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Biochemical Investigations and Immunoblot Analyses of Two Unrelated Patients with an Isolated Deficiency in Complex II of the Mitochondrial Respiratory Chain

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Mitochondrial respiratory chain defects involving complex II are comparatively rare. We report the biochemical findings in two unrelated patients who both have an isolated complex II deficiency (40–50% of control values). Western blot analysis of mitochondrial fractions showed different findings between the two patients. In one patient there was a decrease in the levels of both the Fp and Ip subunits whereas in the other patient the levels of all immunoreactive complex II subunits were normal. This is the first time that an isolated deficiency of complex II activity associated with normal levels of protein subunits, using subunit specific antisera, has been described.

Abnormalities of the mitochondrial respiratory chain are increasingly being recognised as an important cause of neurological disease in man (1). Defects of the mitochondrial genome with abnormalities of complexes I, III, IV and V have been identified in many of these patients. In contrast, defects of complex II are comparatively rare.

Complex II (succinate:ubiquinone oxidoreductase, E.C. 1.3.5.1) is the only respiratory chain enzyme that does not have subunits encoded by mitochondrial DNA. It catalyses the oxidation of succinate to fumarate and transfers the electrons directly to the ubiquinone (UQ) pool (2). Purified complex II can be resolved into two reconstitutively active fractions, a soluble succinate dehydrogenase (SDH) and a membrane-anchoring fraction. SDH consists of a flavoprotein (Fp) subunit (70 kDa) containing the putative active site and the covalently-bound FAD moiety of the enzyme, and an iron-sulphur (Ip) subunit (30 kDa) carrying three dissimilar iron-sulphur clusters. SDH is anchored to the mitochondrial inner membrane by two polypeptides generally known as QPs1 and QPs2 (15 kDa and 13 kDa respectively) which contain a single heme group (cytochrome b_{558}) and also provide the UQ binding sites.

We report the biochemical findings in two unrelated patients who have both an isolated complex II deficiency and present with similar clinical symptoms. In both patients, spectrophotometric and polarographic measurements of succinate oxidation and the activities of the respiratory chain complexes in skeletal muscle mitochondria showed a partial decrease (40–50% of control values) of complex II activity. In contrast, the activities of the other respiratory enzymes were normal in both patients. Western blot analysis of mitochondrial fractions showed different findings between the two patients. In one patient there was a decrease in the levels of both the Fp and Ip subunits whereas in the other patient the levels of all immunoreactive subunits were normal. This is the first time that an isolated deficiency of complex II activity associated with normal levels of protein subunits, using subunit specific antisera, has been described.

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MATERIALS AND METHODS

Case Histories

Patient 1. This girl, the first child of normal consanguineous parents, developed normally until 10 months of age and then began to regress rapidly showing some signs of loss of acquired psychomotor skills. In the blood there was mild hyperlactatemia (levels of 2.69 to 3.47mmol/L; normal levels, 0.63 to 2.44). A CT scan showed a diffuse hypodensity of the white matter in the cerebral hemispheres predominating in both frontal and occipital areas. This aspect was interpreted as compatible with a leukodystrophic process. The child died at 19 months. A detailed clinical description has been previously reported (3).

Patient 2. This 5 year old girl, born of normal non-consanguineous parents, developed normally until 10 months of age (as above) and then began to regress showing loss of acquired psychomotor skills. In the blood there was a hyperlactatemia (4.4 mmol/L). As detailed above for patient 1, patient 2 was described as presenting with a leukodystophy following a CT scan.

Preparation of Mitochondrial Fractions and Muscle Post-600g Supernatants

Skeletal muscle samples were obtained by open biopsy and mitochondrial fractions were prepared as previously described (4). Human skin fibroblasts were cultured under standard conditions (5) and a mitochondrial fraction isolated as described by Lowerson et al. (6). A muscle post-600g supernatant was prepared when only milligram amounts of muscle were available (7).

Biochemistry

The polarographic measurements of substrate oxidation were measured as described previously (3). Rotenone was included in the medium when succinate was used as the substrate. The activities of the individual respiratory chain complexes (3 and 8), succinate-cytochrome c reductase (SCR) (9) and citrate synthase (10) were measured. The protein concentration of mitochondrial fractions was determined by a modification of the Lowry method (11).

Immunoblot Analysis

The proteins in the mitochondrial fractions were separated by SDS-PAGE (12) using 12.5% polyacrylamide gels, transferred to Hybond-PVDF (Amersham) membranes (13) in a buffer comprising 10mM CAPS, 10% methanol (v/v), pH 11 and probed with antisera raised in rabbits to the individual subunits of complex II (a gift from Dr. BAC Ackrell, San Francisco). Immunoreactive peptides were visualised using the Enhanced Chemiluminescence (ECL) system (Amersham).

RESULTS

Biochemistry

Patient 1. A complex II deficiency was identified in the mitochondria isolated from skeletal muscle (approximately 40–50% of control values) based on both the impairment of succinate oxidation (14) and the decrease of succinate-dependent enzyme activities (Table I). The other respiratory chain enzymes were polarographically and spectrophotometrically normal (Table I and 14). This decrease of complex II activity in muscle is equally apparent when the activity of complex

TABLE 1
Enzyme Activities in Mitochondrial Fractions of Patient 1 and Controls[a]

	Muscle		Fibroblasts	
	Patient	Control (n = 15)	Patient	Controls (n = 8)
Complex II	12	50–140	4.5	13–31
Complex IV	294	245–1247	99	47–172
SCR	50	147–402	15	17–58
IV/SCR ratio	5.9	3.2 ± 0.3	6.2	3.0 ± 0.4
IV/II ratio	24.5	9.1 ± 1.5	22.0	5.2 ± 0.5

[a] Results are expressed as nmol DCPIP reduced. min^{-1}. mg $protein^{-1}$ for complex II, nmol cytochrome c oxidized. min^{-1}. mg $protein^{-1}$ for complex IV, and nmol cytochrome c reduced. min^{-1}. mg $protein^{-1}$ for SCR. Values for control ratios are mean ± SD.

IV is expressed as a ratio to that of complex II (indicating a decrease of activity to 38% of control values). The complex II defect was found also in both cultured skin fibroblasts (Table I) and circulating lymphocytes (results not shown).

Patient 2. Polarographic measurement of succinate oxidation in the muscle mitochondrial fraction of the patient was (42 ng atoms .min^{-1} .mg $protein^{-1}$) 39% of control values (109 ± 32; mean ± SD, n = 20). In contrast, the oxidation of pyruvate plus malate (113 ng atoms 0.min^{-1} .mg $protein^{-1}$) was within the normal range (115 ± 42; mean ± SD, n = 20). In addition, spectrophotometric measurement of succinate-cytochrome c reductase (SCR) activity in the mitochondrial fraction of the patient (373 nmol cytochrome c reduced min^{-1} mg of $protein^{-1}$) was 51% of control values (728 ± 122; mean ± SD, n = 20). The impairment of succinate oxidation and the decrease of succinate-dependent enzyme activity was investigated further by the measurement of the activities of the individual respiratory chain complexes in post-600g supernatants prepared from 60 milligrams of a frozen muscle biopsy sample (Table II). The enzyme activities in table II are presented relative to citrate synthase activity rather than protein in order to preclude variability due to protein contamination in these mitochondrial-enriched supernatants. The activities of complexes I, III and IV are higher than control values, whilst that of complex II is much lower (50% of control values). This decrease of complex II activity becomes more apparent when the activities of the other complexes are expressed as a ratio to that of complex II. The ratios indicate a decrease of complex II activity to 30–38% of control values while in contrast the ratios of complexes IV/I and IV/III are normal.

Immunoblot Analysis

Patient 1. Due to limitations in the availability of muscle tissue, immunoblot analysis was performed with the patient's fibroblast mitochondria in which the decrease of complex II activity was also observed (Table I). There was a marked decrease in the steady-state levels of the 70kDa (Fp) and 30kDa (Ip) subunits in the fibroblast mitochondrial fraction of the patient compared with the control (Fig. 1A). In contrast, there was no difference in the level of the 15kDa (QPs1) subunit between patient and control samples. To confirm similar loading, immunoblots were probed with antibodies specific to electron transfer flavoprotein dehydrogenase (ETF-dh).

Patient 2. There was no difference in the steady-state levels of the 70kDa (Fp), 30kDa (Ip) or 15kDa (QPs1) subunits in the muscle mitochondrial fraction of the patient compared with controls

TABLE 2
Activity of Respiratory Chain Enzymes in Muscle Post-600g Supernatants of Patient 2 and Controls[a]

	Patient 2	Controls (mean ± SD, n = 6)
Complex I	0.050	0.037 ± 0.013
Complex II	0.030	0.060 ± 0.014
Complex III	3.35	1.97 ± 0.86
Complex IV	4.82	3.17 ± 1.51
Complex I/II ratio	1.62	0.62
Complex III/II ratio	108.1	32.8
Complex IV/II ratio	155.4	52.8
Complex IV/I ratio	96.0	85.7
Complex IV/III ratio	1.44	1.61

[a] Results are expressed as ratios of activity (nmol NADH oxidized. min^{-1}. for complex I, nmol DCPIP reduced. min^{-1}. for complex II, and an apparent first order rate constant (k. sec^{-1}.) (multiplied by 10^4 for ease of display) for complexes III and IV) compared with the activity of citrate synthase (μmol.min^{-1}.) rather than milligrams of protein (see text for explanation).

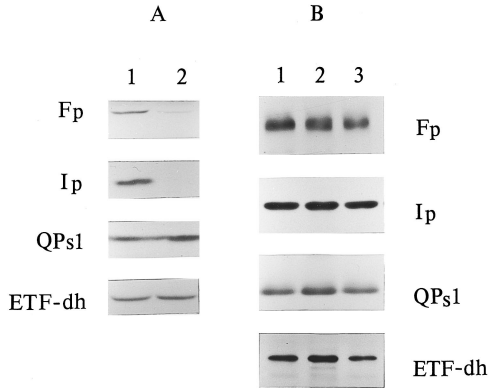

FIG. 1. *Immunoblot analysis of complex II subunits in mitochondrial fractions isolated from patient 1* **(A)** and patient 2 **(B).** (**A**) Lane 1, control (75 μg protein); Lane 2, patient 1 (75 μg protein). (**B**) Lanes 1 and 3, controls (25 μg protein); Lane 2, patient 2 (25 μg protein). Proteins were separated on a 12.5% SDS-polyacrylamide gel and transferred to a PVDF membrane. The filter was probed with antibodies raised against the Fp (70kDa), Ip (30kDa) and QPs1 (15kDa) subunits of complex II and ETF-dehydrogenase (ETF-dh). The decrease of the Fp and Ip subunits in patient 1 is clearly apparent. In contrast, comparable amounts of the complex II subunits are seen in patient 2 and the two controls.

(Fig. 1B). Similar loading of samples onto the gel was confirmed by probing with ETF-dehydrogenase subunit specific antibody.

These results, obtained from the immunoblot analysis of mitochondrial fractions from patients 1 and 2, were observed whether mitochondrial fractions were loaded on gels according to citrate synthase activity of the samples, or on the basis of mitochondrial protein concentration.

DISCUSSION

The studies presented in this paper describe the biochemical investigation of two unrelated patients who have both an isolated complex II deficiency and present with similar clinical symptoms. In both patients, spectrophotometric and polarographic measurements of succinate oxidation and the activities of the respiratory chain complexes in skeletal muscle mitochondria showed a partial decrease (40–50% of control values) of complex II activity. In contrast, the activities of the other respiratory enzymes were normal in both patients.

While defects of complexes I, III and IV, which all have some subunits encoded by mitochondrial DNA, have been extensively reported, patients with defects involving complex II seem to be less common. Of the reported cases of complex II deficiency to date there are only five in which comprehensive studies of the biochemistry of the respiratory chain and levels of immunoreactive subunits of complex II have been performed (15–19). In all these patients, the activity of complex II and SDH was very low (between 9% and 25% of control values) except the two sisters described by Taylor et al. (19) who had a partial deficiency (50% of controls) of complex II. Apart from the patient described by Rivner et al. (16), where an abnormally high activity of complex I was also observed, three (15, 17 and 18) of the other four studies demonstrate that the complex II defect was found to be associated with firstly, a decrease in the activities of the other complexes and secondly, with abnormally low amounts of immunoreactive complex II peptides. Therefore this is very different from the biochemical defect in our patients and the case described by Taylor et al. (19), in which the abnormality is restricted to complex II alone. Only one of the two cases (patient 1) with isolated complex II deficiency that we describe in the present study is associated with a marked decrease in immunoreactive complex II peptides, namely the Fp and Ip subunits. In contrast and to our surprise, the specific enzyme defect in patient 2 is clearly associated with normal amounts of immunoreactive complex II peptides. As Taylor et al. observed an overexpression of the Fp subunit in their patients, our demonstration of a specific abnormality of complex II activity

associated with normal levels of protein subunits, using subunit specific antisera, is a novel feature and has not previously been described. The immunoblot analysis by Rivner et al. (16) used holoenzyme antibody and on close examination there is clearly a partial decrease in at least the Fp and Ip subunits; furthermore the complex II deficiency in this patient is associated with also an abnormality of complex I activity.

The underlying genetic defect of patient 1 has been identified (14) as a mutation in the Fp subunit gene and this represents the first report of a nuclear mutation causing a mitochondrial respiratory chain deficiency in humans. Northern blot analysis of the patient's RNA showed a normal abundance of both the Fp and Ip transcripts (results not shown). This contrasts with the immunoblot data from patient 1 which shows a decrease in the levels of both the Fp and Ip subunits even though the mutation causes an amino acid change only in the Fp subunit. This is not surprising since mutational studies of the Fp gene in yeast have shown that a decrease in the level of the Fp protein subunit is associated with a decrease in the Ip subunit (20). The underlying defect in patient 2 has not yet been identified. Recent studies have indicated a mutation in the putative non-covalent AMP binding domain in the Fp gene from both patients described by Taylor et al. (M. Birch-Machin, unpublished results).

The clinical presentation of complex II deficiency is very variable. A myopathic presentation associated with muscle weakness and exercise intolerance has been a prominent feature (17,18,21,22) although patients have also presented with Kearns-Sayre Syndrome (KSS) and progressive ophthalmoplegia (16), encephalopathy (15), cardimyopathy (23) and Leigh's disease (24). In addition, the age at onset of clinical symptoms differs among patients and ranges from childhood to early adulthood (14–18, 21–24) and even to late adulthood (19). With this in mind, it is interesting to observe that patients 1 and 2 of the present study both shared simliar clinical symptoms, namely a loss of acquired psychomotor skills, an increase level of blood lactate and leukodystrophy. In addition, the age of onset for these clinical symptoms was the same (i.e. 10 months) in both patients.

In accordance with the variable clinical expression of complex II deficiency reported in the literature, the biochemical expression of the disease shows a marked tissue-to-tissue variation. For example, the enzyme defect in the patient described by Taylor et al. (19) was expressed in muscle and platelets but not in cultured fibroblasts or immortalised lymphocytes. This is in contrast to case 1 of the present study in which the enzyme defect was detected in all those tissues tested, namely, muscle, circulating lymphocytes and cultured fibroblasts. Although both studies report an isolated complex II deficiency of a partial nature (40–50% of controls), there is a considerable difference in the results from the immunoblot analysis of the complex II subunits between the two patients. In particular the decrease in the level of the Fp subunit in patient 1 contrasts with an overexpression of the same subunit in the patients reported by Taylor et al. The extent of the tissue expression of the decreased complex II activity in patient 2 of the present study remains to be identified but it should be interesting to discover whether there is an association with the different immunoblot results observed between patients 1 and 2.

Recent studies have indicated that there might be a duplication of the Fp subunit gene in the human genome (3q29 and 5p15) (14). The gene localized on chromosome 5 is the only one expressed in human-hamster somatic cell hybrids. Yet if both genes are expressed, their tissue-specific and/or developmentally regulated expression could be highly relevant to the clinical heterogeneity of complex II deficiencies in humans.

In conclusion we describe two patients who have both an isolated complex II defect and present with similar clinical symptoms but in contrast immunoblot analysis of the complex II subunits showed different findings between the two patients. In one of the patients, the association of a complex II abnormality with normal levels of protein subunits is a novel observation. These findings together with the heterogeneous pattern of clinical presentations, organ involvement,

immunoblot analysis and biochemical findings in other complex II deficiencies is suggestive of the genetic heterogeneity of complex II deficiencies.

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