Disproportionate Deficiency of Iron-Sulfur Clusters and Subunits of Complex I in Mitochondrial Encephalomyopathy

TAKASHI ICHIKI, MASASHI TANAKA, MASANORI KOBAYASHI, NARUJI SUGIYAMA,
HIROSHI SUZUKI, MORIMITSU NISHIKIMI, TOMOKO OHNISHI, IKUYA NONAKA,
YOSHIRO WADA, AND TAKAYUKI OZAWA

*Department of Pediatrics [T.I., M.K., N.S., Y.W.], Nagoya City University Medical School, Nagoya 467, Japan,
Department of Biomedical Chemistry [M.T., H.S., M.N., T. Ozawa], Faculty of Medicine, University of Nagoya,
Nagoya 466, Japan, Department of Biochemistry and Biophysics [T. Ohnishi], University of Pennsylvania,
Philadelphia, Pennsylvania 19104, and National Center for Neurology and Psychiatry [I.N.], Kodaira, Tokyo
187, Japan*

ABSTRACT. To investigate the molecular abnormality in the mitochondria from various tissues of an autopsied patient exhibiting mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes, we have examined the enzymatic activity, iron-sulfur cluster, and subunit composition of the NADH-ubiquinone oxidoreductase (complex I). Rotenone-sensitive NADH-cytochrome *c* reductase activity was found to be decreased in all the tissues examined. A detailed study of the liver mitochondria has shown that NADH-ubiquinone oxidoreductase activity was greatly diminished. Analysis of the electron paramagnetic resonance spectra of the liver submitochondrial particles revealed a disproportionate deficiency of iron-sulfur clusters in the complex I segment of the respiratory chain. Signals from the clusters N-2 and N-3 diminished more drastically than those from clusters N-1b and N-4. Immunoblotting analysis showed that the 75-kD, 51-kD, and several other subunits were markedly diminished among multiple subunit polypeptides of complex I. These findings suggest that the underlying bases for mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes are defects, at least, in the complex I subunits containing a flavin and/or iron-sulfur cluster(s), which resulted in deficiencies of some iron-sulfur clusters. (*Pediatr Res* 25:194–201, 1989)

Abbreviations

MELAS, mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes
complex I, NADH-ubiquinone oxidoreductase
complex II, succinate-ubiquinone oxidoreductase
complex III, ubiquinol-cytochrome *c* oxidoreductase
complex IV, cytochrome *c* oxidase
complex V, F₀F₁-ATPase
EPR, electron paramagnetic resonance
SMP, submitochondrial particles

Mitochondrial cytopathy is a multisystem disorder that affects the skeletal muscles, brain, heart, kidney, liver, and other organs in various combinations (1). A distinctive syndrome in mitochondrial cytopathy is MELAS (2). Recently, it has been recognized that deficiency of the respiratory chain enzymes is one of the causes of mitochondrial cytopathies (3, 4). The biochemical defect in MELAS has been reported to reside in NADH-ubiquinone oxidoreductase (complex I) (5–10). In the previous studies (11, 12), we have demonstrated that variation in the degree of deficiency of complex I activity among patients with MELAS or among tissues of a patient with MELAS is based on variation in the defects of complex I subunits. However, the relationship between the decreased enzymic activity and the defects of complex I subunits has not been fully elucidated. In the present report, we studied the molecular bases of complex I deficiency in a patient with MELAS using enzymologic, EPR spectroscopic, and immunochemical methods. The results demonstrated that the molecular integrity of complex I was impaired in this disorder.

CASE REPORT

The patient's detailed history has already been reported (8, 9, 11). He had a normal prenatal and perinatal history. He was completely asymptomatic and fully active until age 3 when he could not run swiftly. At age 7, he was admitted to a hospital because of cardiac failure. Electrocardiography showed flat and inverted T waves in leads V₄–V₆. But the cardiac symptoms disappeared later. At age 10, he had generalized tonic and clonic convulsions about 3 times a day. He was readmitted to the hospital because of seizures, muscle weakness, and impaired visual acuity. The blindness improved spontaneously 1 mo later. At age 12, he complained of blindness again, which persisted thereafter. At age 13, he was hospitalized for biochemical studies. His height and weight were below the 3rd percentile. There was bilateral ptosis nonresponsive to edrophonium chloride (Tensilon). There was no ophthalmoplegia, myoclonus, cardiomyopathy, heart block, or retinal pigmentation. His symptoms did not correspond to those of Kearns-Sayre syndrome (9), although this patient was originally reported as a case of Kearns-Shy syndrome (13). He had diffuse limb weakness, weak tendon reflexes, and an intention tremor. A computed tomographic scan of the head revealed cerebral and cerebellar atrophy, with a low-density area in the occipital region and a high-density area in the basal ganglia. Cerebral blood flow studies proved normal.

Received April 28, 1988; accepted October 18, 1988.

Correspondence Prof. Takayuki Ozawa, Department of Biomedical Chemistry, Faculty of Medicine, University of Nagoya, 65 Tsuruma-cho, Showa-ku, Nagoya 466, Japan.

Supported by the Grants-in-Aid for General Scientific Research (62570128) to M. T., and for Scientific Research on Priority Areas (62617002) to T. Ozawa, from the Ministry of Education, Science and Culture, Japan, by Grant 86-02-39 from the National Center for Nervous, Mental and Muscular Disorders of the Ministry of Health and Welfare, Japan, to T. Ozawa, and by National Institutes of Health Grant GM 30736 to T. Ohnishi.

Blood lactate levels were 26.8 to 61.1 mg/dl (normal <18 mg/dl) with a high lactate/pyruvate ratio. Cerebrospinal fluid lactate concentration was 55.2 mg/dl (normal <15 mg/dl). Serum amino acid values were within normal limits except alanine (6.91 mg/dl, normal <5.0 mg/dl). Creatinine clearance was 29.3 ml/min (normal >70 ml/min). Liver function was almost normal. Total carnitine content in plasma was 32.2 μ mol/liter (normal, 67 ± 11 μ mol/liter, mean $\pm$ SD, $n = 15$), and free carnitine content was 15.5 μ mol/liter (normal, 52 ± 10 μ mol/liter, mean $\pm$ SD, $n = 15$). A muscle biopsy showed a number of ragged-red fibers when the modified Gomori trichrome stain was used for histochemical study. On electron microscopy, these muscle cells were seen to contain an increased amount of glycogen granules and an increased number and size of abnormal mitochondria containing characteristic paracrystalline inclusion bodies. On biochemical studies of mitochondria isolated from biopsied skeletal muscle, rotenone-sensitive NADH-cytochrome *c* reductase activity was markedly decreased in the patient, being 14 nmol/min/mg of mitochondrial protein (controls, 249 ± 174 nmol/min/mg of mitochondrial protein, mean $\pm$ SD, $n = 10$). At age 14, he died suddenly. The autopsy revealed no hypertrophy of the cardiac muscle.

MATERIALS AND METHODS

Control and patient organs were obtained by autopsy after informed consent and signed permission were given. Tissues for biochemical analysis were frozen immediately and stored at -80°C .

Biochemical Studies. Preparation of mitochondria. The following procedures were carried out at 4°C . For frozen specimens, the procedure of Bookelman *et al.* (14) was followed. Heart, skeletal muscle, liver, and kidney were placed in a solution of ice-cold 0.25 M sucrose, 2 mM EDTA, 10 mM Tris-HCl, and 50 IU of heparin/ml, pH 7.4 (SETH medium). After being thawed, the tissues were cut into small pieces, and suspended in SETH medium (10 ml/g of wet wt). Homogenization was performed with a loose-fitting motor-driven glass-Teflon homogenizer and then with a tight-fitting homogenizer. The homogenates were centrifuged at $600 \times g$ for 10 min. The supernatants were centrifuged at $14,000 \times g$ for 15 min, and the resulting pellets were resuspended in SET medium (SETH medium minus heparin) and centrifuged $14,000 \times g$ for 15 min. The final pellets were suspended in SET medium. Brain mitochondria were isolated according to method I described by Lai and Clark (15).

Enzyme assays. NADH-cytochrome *c* reductase activity in freeze-thawed mitochondria was measured at 30°C by monitoring the reduction of cytochrome *c* at 550 nm in a final vol of 1.0 ml containing 0.1 mM NADH, 17 μ M cytochrome *c*, 0.3 mM potassium cyanide, 50 mM phosphate buffer, pH 7.5. Rotenone-sensitive NADH-cytochrome *c* reductase activity was calculated from the difference in rates of cytochrome *c* reduction in the absence and presence of 1.5 μ M rotenone (16). The activity of rotenone-sensitive NADH-cytochrome *c* reductase in the freeze-thawed mitochondria is approximately 70% of that in the sonicated mitochondria. Succinate-cytochrome *c* reductase was measured at 30°C by monitoring the reduction of cytochrome *c* at 550 nm. The assay was performed in a final vol of 1.0 ml containing 0.5 M sodium succinate, 30 mM sodium cyanide, 0.1 mM cytochrome *c*, 1.5 μ M rotenone, 0.17 M phosphate buffer, pH 7.4 (16). Complex IV activity was measured by the method of Cooperstein and Lazarow (17). The oxidation of ferrocytochrome *c* was measured spectrophotometrically at 30°C in 1.0 ml of 30 mM phosphate buffer, pH 7.4, containing 17 μ M ferrocytochrome *c*. NADH-ferricyanide reductase activity was measured at 30°C in 1.0 ml of 40 mM Tris-HCl, pH 7.5, containing 1.6 mM potassium ferricyanide and 0.15 mM NADH by monitoring the reduction of ferricyanide at 410 nm (18). Complex I activity was measured at 30°C in 1.0 ml of 20 mM potassium phosphate buffer, pH 8.0, containing 2 mM $\text{Na}_2\text{S}_2\text{O}_8$

(neutralized), 0.1 mM coenzyme Q_1 , 0.225 mg/ml soybean phospholipid, and 0.15 mM NADH by monitoring the oxidation of NADH at 340 nm (19). Rotenone was added at a final concentration of 15 μ M when included in the reaction mixture. Protein in mitochondrial preparations was measured by the method of Lowry *et al.* (20) using BSA as the standard.

EPR Studies. Preparation of submitochondrial particles. SMP were prepared from frozen liver mitochondria as described by Moreadith *et al.* (21), and suspended to 41 mg/ml in 250 mM sucrose, 50 mM HEPES, pH 7.5.

EPR spectroscopy. EPR measurements were conducted with a spectrometer (E109; Varian Associates Instrument Group, Palo Alto, CA). Sample temperature was controlled by use of a variable temperature, helium flow cryostat system (LTD-d-110; Air Products & Chemicals, Inc., Allentown, PA). The temperature was monitored with an Allen-Bradley (Milwaukee, WI) type carbon resistor located 1 cm below the sample. The magnetic field was calibrated with a pitch gauss value standard (Varian Associates).

Immunologic Studies. Purification of mitochondrial electron-transfer complexes. Complex I (18), complex III (22), complex IV (23), and F_1 -ATPase (the F_1 -part of complex V) (24) were isolated from beef heart mitochondria as described.

Immunization. Antibodies against the mitochondrial enzymes were raised in rabbits by intradermal injections of 0.5–1 mg of enzyme protein in 1 ml of saline emulsified with 1 ml of Freund's complete adjuvant. Booster injections of the same mixture were administered 3, 5, and 7 wk later. Blood was withdrawn from the carotid artery 1 wk after the last injection.

Electrophoresis. SDS-PAGE was carried out using 9.38–18.75% linear acrylamide-gradient gels ($140 \times 140 \times 0.75$ mm) essentially as described by Kadenbach *et al.* (25).

Electroblotting. Mitochondrial proteins separated in the gel were electrophoretically transferred to a Durapore GVHP filter (Millipore Continental Water Systems, Bedford, MA) essentially according to the method of Towbin *et al.* (26) with addition of 0.1% SDS to the electrode buffer. The binding of antibodies to the subunits of the mitochondrial enzymes on the filter was detected by the peroxidase-antiperoxidase method, as described previously (27, 28). The immunoblot strips were analyzed densitometrically using a CS-930 chromatoscanner (Shimadzu Corporation, Kyoto, Japan).

Immunohistochemistry. For immunohistochemical studies, muscle biopsy specimens were frozen in liquid nitrogen-cooled isopentane and cross-sectioned to a 10- μ m thickness in a cryostat. Sections were incubated for 5 min at room temperature with 0.1% Triton X-100, and washed twice with cold PBS. They were treated for 20 min with 0.03% H_2O_2 in methanol to block endogenous peroxidase activity. After being washed twice with cold PBS, the sections were overlaid with 10% normal goat serum for 20 min in a humidified chamber. Then the sections were covered with polyclonal antibodies (1:50 dilution) against complexes I, III, IV, and V for 30 min at 37°C . The immunohistochemical staining for the complexes were performed by the avidin-biotin complex method essentially according to Sato *et al.* (29).

RESULTS

Biochemical Studies. Enzyme assays. The activities of mitochondrial enzymes, measured in mitochondrial preparation from frozen tissues, are shown in Table 1. Rotenone-sensitive NADH-cytochrome *c* reductase (complex I–III) was used as an estimate of complex I activity. The enzymic activity was markedly decreased in the mitochondria from various tissues of the patient, ranging from 15% of control in the liver mitochondria to 42% in the brain mitochondria, whereas succinate-cytochrome *c* reductase (complex II–III) was normal. These results indicate that the severity of deficiency in complex I activity varies from tissue to tissue. Complex IV activity was lower than the control range

in all the tissues examined except for kidney. Further examination of complex I activity in liver mitochondria showed that NADH-ferricyanide reductase activity was 53% of the control value, whereas complex I activity was strikingly decreased to 11% (Table 2).

EPR spectroscopy. Figure 1 shows the EPR spectra of iron-sulfur clusters on the substrate side of the rotenone inhibition site of the respiratory chain in control and MELAS SMP. These signals have been resolved under varying EPR conditions, utilizing their different spin relaxation behavior (30). EPR spectra are expressed in the first-derivative form of the absorption spectra. However, relative concentrations of iron-sulfur clusters in the control and MELAS SMP can be compared using the peak height or trough depth of counterpart signals as long as their spectra were recorded under the same EPR conditions. At 26°K, only EPR signals arising from the cluster N-1b ($g_x = 1.92$, $g_y = 1.93$, $g_z = 2.025$) are detected together with the iron-sulfur cluster in the mitochondrial outermembrane ($g_{x,y,z} = 1.89$, 1.94, 2.01) (Fig.

1A) (31). The same amplitude of the $g_x = 1.89$ signal was found in the normal control (*solid line*) and the MELAS patient (*broken line*) spectra, which indicates that no significant change occurred in the concentration of the iron-sulfur cluster in the mitochondrial outermembrane. Therefore, the cluster N-1b in the liver SMP of the normal control (*solid line*) and the MELAS patient (*broken line*) can be selectively compared by use of the $g = 1.93$ peak to peak amplitude, after subtracting the g_y contribution from the outermembrane iron-sulfur cluster. Cluster N-1b in the patient's SMP decreased to approximately $\frac{1}{2}$ of the control.

Table 2. NADH-ferricyanide reductase and rotenone-sensitive complex I activities in liver mitochondria*

Enzyme	Patient	Control
NADH-ferricyanide reductase	1074	1762
Complex I (rotenone-sensitive)	1.7	15.0

* Activities are expressed as nmol/min/mg of mitochondrial protein.

Table 1. Enzymic activities in mitochondria from various tissues*

Enzyme		Heart		Skeletal muscle		Liver		Kidney		Brain	
Complex I-III											
Patient	(%)	87	(41)	39	(20)	27	(15)	60	(41)	16	(42)
Controls	(n)	213 ± 45	(4)	191 ± 121	(9)	180 ± 114	(4)	147 ± 80	(4)	38 ± 16	(3)
Range		175-277		104-487		90-347		72-257		23-55	
Complex II-III											
Patient	(%)	347	(65)	668	(101)	166	(99)	266	(114)	82	(58)
Controls	(n)	571 ± 230	(6)	664 ± 190	(9)	168 ± 45	(6)	234 ± 27	(4)	142 ± 64	(3)
Range		315-946		438-1090		112-228		212-273		98-215	
Complex IV											
Patient	(%)	804	(43)	912	(53)	311	(69)	298	(67)	212	(60)
Controls	(n)	1879 ± 319	(4)	1720 ± 681	(8)	449 ± 160	(4)	448 ± 144	(4)	351 ± 150	(3)
Range		1426-2115		1008-2813		389-677		285-628		215-512	

* Activities are expressed as nmol/min/mg of mitochondrial protein. Control values are mean ± SD. Complex I-III, rotenone-sensitive NADH-cytochrome c reductase; complex II-III, succinate-cytochrome c reductase.

TO-996a

Fig. 1. EPR spectra of iron-sulfur clusters in the complex I segment of the electron-transport chain. SMP from the control (*solid line*) and from the patient (*broken line*) were preincubated with rotenone (0.1 nmol/mg of protein) for 10 min before reduction with 4 mM NADH. Spectra A, B, and C were measured at sample temperatures of 26, 13, and 9°K with microwave power levels of 2, 2, and 5 mW, respectively. The EPR recording conditions were: modulation amplitude, 1.25×10^{-3} tesla; time constant, 0.128 s; scanning rate, 10^{-2} tesla/min.

Fig. 2. EPR spectra of submitochondrial particles reduced with 5 mM dithionite and recorded at two different temperatures. Sample temperatures, microwave power, and other EPR conditions were the same as described in Figure 1.

Similarly, at 13°K, cluster N-2 can be measured by the signal with the negative peak at the g_y value of 1.92 (Fig. 1B), which has revealed that the N-2 signal in the MELAS patient's SMP decreased to approximately $\frac{1}{3}$ of the control level. At this temperature, the overlapping EPR signal from the cluster S-3 of complex II obscured the $g_z = 2.05$ signal of the cluster N-2.

At 9°K, clusters N-3 ($g_{x,y,z} = 1.86, 1.93, 2.04$) and N-4 ($g_{x,y,z} = 1.88, 1.93, 2.11$) are selectively observed (Fig. 1C) and can be compared with those of the control system by use of the $g_x = 1.86$ (N-3 cluster) and 1.88 (N-4 cluster) signals. Again, cluster N-3 was decreased to $\frac{1}{3}$ of the control, while cluster N-4 diminished to $\frac{1}{2}$.

The difference between the control and MELAS patient's spectra is given below the individual spectra in Figure 1 and

Fig. 4. Densitograms of the immunoblots of complex I subunits. C, control mitochondria (120 μ g protein); P, patient's mitochondria (120 μ g protein). The figures are the molecular masses in kD of subunits (32).

Fig. 3. Immunoblots for complex I subunits in mitochondria from various tissues. BHM, beef heart mitochondria (25 μ g protein); C, control mitochondria (60 μ g protein); P, patient's mitochondria (60 μ g protein). The molecular masses of complex I subunits and standard proteins were indicated in kD on the left and right side of the lane for BHM, respectively. Arrowheads indicate the subunits whose density is markedly decreased.

shows the resolved EPR spectra of the iron-sulfur clusters in the complex I segment of the respiratory chain. The results of these studies indicate either that the iron-sulfur clusters in the complex I, which are reducible by NADH in the presence of rotenone, are greatly diminished or that the clusters are present in normal amounts but are not totally reducible by NADH.

Figure 2 presents EPR spectra of the iron-sulfur clusters in the control and MELAS patient's SMP, which were reduced with dithionite and recorded at two different temperatures. Dithionite is a nonspecific reducing agent which chemically reduces all iron-sulfur clusters of SMP. It was used to determine whether the deficiency of one or more of the clusters was due primarily to the failure of NADH to reduce them enzymatically. Figure 2A presents (solid line) EPR signals of the control SMP observed at 26°K, which elicits a spectral line shape from the N-1b, S-1, and the outermembrane iron-sulfur cluster. In contrast, the spectrum from MELAS patient SMP (broken line) gave rise to a spectrum dominated by the cluster S-1 line shape with the g_x value of 1.91. The $g_x = 1.92$ signal of the cluster N-1b is not discernible. The amplitudes of the $g_x = 1.93$ signal in the control and the MELAS patient SMP are about the same, which indicates that in the MELAS system, the content of the cluster S-1 was increased relative to the counterpart in the control system. Figure 2B shows decreased signal intensity of clusters N-2 ($g = 2.05$), N-3 ($g_x = 1.86$), and N-4 ($g_x = 2.11$) consistent with observations in Figure 1C. The EPR signals at $g_z = 2.18$ and $g_x = 1.88$ (Fig. 2B) arising from the electron transferring flavoprotein-ubiquinone oxidoreductase indicate that this cluster was not significantly different or even somewhat enhanced in the MELAS patient than the control SMP. The combined results of the EPR studies presented in Figures 1 and 2 demonstrate that the observed deficiency of iron-sulfur clusters in the mitochondria from the MELAS patient was not a generalized deficiency of all mitochondrial iron-sulfur clusters but was limited to those of complex I. Furthermore, it is noted that four distinct iron-sulfur clusters in complex I were disproportionately decreased.

Immunologic Studies. Immunoblotting. The electron-transfer chain is divided into four segments: complexes I, and II, III, and IV. Each segment is a multi-subunit enzyme; there are 25 unlike subunits in complex I, 4–5 in complex II, 9–10 in complex III, 7–8 in complex IV, and 12–14 in complex V (32). Immunoblot analysis was performed in order to determine which of the subunits in each complex was affected. In Figure 3 the subunit profiles of complex I of the patient's mitochondria from various tissues are compared with those of complex I of the normal human mitochondria and beef heart mitochondria. The content of complex I subunits was markedly decreased in the patient's mitochondria from the skeletal muscle, liver, kidney, and brain, but it was not so much decreased in the heart mitochondria. The difference in immunoblot pattern among the tissues seems to be due to the difference in the contents of respiratory complex subunits among the crude mitochondrial preparations from the tissues. There were no essential differences in their electrophoretic mobility and relative amounts of the subunit polypeptides. No differences in immunoblot pattern were observed between the mitochondria from autopsied muscle tissue and those from biopsied tissue, indicating that there is no appreciable proteolytic degradation of polypeptides even in the frozen-stored autopsied tissues. The content of subunits in complex I was decreased in parallel with the activity, indicating that the decreased activity of complex I in each tissue is due to a deficiency of subunits of the complex. Severe deficiency was observed in the 75-kD subunit in the iron-sulfur protein fraction, the 51-kD subunit in the flavoprotein fraction, and some of the lower-mol wt-subunits in the hydrophobic fraction. The densitometric scan of the blots in the skeletal muscle mitochondria indicated disproportionate and severe deficiency of the 75-kD, 51-kD, and several lower-mol wt-polypeptides (Fig. 4). To investigate possible abnormality in other components of the mitochondrial oxidative phosphorylation system, immunoblotting experiments were performed using antibodies against complexes III, IV, and V. The immunochemically detectable subunits of complex IV were reduced in the mito-

Fig. 5. Immunoblots for complex IV subunits in mitochondria from various tissues. BHM, beef heart mitochondria (25 μ g protein); C, control mitochondria (60 μ g protein); P, patient's mitochondria (60 μ g protein). The subunits which are markedly decreased in content in patient's mitochondria are indicated by arrowheads.

chondria from the skeletal muscle and liver (Fig. 5), whereas decrease in the subunits was slight in the heart, kidney, and brain. Subunit 2 appeared to be most markedly decreased among the complex IV subunits in the patient's mitochondria from the skeletal muscle. In complexes III and V, the content of subunits was not appreciably decreased in all the tissues examined (data not shown).

Immunohistochemistry. Ragged-red fibers were prominent in the patient's skeletal muscle (Fig. 6A). The histochemistry for complex IV activity (Fig. 6B) showed scattered, poorly stained fibers, some of which corresponded to the ragged-red fibers. The ragged-red fibers were heavily immunostained using anticomplex I antibody (Fig. 6C) and less heavily immunostained using anticomplex IV antibody (Fig. 6D). In contrast, the non-ragged-red fibers in the patient's muscle were less intensely immunostained with anticomplex I antibody (Fig. 6C) than the muscle fibers from a normal individual (Fig. 6E). These findings indicate that the immunologically detectable subunits of complex I were deficient in the patient's muscle fibers except the ragged-red fibers. Staining for complex IV activity (Fig. 6B) was decreased in the ragged-red fibers despite the accumulation of immunoreactive substance of complex IV (Fig. 6D), indicating that the enzyme proteins were nonfunctional in these fibers.

DISCUSSION

In the present investigation, we have studied the molecular bases of complex I deficiency in a patient with MELAS by enzymologic (Tables 1 and 2), EPR spectroscopic (Figs. 1 and 2), and immunochemical methods (Figs. 3 and 4) and have found that deficiencies of iron-sulfur clusters and subunits in complex I are responsible for the decreased enzymic activity of the complex in various tissues. Moreadith *et al.* (21, 33) reported an almost total loss of the iron-sulfur clusters in complex I associated with a selective absence of at least two (75-kD and 13-kD) subunits comprising the iron-sulfur protein fragment of the complex in an infant with congenital lactic acidosis. The difference in molecular defect of complex I between the present patient with MELAS and the patient with congenital lactic acidosis may explain the difference in clinical severity between two patients.

Electrons from NADH are first transferred to flavin mononucleotide and low potential iron-sulfur clusters (N-1, N-3, and N-4), and finally to ubiquinone via the high potential N-2 cluster in the hydrophobic fraction (32) (Table 3). In the liver mitochondria of the present patient, enzymatic activity measurements demonstrated a marked decrease (11% of the control) in complex I in contrast with a milder decrease (53% of the control) in NADH-ferricyanide reductase activity (Table 2). Because the latter activity can be ascribable not only to the flavoprotein moiety of complex I (34) but also to flavoproteins in the outer-membrane (16), we cannot estimate precisely the deficiency of complex I by this activity. In contrast, the former activity requires the whole structure of complex I (18), and thus serves as a better indicator of complex I deficiency. Moreadith *et al.* (33) reported

distinct abnormalities in kinetics of NADH-ferricyanide reductase activity in a case of congenital complex I deficiency. Similar abnormality might be observed in the present case if the enzymic activity were kinetically analyzed. EPR spectroscopy on liver SMP (Figs. 1 and 2) demonstrated that the decrease in enzymic activity of complex I is based on a disproportionate deficiency of iron-sulfur clusters; clusters N-2 and N-3 being markedly decreased (each to about 1/3 of the control), and clusters N-1 and

Fig. 6. Histochemistry and immunohistochemistry of the skeletal muscle from patient. A, Gomori's modified trichrome stain. Arrowheads indicate ragged-red fibers. B, activity staining for complex IV. C, immunostaining for complex I. D, immunostaining for complex IV. E and F are immunostaining of normal skeletal muscle for complexes I and IV, respectively (Bar = 100 μ m, A-F).

Table 3. Polypeptide composition of fractions of complex I and putative localization of prosthetic groups*

Fraction	Polypeptide	Prosthetic group
Flavoprotein	51 kD	Flavin mononucleotide
	24 kD	N-3 cluster [4Fe-4S]
	9 kD	N-1b cluster [2Fe-2S]
Iron-sulfur protein	75 kD	N-1a cluster [2Fe-2S]
	49, 30, 13 kD	N-4 cluster [4Fe-4S]
	18, 15 kD	
Hydrophobic protein	16 polypeptides (67, 51, 39, 36, 19, 13, 11 kD)†	N-2 cluster [4Fe-4S]

* The putative localization of prosthetic groups are based on the review by Hatefi (32).

† Subunits encoded by mitochondrial DNA (38, 39).

N-4 being less markedly decreased (each to approximately 1/2 of the control).

Immunochemical analysis (Figs. 3 and 4) further elucidated molecular basis for deficiency of enzymatic activity and iron-sulfur clusters. Although the assignment of specific redox centers to subunits (35) is in some cases still tentative, we can correlate the EPR data to the immunochemical data.

The flavoprotein fragment of complex I are composed of three polypeptides: the 51-kD polypeptide containing flavin mononucleotide and the N-3 iron-sulfur cluster, the 24-kD polypeptide containing the N-1b cluster, and the 9-kD polypeptide (35). Although the decrease in NADH-ferricyanide reductase activity that is ascribable to this flavoprotein fragment could not be demonstrated, probably due to the presence of the flavoproteins in the outer membrane that have the same enzymic activity, we could demonstrate a concomitant reduction in the 51-kD polypeptide and the N-3 iron-sulfur clusters.

It is noteworthy, however, that the decreased activity was not ascribable to a deficiency of a single iron-sulfur cluster or to a defect of a single immunochemically detectable subunit but to deficiencies of multiple iron-sulfur clusters and subunits. Therefore, we speculate that disproportionateness observed in the EPR spectroscopy and Western blotting manifests impaired molecular integrity of complex I, which may also result from a defect in a subunit(s) essential for the assembly of the complex.

The immunohistochemical study on the skeletal muscle (Fig. 6) demonstrated that a large amount of immunoreactive substance of complex I accumulated in the ragged-red fibers. The increased number of mitochondria in the ragged-red fibers is regarded as a result of stimulated proliferation of mitochondria in response to the decreased energy supply in these cells. Because deficiency of complex I subunits is not complete but disproportionate, the increased immunoreactivity in the ragged-red fibers is ascribable to the residual subunits in defective mitochondria that proliferated in the fibers.

We observed that mild decreases in activity (Table 1) and content (Fig. 5) of subunits in complex IV were associated with complex I deficiency. The mild defect in complex IV might be an epiphenomenon of complex I deficiency. Alternatively, an abnormality might cause defects in complexes I and IV simultaneously to a different extent. Combined defects in complexes I and IV have been reported in other cases (36, 37). In our previous patient with mitochondrial myopathy (28), we observed that a mild decrease in content of complex I subunits was accompanied by a severe defect in subunit 2 of complex IV. As both complexes I and IV contain mitochondrially encoded subunits (38, 39), the combined defects of the complexes might be due to an abnormality in the mitochondrial DNA (40, 41). The finding that subunit 2, a mitochondrially encoded subunit, was most markedly decreased among the complex IV subunits in the patient's mitochondria supports this hypothesis. Studies on abnormalities in mitochondrial and nuclear genes using cloned mitochondrial DNA and cDNA for nuclearly encoded subunits (42, 43) will further elucidate the mechanism of defects in the electron-transfer complexes in MELAS.

REFERENCES

- Egger J, Lake BD, Wilson J 1981 Mitochondrial cytopathy: a multisystem disorder with ragged red fibers on muscle biopsy. *Arch Dis Child* 56:741-752
- Pavakis SG, Phillips PC, DiMauro S, DeVivo DC, Rowland LP 1984 Mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes: a distinctive clinical syndrome. *Ann Neurol* 16:481-488
- Sengers RCA, Stadhouders AM, Trijbels JMF 1984 Mitochondrial myopathies: clinical, morphological and biochemical aspects. *Eur J Pediatr* 141:192-207
- DiMauro S, Bonilla E, Zeviani M, Nakagawa M, DeVivo DC 1985 Mitochondrial myopathies. *Ann Neurol* 17:521-538
- Morgan-Hughes JA, Darveniza P, Landon DN, Land JM, Clark JB 1979 A mitochondrial myopathy with a deficiency of respiratory chain NADH-CoQ reductase activity. *J Neurol Sci* 43:27-46
- Morgan-Hughes JA, Hayes DJ, Clark JB, Landon DN, Swash M, Stark RJ, Rudge P 1982 Mitochondrial encephalomyopathies: biochemical studies in two cases revealing defects in the respiratory chain. *Brain* 105:553-582
- Tanaka M, Nishikimi M, Suzuki H, Ozawa T, Nishizawa M, Tanaka K, Miyatake T 1986 Deficiency of subunits in heart mitochondrial NADH-ubiquinone oxidoreductase of a patient with mitochondrial encephalomyopathy and cardiomyopathy. *Biochem Biophys Res Commun* 140:88-93
- Kobayashi M, Morishita H, Sugiyama N, Yokochi K, Nakano M, Wada Y 1986 Mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes syndrome and NADH-CoQ reductase deficiency. *J Inherited Metab Dis* 9:301-304
- Kobayashi M, Morishita H, Sugiyama N, Yokochi K, Nakano M, Wada Y, Hotta Y, Terauchi A, Nonaka I 1987 Two cases of NADH-coenzyme Q reductase deficiency: relationship to MELAS syndrome. *J Pediatr* 110:223-227
- Nishizawa M, Tanaka K, Shinozawa K, Kuwabara T, Atsumi T, Miyatake T, Ohama E 1987 A mitochondrial encephalomyopathy with cardiomyopathy: a case revealing a defect of complex I in the respiratory chain. *J Neurol Sci* 78:189-192
- Ichiki T, Tanaka M, Nishikimi M, Suzuki H, Ozawa T, Kobayashi M, Wada Y 1988 Deficiency of subunits of Complex I and mitochondrial encephalomyopathy. *Ann Neurol* 23:287-294
- Tanaka M, Nishikimi M, Suzuki H, Ozawa T, Ichiki T, Kobayashi M, Wada Y 1987 Variation in the levels of Complex I subunits among tissues in a patient with mitochondrial encephalomyopathy and renal dysfunction. *Biochem Int* 14:735-739
- Yoda S, Terauchi A, Kitahara F, Akabane T 1984 Neurologic deterioration with progressive CT changes in a child with Kearns-Shy syndrome. *Brain Dev* 6:323-327
- Bookelman H, Trijbels JMF, Sengers RCA, Janssen AJM, Veerkamp JH, Stadhouders AM 1978 Pyruvate oxidation in rat and human skeletal muscle mitochondria. *Biochem Med* 20:395-403
- Lai JCK, Clark JB 1979 Preparation of synaptic and nonsynaptic mitochondria from mammalian brain. *Methods Enzymol* 55:51-60
- Sottocasa GL, Kuylentier B, Ernster L, Bergstrand A 1967 An electron-transport system associated with the outer membrane of liver mitochondria: a biochemical and morphological study. *J Cell Biol* 32:415-438
- Cooperstein SJ, Lazarow A 1951 A microspectrophotometric method for the determination of cytochrome oxidase. *J Biol Chem* 189:665-670
- Hatefi Y 1978 Preparation and properties of NADH: ubiquinone oxidoreductase (Complex I), EC 1.6.5.3. *Methods Enzymol* 53:11-14
- Hatefi Y, Rieske JS 1967 Preparation and properties of DPNH-coenzyme Q reductase (Complex I of the respiratory chain). *Methods Enzymol* 10:235-239
- Lowry OH, Rosebrough NJ, Farr AL, Randall RJ 1951 Protein measurement with the Folin phenol reagent. *J Biol Chem* 193:265-275
- Moreadith RW, Batshaw ML, Ohnishi T, Kerr K, Knox B, Jackson D, Hruban R, Olson J, Reynafarje B, Lehninger AL 1984 Deficiency of the iron-sulfur clusters of mitochondrial reduced nicotinamide-adenine dinucleotide-ubiquinone oxidoreductase (Complex I) in an infant with congenital lactic acidosis. *J Clin Invest* 74:685-697
- Ozawa T, Tanaka M, Shimomura Y 1983 Crystallization of cytochrome *b_c* complex. *Proc Natl Acad Sci USA* 80:921-925
- Tanaka M, Ozawa T 1982 Molecular profile of highly purified beef heart cytochrome oxidase. *Biochem Int* 5:67-75
- Vogel G 1979 An improved method for the isolation of mitochondrial ATPase (F₁) from beef heart. *Methods Enzymol* 55:317-319
- Kadenbach B, Jarausch J, Hartmann R, Merle P 1983 Separation of mammalian cytochrome *c* oxidase into 13 polypeptides by a sodium dodecyl sulfate-gel electrophoretic procedure. *Anal Biochem* 129:517-521
- Towbin H, Staehelin T, Gordon J 1979 Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci USA* 76:4350-4354
- Tanaka M, Nishikimi M, Suzuki H, Ozawa T, Okino E, Takahashi H 1986 Multiple cytochrome deficiency and deteriorated mitochondrial polypeptide composition in fatal infantile mitochondrial myopathy and renal dysfunction. *Biochem Biophys Res Commun* 137:911-916
- Tanaka M, Miyabayashi S, Nishikimi M, Suzuki H, Shimomura Y, Ito K, Narisawa K, Tada K, Ozawa T 1988 Extensive defects of mitochondrial electron-transfer chain in muscular cytochrome *c* oxidase deficiency. *Pediatr Res* 24:447-454
- Sato T, Anno M, Arahata K, Nakamura S, Mukoyama M, Nakamura H, Ozawa T 1986 Immunocytochemical demonstration of cytochrome *c* oxidase and Complex III in tissues from patients with mitochondrial encephalomyopathy. *Muscle Nerve* 9 (suppl):183
- Ohnishi T 1979 Mitochondrial iron-sulfur flavodehydrogenases. In: Capaldi RA (ed) *Membrane Proteins in Energy Transduction*. Marcel Dekker, New York, pp 1-87
- Bäckström D, Lorusso M, Anderson K, Ehrenberg A 1978 Characterization of the iron-sulfur protein of the mitochondrial outer membrane partially purified from beef kidney cortex. *Biochim Biophys Acta* 502:276-288
- Hatefi Y 1985 The mitochondrial electron transport and oxidative phosphorylation system. *Annu Rev Biochem* 54:1015-1069
- Moreadith RW, Cleeter MWJ, Ragan CI, Batshaw ML, Lehninger AL 1987 Congenital deficiency of two polypeptide subunits of the iron-protein fragment of mitochondrial complex I. *J Clin Invest* 79:463-467
- Galante YM, Hatefi Y 1978 Resolution of Complex I and isolation of NADH

- dehydrogenase and an iron-sulfur protein. *Methods Enzymol* 53:15-21
35. Ohnishi T, Ragan CI, Hatefi Y 1985 EPR studies of iron-sulfur clusters in isolated subunits and subfractions of NADH-ubiquinone oxidoreductase. *J Biol Chem* 260:2782-2788
 36. Roodhooft AM, Van Acker KJ, Martin JJ, Ceuterick C, Scholte HR, Luyt-Houwen IEM 1986 Benign mitochondrial myopathy with deficiency of NADH-CoQ reductase and cytochrome *c* oxidase. *Neuropediatrics* 17:221-226
 37. Tanaka M, Nishikimi M, Suzuki H, Ozawa T, Koga Y, Nonaka I 1987 Partial deficiency of subunits in Complex I or IV of patients with mitochondrial myopathies. *Biochem Int* 14:525-530
 38. Anderson S, Bankier AT, Barrell BG, deBruijn MHL, Coulson AR, Drouin J, Eperon IC, Nierlich DP, Roe BA, Sanger F, Schreier PH, Smith AJH, Staden R, Young IG 1981 Sequence and organization of the human mitochondrial genome. *Nature* 290:457-465
 39. Chomyn A, Cleeter MWJ, Ragan CI, Riley M, Doolittle RF, Attardi G 1986 URF6, last unidentified reading frame of human mtDNA, codes for an NADH dehydrogenase subunit. *Science* 234:614-618
 40. Holt IJ, Harding AE, Morgan-Hughes JA 1988 Deletions of muscle mitochondrial DNA in patients with mitochondrial myopathies. *Nature* 331:717-719
 41. Ozawa T, Yoneda M, Tanaka M, Ohno K, Sato W, Suzuki H, Nishikimi M, Yamamoto M, Nonaka I, Horai S 1988 Maternal inheritance of deleted mitochondrial DNA in a family with mitochondrial myopathy. *Biochem Biophys Res Commun* 54:1240-1247
 42. Nishikimi M, Suzuki H, Ohta S, Sakurai T, Shimomura Y, Tanaka M, Kagawa Y, Ozawa T 1987 Isolation of a cDNA clone for human cytochrome *c*₁ from a λ gt11 expression library. *Biochem Biophys Res Commun* 145:34-39
 43. Nishikimi M, Suzuki H, Yamaguchi M, Matsukage A, Yoshida MC, Ozawa T 1988 Assignment of the human cytochrome *c*₁ gene to chromosome 8. *Biochem Int* 16:655-660