Biochemical and molecular aspects of human mitochondrial respiratory chain disorders

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Functional mitochondrial respiratory chain and oxidative phosphorylation systems are obligatory requirements for the normal function of most eukaryotic cells. When a defect of one of these systems occurs in humans, it results in a variety of clinical symptoms according to the severity of the defect and the tissues involved. Defects of all five multisubunit complexes comprising the respiratory chain and oxidative phosphorylation systems have been reported [1–5], with some patterns emerging [6], but generally no clear picture correlating the clinical symptoms and the biochemical or molecular defects [7].

We have investigated 58 cases with various neuromuscular disorders using standard biochemical techniques [6]. Ragged red muscle fibres (> 2% of fibres), a histochemical hallmark of mitochondrial myopathy, were present in all cases studied (except one — EP, this study), and the absence of staining for cytochrome oxidase activity (> 2% of fibres) was observed in the muscle fibres of 45 cases. These cases have been grouped according to the results of polarographic analysis on freshly isolated muscle mitochondria: in 22 cases oxygen utilization rates with NADH-linked substrates were decreased, but normal with succinate (normal respiratory chain). In 21 cases oxygen utilization rates with succinate were also decreased, but normal with ascorbate plus TMPD (complex I–III defect); in 11 cases oxygen utilization rates with all substrates decreased (complex I–IV defect); in one case all rates were decreased, but the addition of uncoupler restored the rates to normal (complex V defect), and in 12 cases all rates were normal (normal respiratory chain).

Spectrophotometric analyses of rotenone-sensitive NADH–CoQ reductase, antimycin A-sensitive succinate–cytochrome-c reductase and cytochrome oxidase activities were used to confirm the deficiencies in many of the patients investigated. These data were in broad agreement with the polarographic data, the major exception being that of cytochrome oxidase activity, which was occasionally decreased when ascorbate plus TMPD oxygen utilization rates were normal.

Biochemical analyses of the mitochondria from three representative cases of complex I deficiency are shown in Table 1. YD, presented with a myopathy only, had markedly decreased oxygen utilization using NADH-linked substrates, FB, presented with Kearns Sayre syndrome (a multisystem disorder affecting muscle, heart and ocular systems), had moderately decreased oxygen utilization using NADH-linked substrates and MHO, presented with chronic progressive external ophthalmoplegia (CPEO) and myopathy, had mildly decreased oxygen utilization using NADH-linked substrates. The complex I deficiencies were confirmed by the decrease in rotenone-sensitive NADH–CoQ reductase activity. In all three cases, oxygen utilization rates using succinate and ascorbate plus TMPD and the activity of succinate–cytochrome-c reductase were normal; however, cytochrome oxidase activity was slightly decreased in FB (Table 1). Low temperature cytochrome analysis of the isolated mitochondria revealed decreased levels of cytochromes a, a1, in both YD and FB (Table 1). Normal respiratory rates using succinate and ascorbate plus TMPD in both patients suggested the deficiencies of the complex IV parameters were not having a major effect on the overall electron transport of the mitochondrial respiratory chain, in comparison to the complex I defect.

Polyclonal antibodies raised to bovine complex I (from C. I. Ragan) were used to detect complex I subunits in muscle mitochondria isolated from these patients, after the proteins had been separated on SDS/PAGE and blotted onto nitrocellulose [1]. There were normal amounts of cross-reactive subunits in MHO, a mild deficiency of all subunits in FB, and a general deficiency of all subunits, associated with a disproportionate deficiency of the 24 and 13 kDa subunits, in YD (Fig. 1). These patterns of complex I subunit cross-reactivity were characteristic of those observed in all the patients with a complex I deficiency (as defined polarographically); a normal pattern of cross-reactive subunits was observed in five cases; a general deficiency of all subunits, mild to severe loss of cross-reactivity, was observed in nine cases; and a general deficiency associated with a more severe loss of either the 24 and/or 13 kDa subunits was observed in four cases. The severity of the complex I subunit deficiencies broadly paralleled the severity of the biochemical abnormality.

There are many possible mechanisms for these subunit deficiencies including: (a) the deficiency of a specific subunit, a product of either the nucleus or mtDNA, resulting in the non-assembly of the complex and consequent degradation; (b) a problem with the transport of the subunits into the mitochondrion; and (c) a defect of transcription/translational modification of a family of proteins. The antibodies used only consistently cross-reacted with nine subunits of complex I, with a general lack of cross-reactive subunits in YD and FB.

Table 1. Polarographic, low temperature cytochrome and enzyme analysis of isolated muscle mitochondria from patients YD, FB, MHO and controls

<table>
<thead>
<tr>
<th>Enzymes &amp; Parameters</th>
<th>Patients</th>
<th>Controls</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>YD</td>
<td>FB</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pyruvate</td>
<td>11</td>
<td>24</td>
</tr>
<tr>
<td>Glutamate</td>
<td>11</td>
<td>16</td>
</tr>
<tr>
<td>Succinate</td>
<td>144</td>
<td>97</td>
</tr>
<tr>
<td>Ascorbate + TMPD</td>
<td>220</td>
<td>191</td>
</tr>
</tbody>
</table>

Polarographic data, state 3 respiration rates (nmol of O2/min per mg of mitochondrial protein): cytochrome content (nmol/mg of mitochondrial protein); enzyme activities (nmol/min per mg of mitochondrial protein); cytochrome oxidase activity, first order rate constant, k (per min per mg of mitochondrial protein). Controls are mean ± s.d. with the number of controls in parentheses.
complex I, none of which are known mtDNA-encoded subunits, making the interpretation of this data difficult.

Polyclonal antibodies raised to bovine complex III and bovine complex IV (from V. Darley-Usmar) were also used to analyse the Western blots of the patients' separated mitochondrial proteins. The mitochondria from YD and FB contained normal amounts of cytochrome c_{1}, the Rieske iron–sulphur protein and the two core subunits of complex III, but there was a deficiency of subunit VI of complex III in FB (data not shown). This was a finding in three other cases of complex I abnormality, all with a moderate to severe functional complex I abnormality, but no associated functional abnormality of complex III.

In the mitochondria from YD there was a deficiency of subunit II of complex IV (COX II) in association with a deficiency of the other cross-reactive subunits (IV, Va, Vb, Vla), but in FB the complex IV subunits were normal (data not shown). COX II is a product of the mtDNA, and its selective deficiency in YD means there is a specific deficiency of a nuclear-encoded complex I subunit (either 24 and 13 kDa subunits) and a defect of a mtDNA product in the same patient. This was also found in two other patients with similar biochemical features.

mtDNA deletions have been shown to occur in a significant number of patients with mitochondrial myopathy [6]. Both FB and MHO were found to have a deletion affecting variable proportions of their mtDNA, but YD did not have any detectable mtDNA abnormality. This, however, does not preclude a regulatory abnormality or point mutation of the mtDNA in YD causing the COX II deficiency.

A 14-year-old girl (EP) who presented with muscle weakness and exercise intolerance from early infancy was found to have several unusual features. Histochemically, her muscle showed no ragged red fibres with the modified Gomori trichrome stain, there was an absence of staining for succinate dehydrogenase and 9% of the fibres failed to stain for cytochrome oxidase. Her muscle mitochondria contained unusual irregular dense granular inclusions in the matrix, rich in iron, phosphorus and sulphur. Polarographic analysis of freshly isolated skeletal muscle mitochondria revealed decreased rates with pyruvate (37 nmol of O/min per mg), glutamate (56 nmol of O/min per mg) and succinate (31 nmol of O/min per mg), but a normal rate with ascorbate plus TMPD (176 nmol of O/min per mg), suggesting a defect of complexes I–III. In agreement with this, both rotenone-sensitive NADH-cytochrome-c reductase (68 nmol/min per mg; control 189 ± 42; n = 4) and antimycin A-sensitive succinate-cytochrome-c reductase (7.5 nmol/min per mg) were decreased. Succinate dehydrogenase activity was also markedly decreased (14 nmol/min per mg; control 109 ± 11; n = 3), in agreement with the absence of any histochemical staining of the muscle for this enzymic activity; cytochrome oxidase activity was decreased (first-order rate constant, k = 10.4/min per mg) and cytochrome aa3 level was slightly decreased (0.30 nmol/mg).

Western blot analyses using polyclonal antibodies against complex I (from C. I. Ragan), complex II (from J. G. Lindsey), complex III and complex IV (from V. Darley-Usmar) revealed: a general deficiency of all cross-reacting complex I subunits (data not shown); a severe deficiency of the 27.2 kDa subunit and a mild deficiency of the 72 kDa subunit of succinate dehydrogenase (Fig. 2a); a specific deficiency of the Rieske iron–sulphur protein in complex III (Fig. 2b) and normal complex IV cross-reacting subunits (data not shown). An antibody raised specifically to the Rieske iron–sulphur protein of complex III was found to cross-react with three subunits in control human muscle mitochondria, of apparent molecular mass 22.5, 23.3 and 26 kDa (Fig. 2c). In the mitochondria isolated from EP, the large subunit was absent and the two lower ones were markedly decreased; however, in the muscle homogenate, only the larger subunit cross-
The Rieske iron–sulphur protein \textit{Neurospora crassa} [8] has been shown to be synthesized in the cytoplasm as a precursor (27.9 kDa) and during its importation is processed first to an intermediate and finally to the mature (24.7 kDa) form. The 26 kDa subunit in the mitochondrial fraction of the human controls was also present in rat liver mitochondria and could be removed by proteinase K treatment, suggesting it is associated with the outer mitochondrial membrane. The 26 and 22.5 kDa bands were cut from an SDS/PAGE separation of control muscle homogenate and mitochondria sample, respectively, and digested with protease V8. The proteins were separated on SDS/PAGE and immunoblotted with the specific Rieske iron–sulphur antibody. Two cross-reactive bands appeared in both the mitochondrial and homogenate fractions, with the same apparent relative molecular mass, suggesting that the high molecular mass protein in the homogenate and the lower molecular mass protein in mitochondria are structurally related.

This data is consistent with the 26, 23.3, and 22.5 kDa subunits being the precursor, intermediate and mature forms of the Rieske iron–sulphur protein and suggests that, in the case of EP, the precursor is synthesized in normal amounts, but does not associate with or incorporate into the mitochondria normally. This could be due to (i) a change in the presequence of the precursor; (ii) an altered structure of the protein affecting its passage through the membranes or (iii) a defect of the transport receptor.

The 27.2 kDa subunit of complex II was specifically deficient in both the muscle mitochondria and homogenate of EP, suggesting that this subunit was deficient owing to decreased synthesis or increased degradation, rather than defective transport. Work to investigate the biosynthesis of both the Rieske iron–sulphur protein of complex III and the 27.2 kDa subunit of complex II in EP is currently in progress.

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\textbf{Mitochondrial myopathies: genetic defects}

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Introduction

The term mitochondrial myopathy (MM) is applied to a clinically, biochemically and genetically heterogeneous group of diseases which share the common feature of major mitochondrial structural abnormalities in skeletal muscle. They are often associated with defects of the respiratory chain and oxidative phosphorylation system. Their clinical and biochemical features are described in the two preceding papers and have also been reviewed elsewhere [1, 2]. The majority of cases of MM are non-familial; 18% of probands had similarly clinically affected relatives in a series of 71 patients [3]. No consistent pattern of inheritance was evident for any of the clinical syndromes or identified defects of mitochondrial metabolism, in either this study or other reports. Some pedigrees suggest autosomal recessive or dominant inheritance; no convincing X-linked pedigrees have been described.

It is clear that, when individuals are affected in more than one generation, maternal transmission to offspring is far more frequent than paternal transmission (in a ratio of approximately 9:1) [3], and large pedigrees exclusively exhibiting maternal transmission have been reported [4, 5].

Abbreviations used: MM, mitochondrial myopathy; ND, NADH dehydrogenase; CNS, central nervous system; H, heavy; L, light.

Hudson and colleagues [5] and Egger & Wilson [6] suggested that the excess of maternal transmission of MM could be explained on the basis of mitochondrial inheritance, as mtDNA is exclusively maternally inherited. Support for this hypothesis comes from the fact that the majority of patients with MM have biochemical defects localized to complex I, III, or IV of the respiratory chain, all of which contain subunits encoded by mtDNA.

Mammalian mitochondria each contain 5–10 circular DNA molecules which are double stranded and about 16.6 kb in length, contributing about 1% of total cellular DNA. Human mtDNA has been sequenced [7]; it differs from nuclear DNA to some extent in its genetic code and also because it contains very little non-coding sequence. Each strand of mtDNA is transcribed from a single promoter site and then processed. The heavy (H) strand transcripts consist of two ribosomal RNAs, 14 tRNAs, and 12 protein-coding sequences, and the light (L) strand codes for eight tRNAs and one protein coding sequence. The mitochondrial protein-coding transcripts are not capped at their 5' end, but their 3' ends are polyadenylated by mitochondrial poly(A) polymerase. Mitochondria divide at a rate appropriate to that of division of their parent cells, and in most instances mtDNA molecules also replicate with every cell cycle [7–10]. mtDNA encodes for 13 of the 67 or so subunits of the mitochondrial respiratory chain and oxidative phosphorylation system: seven subunits of complex I (NADH dehydrogenase; ND); cytochrome \textit{b} (complex III); subunits I, II, and III of cytochrome oxidase (complex IV) and subunits 6 and 8 of ATP synthetase [7, 11, 12]. The nuclear genome encodes the remaining polypeptides in the respiratory chain, and also controls their transport into mitochondria by synthesizing leader peptides which appear to direct the proteins to sites of