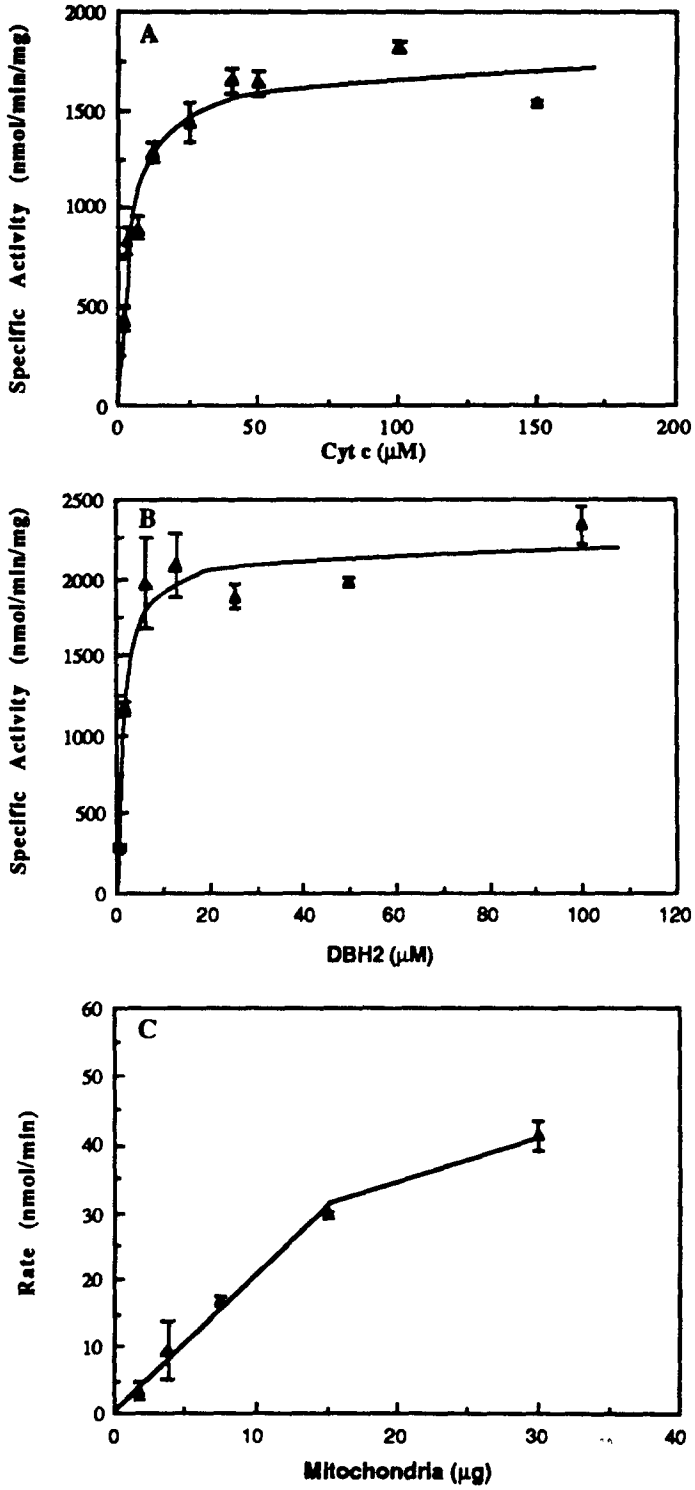

Fig. 3. Kinetics of DBH_2-cytochrome *c* assay on human muscle mitochondria. (A) Saturation curve for substrate cytochrome *c* at 50 μM DBH_2. (B) Saturation curve for substrate DBH_2 at 50 μM of cytochrome *c*. Mitochondria isolated from category I specimens and using mitochondrial protein concentrations between 3.75 and 15 μg/ml. (C) Relationship between the initial rate of cytochrome *c* reduction and mitochondrial protein concentration at substrate concentrations of 50 μM DBH and 50 μM cytochrome *c*. Duplicates or triplicates were done for each data point. Antimycin-insensitive activities were subtracted from each point.

I + III, and III assays being significant at the $P < 0.05$ level. The 5–95% confidence limits were wide for both groups (Table I). Differences due to the ages of the donors or the muscle group used were less significant than those resulting from procedural differences. Since protein yields were comparable for both techniques (0.8 mg protein/g wet weight) and enzyme activities were expressed as nmol substrate oxidized or reduced per

TABLE I

OXPHOS activities of mitochondria isolated by different procedures

Mitochondria were isolated from fresh Category I and II specimens using either the scissor-Nagase or the tissue slicer technique. OXPHOS enzyme activities were assayed on sonicated mitochondria. Specific activity, nmol/min per mg; S.D., standard deviation; 5–95% CI, 5–95% confidence interval; *N*, sample size.

Assay	*N*	Samples	Median	Mean ± S.D.	Range	5–95% CI
I	31	$II_{scissor\text{-}Nagase}$	70	80 ± 38	30 – 167	1.1 – 159
	17	II_{slicer}	149	156 ± 67	66 – 337	9.8 – 303
	8	I_{slicer}	215	206 ± 62	75 – 279	51 – 361
I + III	31	$II_{scissor\text{-}Nagase}$	99	122 ± 69	26.5 – 271	0 – 266
	17	II_{slicer}	145	179 ± 120	54 – 486	0 – 440
	8	I_{slicer}	232	280 ± 139	148 – 540	0 – 629
II + III	31	$II_{scissor\text{-}Nagase}$	366	423 ± 198	86 – 951	13 – 833
	17	II_{slicer}	484	525 ± 234	239 – 1246	15 – 1035
	8	I_{slicer}	697	704 ± 173	480 – 930	270 – 1138
III	31	$II_{scissor\text{-}Nagase}$	947	1119 ± 546	512 – 2833	0 – 2253
	17	II_{slicer}	1500	1501 ± 534	712 – 2795	338 – 2666
	8	I_{slicer}	1668	1783 ± 580	884 – 2854	328 – 3238
IV	31	$II_{scissor\text{-}Nagase}$	653	683 ± 359	104 – 1544	0 – 1429
	17	II_{slicer}	862	848 ± 393	228 – 1477	0 – 1704
	8	I_{slicer}	1579	1568 ± 229	1301 – 1926	994 – 2142
V	29	$II_{scissor\text{-}Nagase}$	250	298 ± 161	65.3 – 573	0 – 634
	15	II_{slicer}	318	357 ± 204	135 – 915	0 – 810
	8	I_{slicer}	460	445 ± 118	256 – 573	149 – 741

milligram protein, these results suggest that low enzyme activities from mitochondria isolated using scissor-Nagase technique were due to inactivation of enzyme activities by Nagase.

Two sources of muscle were then compared for OXPHOS activity in mitochondria isolated by the slicer technique: fresh biopsy specimens collected under local anesthesia (I_{slicer}) and excess surgery tissue collected under general anesthesia (II_{slicer}). The OXPHOS enzyme levels were consistently higher for Category I_{slicer} than for Category II_{slicer} samples with mean Complex IV enzyme activity being significantly higher ($P < 0.05$). The 5–95% confidence intervals were narrower for category I_{slicer} than for category II_{slicer} for all assays except Complex I + III and III. These analyses indicate that preparation of human mitochondria by the tissue slicer technique gives higher mean enzyme activities than does the scissor-Nagase technique, and that less variable data are obtained with muscle biopsy specimens obtained by local anesthesia than surgical specimens obtained using general anesthesia.

III-C. Sonication and freezing affect OXPHOS disease muscle mitochondria differently as compared with controls

The mitochondrial OXPHOS specific activities were compared for fresh versus frozen tissue and following mitochondrial sonication versus freeze-thaw. Samples from Category II_{slicer} and four patients with MM were examined.

No single procedure can give maximum OXPHOS activities for all OXPHOS assays. Mean enzyme activities of Category II_{slicer} mitochondria isolated from fresh

TABLE II

OXPHOS activities of control mitochondria under different disruption conditions

Mitochondria were isolated from fresh or frozen Category II specimens using tissue slicer technique and OXPHOS enzyme activities were assayed with either 6-s sonicated or three-cycle freeze-thawed mitochondria. The results were reported as mean ± S.D. $n = 5$ for all assays.

Disruption conditions	Specific activities (nmol/min/mg)					
	I	I + III	II + III	III	IV	V
Fresh sonicated	162 ± 88	154 ± 67	580 ± 223	1794 ± 490	1087 ± 313	310 ± 80
Fresh freeze-thawed	58 ± 14	164 ± 93	813 ± 207	852 ± 269	1443 ± 287	180 ± 49
Frozen sonicated	121 ± 41	123 ± 27	235 ± 79	1509 ± 520	617 ± 378	209 ± 59
Frozen freeze-thawed	76 ± 25	125 ± 11	467 ± 126	1167 ± 319	989 ± 430	180 ± 45

TABLE III

The effect of sonication on OXPHOS disease patients' mitochondria Complex I and IV enzyme activities

Mitochondria were isolated from fresh muscle biopsies from OXPHOS disease patients and assayed for Complex I and IV enzyme activities after 6-s sonication or three-cycles of freeze-thawing. The control data are from Table II. Specific activity, nmol/min per mg; FT, freeze-thawing and Son, sonication.

MM	Complex I		Complex IV		
	FT	Son	FT	Son	% of activity remaining
MERRF I	24	34	692	139	20
II	6.3	21.2	751	68.5	9
MELAS	30	29	578	37	6
Kearns-Sayre	-	-	936	146	16
Control II_{slicer}	58 ± 14	162 ± 88	1443 ± 287	1087 ± 313	75

or frozen tissue, and disrupted by sonication or freeze-thawing are shown in Table II. Comparison of the effects of sonication or freeze-thawing of mitochondria isolated from fresh tissue or frozen tissue revealed that the differences were specific for the Complexes and independent of tissue preparations. Sonication of mitochondria gave significantly higher specific activities than freeze-thawing for Complexes I and III ($P < 0.05$), whereas freeze-thawing gave significantly higher specific activities than sonication for Complexes II + III and IV. Sonicated and freeze-thawed mitochondria gave equivalent values for the Complex I + III assay and sonicated mitochondria gave higher activity than freeze-thawed mitochondria from fresh tissue for Complex V. However, freeze-thawed and sonicated mitochondria gave about the same Complex V enzyme activity for frozen tissue (Table II).

Comparison of OXPHOS enzyme activities between fresh and frozen tissue (Table II) revealed that sonicated mitochondria generally gave higher activity for fresh tissue than for frozen tissue, though the difference was only significant for Complex II + III ($P < 0.05$). Freeze-thawed mitochondria from fresh tissue gave lower Complex I and III activities than mitochondria from frozen tissue but higher Complex II + III and IV activities.

Thus, sonication stimulates enzyme activity for Complexes I, III and V assays in either fresh or frozen tissue, reduces activity for Complexes II + III and IV assays and has no effect on the Complex I + III assay. Furthermore, enzyme activities of sonicated or freeze-thawed mitochondria are generally higher from fresh rather than frozen tissue, the only exceptions being from Complex I and III in freeze-thawed mitochondria isolated from frozen tissue. These data are consistent with the hypothesis that mitochondrial membrane disruption is essential for obtaining maximum Complex I and III activities, presumably to permit access of substrates to the enzymes. By contrast, disruption is deleterious to the Complex II + III assay this decreased activity is possibly the product of the separation of the two components of the linked assay. For Complex IV, the enzyme appears to be more labile than the other enzymes.

Mitochondria from OXPHOS disease patients are prone to inactivation. Complex I and IV enzyme activities of mitochondria from patients with MERRF, MELAS and KSS were compared with those of control mitochondria under different disruption conditions (Table III). The Complex I activities of the controls were enhanced about 3-fold by sonication, but the activity remained low for the MERRF and MELAS patients, suggesting a primary defect in Complex I. The complex IV enzyme activity for the controls was reduced 25% by sonication relative to freeze-thawed mitochondria but for the patients it dropped 80–94%. This suggests that the Complex IV in patients' mitochondria is more labile than that in control mitochondria. Consequently, the mechanical disruption necessary to make Complex I activity accessible in controls is potentially deleterious to the Complex IV activity of diseased mitochondria.

OXPHOS activities for patients when mitochondria were isolated from frozen tissue. The enzyme activities of mitochondria from controls were not significantly different between fresh and frozen tissue (Table II). However, the Complex IV activity of MM patients was more sensitive to inactivation by sonication when isolated from frozen tissue (Fig. 4B).

The progressive reduction in Complex IV activity for II_{slicer} control mitochondria in fresh versus frozen mitochondria and freeze-thawed versus sonicated mitochondria confirms the lability of this Complex and the increasing disruptive nature of these procedures in controls (Fig. 4A). In the MELAS patient, the Complex IV activity in freeze-thawed mitochondria was comparable when mitochondria were isolated from fresh or frozen tissue. However, sonication of mitochondria isolated from fresh tissue reduced Complex IV activity about 75% but totally inactivated mitochondria isolated from frozen tissue (Fig. 4B). This supports the concept that

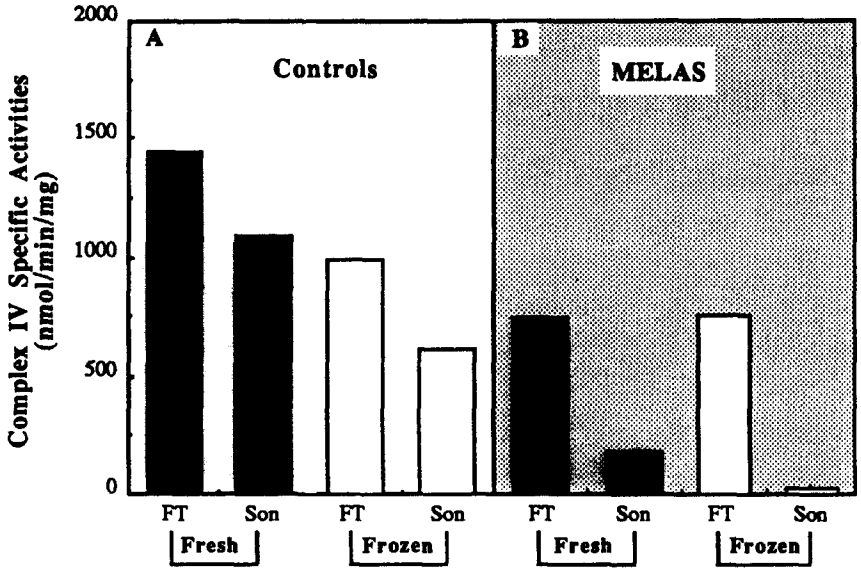

Fig. 4. Comparison of Complex IV enzyme activities between controls and a MELAS patient. Mitochondria were isolated from fresh (black bars) or frozen (open bars) samples using the tissue slicer and sonicated or freeze-thawed before assay for Complex IV activity. Control data were from Table II.

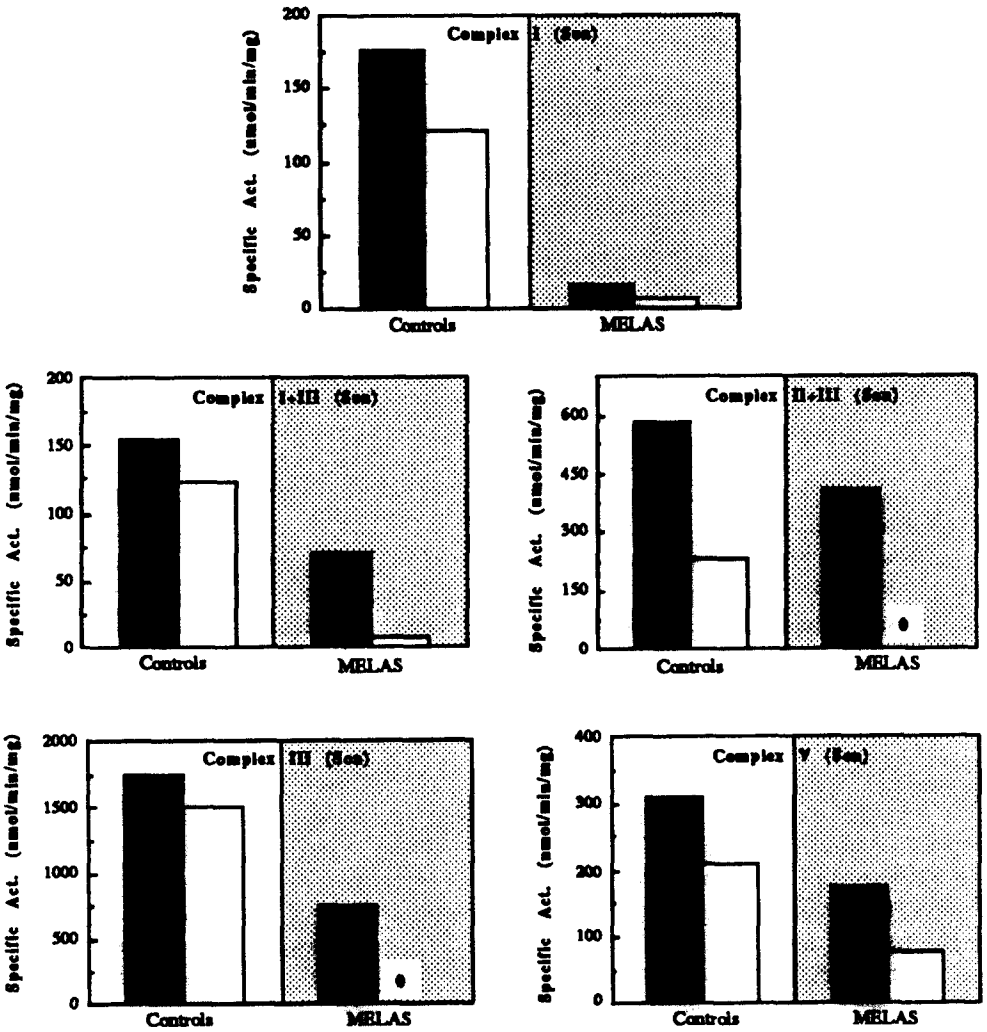

Fig. 5. Effects of using frozen tissue on sonicated mitochondria enzyme activities for controls and a MELAS patient. The patient was the same as Table III. Control values were from Table II. Black bars, mitochondria from fresh tissue; open bars, mitochondria from frozen tissue; Son, sonication.

mitochondrial myopathy patient Complex IV activity is more labile than normal individual activity and that this has the most deleterious effect in sonication of mitochondria isolated from frozen tissue.

A similar loss of activity in mitochondria isolated from frozen tissue relative to fresh tissue was observed for the other enzyme complexes (Fig. 5). In contrast to the insignificant differences in enzyme activities between fresh and frozen tissue for controls, there were dramatic reductions of enzyme activities between fresh and frozen tissue in sonicated patient mitochondria for the Complexes I + III, II + III and III assays (Fig. 5). These analyses indicate that patient OXPHOS complexes are considerably more labile than those of normals. Consequently, isolating mitochondria from frozen tissue and subsequent sonication can inactivate a significant proportion of the residual patient OXPHOS enzyme activity.

IV. Discussion

Our studies indicate that a number of experimental procedures routinely used in analyzing mitochondrial OXPHOS enzyme activity in human skeletal muscle can have significant effects on respiratory complex specific activities. This is particular problematic in patients with OXPHOS diseases whose mitochondria appear to be more labile than those of normal controls. Hence, methodological variation in OXPHOS assays may in part account for recent observations that OXPHOS defects frequently do not correlate with the mtDNA genetic mutations [6–8]. Additional factors could include environmental factors, post-translational modification and additional genetic factors.

The lability of MM patient mitochondria appears to be particular important for Complex IV which is prone to inactivation by mechanical disruption (sonication). The marked lability of patient Complex IV raises the possibility that defects in Complex IV may be reported when other more subtle defects may be the primary cause of the diseases. For example, mitochondrial lability may account for the report of a primary Complex IV defect in an ocular myopathy patient with a mtDNA deletion in which all three subunits of Complex IV remained intact [6].

Additional difficulties may be encountered because of the high variability of the traditional Complex I + III and II + III assays. The Complex I + III assay variability results from a rotenone-insensitive oxidase in the outer mitochondrial membrane [24] whose activity must be subtracted from the total activity to reveal the rotenone-sensitive activity of Complex I. Since the rotenone-insensitive activities are often very high relative to the rotenone-sensitive activity, the 5–95% confidence intervals are great and partial Complex I deficiency in patients are difficult to detect. Our Complex I assay avoids this additional oxidase and gives more reliable results. Variability in the linked Complex II + III assay seems to be a product of the association of Complexes II and III. This problem has also been overcome by our specific Complex III assay.

By utilizing these OXPHOS assays and paying close attention to the Complex IV lability, we have been able to correlate the enzymatic dysfunction of Complexes I, IV and V with the corresponding deletion of these subunits in the mtDNA of a KSS patient [16]. We have also observed major Complex I and IV defects in MERRF [13] and MELAS patients. Evidence that this is the primary defect will need to await identification of the mtDNA molecular defect in these diseases.

In conclusion, our data indicate that multiple variables must be kept in mind when OXPHOS enzymology is performed on patients with OXPHOS diseases. For optimizing OXPHOS activities in patients and controls, and for narrowing the 5–95% confidence limits of controls, mitochondria should be isolated from a fresh biopsy performed under local anesthesia. In addition to using coupled OXPHOS assays, each respiratory Complex should be assayed separately to compensate for unforeseen interactions between complexes. Since no single protocol appears to be optimal for all cases and all enzymes, both freeze-thawing and sonication should be used to disrupt mitochondria from MM patients prior to assay of the OXPHOS complexes to provide maximum access of substrates while minimizing the secondary inactivation of enzymes. Hence, a combination of procedures for mitochondrial isolation may be necessary to provide the most accurate biochemical representation of a patient's mitochondrial OXPHOS defect.

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