# Mitochondrial Myopathy with Succinate Dehydrogenase and Aconitase Deficiency

## Abnormalities of Several Iron-Sulfur Proteins

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## Abstract

**Recently, we described a patient with severe exercise intolerance and episodic myoglobinuria, associated with marked impairment of succinate oxidation and deficient activity of succinate dehydrogenase and aconitase in muscle mitochondria (1). We now report additional enzymatic and immunological characterization of mitochondria. In addition to severe deficiency of complex II, manifested by reduction of succinate dehydrogenase and succinate:coenzyme Q oxidoreductase activities to 12 and 22% of normal, respectively, complex III activity was reduced to 37% and rhodanese to 48% of normal. Furthermore, although complex I activity was not measured, immunoblot analysis of complex I showed deficiency of the 39-, 24-, 13-, and 9-kD peptides with lesser reductions of the 51- and 18-kD peptides. Immunoblots of complex III showed markedly reduced levels of the mature Rieske protein in mitochondria and elevated levels of its precursor in the cytosol, suggesting deficient uptake into mitochondria. Immunoreactive aconitase was also low. These data, together with the previous documentation of low amounts of the 30-kD iron-sulfur protein and the 13.5-kD subunit of complex II, compared to near normal levels of the 70-kD protein suggest a more generalized abnormality of the synthesis, import, processing, or assembly of a group of proteins containing iron-sulfur clusters. (*J. Clin. Invest.* 1993. 92:2660–2666.) Key words: myoglobinuria • electron transport • nicotinamide adenine dinucleotide (reduced form) dehydrogenase • ubiquinol-cytochrome *c* reductase • thiosulfate sulfurtransferase**

## Introduction

The mitochondrial myopathies and encephalomyopathies represent a diverse group of disorders frequently associated with deficiencies of complexes I–IV of the mitochondrial respiratory chain (Fig. 1, references 2 and 3). They include both nuclear and mitochondrial encoded defects, reflecting the dual genetic origin of these complex proteins. Multiple deficiencies, as well as isolated deficiencies, of complexes I–IV have been described. Recent work aimed at elucidating the molecular basis of these diseases has documented point mutations of mitochondrial DNA in some patients and deletions of mitochondrial DNA in others (4–6). However, the molecular basis of the nuclear encoded defects remain to be identified. Such defects could include mutations of specific subunits of complexes I–IV, defects of recognition and import of precursor proteins into mitochondria or processing of imported proteins to their mature forms, abnormalities of proteins required for expression and processing of mitochondrially encoded subunits, or assembly of the functional complexes containing the flavoprotein cofactors, heme groups, and Fe-S centers that catalyze the redox reactions of the electron transport chain.

We have previously described a 22-yr-old Swedish man with succinate dehydrogenase deficiency, severe exercise intolerance, and muscle fatigability and weakness (1), an autosomal recessive disease first recognized in several families from northern Sweden, with multiple siblings affected (7, 8). In a repeat muscle biopsy from this patient, we have now documented low activity of complex III in addition to complex II, markedly reduced levels of the mature Rieske Fe-S protein of complex III in mitochondria with elevated levels of its precursor in the cytosol, and decreased levels of several subunits of complex I. These data, along with our previous documentation of decreased levels of the 30-kD Fe-S protein of succinate dehydrogenase and the low activity of aconitase, another Fe-S protein, lead us to suggest that this patient has a generalized abnormality of proteins containing Fe-S centers, which may be due to a defect at one of several sites including synthesis, import, processing, or assembly. A preliminary report of these findings has been published (9).

## Methods

*Patient.* The clinical history of this 22-yr-old Swedish man, his response to graded exercise testing, and analysis of a skeletal muscle biopsy have been reported previously (1). Briefly, he had lifelong exercise intolerance with muscle fatigue, dyspnea, and cardiac palpitations, with episodes of increased muscle fatigability and weakness accompanied by painful muscle swelling and pigmenturia. Muscle histochemistry showed severe deficiency of succinate dehydrogenase activity in all fibers, with normal staining of blood vessels. Ultrastructurally, the muscle showed abundant lipid and glycogen, and many mitochondria contained curvilinear or paracrystalline inclusions as well as dark amorphous material, which, by x-ray analysis, was rich in iron.

Biochemical analysis, previously performed on mitochondria isolated from fresh skeletal muscle (1), showed normal oxygen consumption with glutamate plus malate as substrate (82% of the control mean) but markedly low oxygen consumption with succinate plus rotenone (32% of control). This was consistent with severe deficiency of succi-

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Portions of this work have appeared in abstract form (1991. *Am. J. Hum. Genet.* 48 [Suppl]:100*a*).

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*Received for publication 10 December 1992 and in revised form 6 July 1993.*

J. Clin. Invest.

0021-9738/93/12/2660/07 $2.00
Volume 92, December 1993, 2660–2666

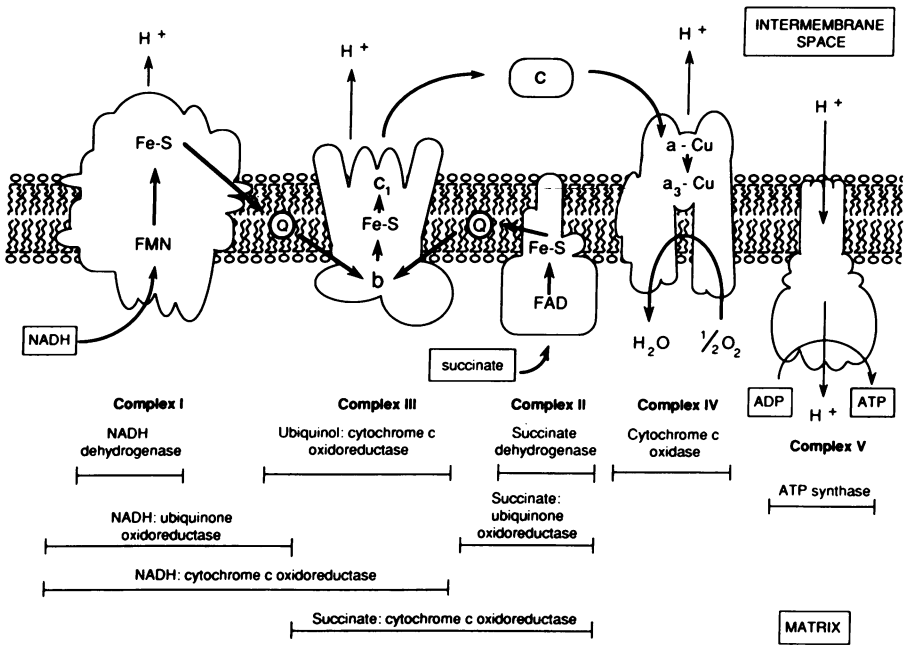


*Figure 1.* Schematic representation of the mitochondrial respiratory chain.

nate dehydrogenase and succinate:cytochrome *c* oxidoreductase (complex II plus III) activities (both 19% of normal) (Fig. 1). Mitochondrial aconitase activity (17% of normal) was also low. NADH dehydrogenase was slightly reduced (70% of normal) although NADH:cytochrome *c* oxidoreductase (complex I plus III) was normal. Cytochrome *c* oxidase (complex IV) was also normal, as were cytochromes *b*, $c + c_1$, and $aa_3$. The other citric acid cycle enzyme activities including citrate synthase, NADH:isocitrate dehydrogenase, fumarase, and malate dehydrogenase, on the other hand, were all approximately two to four times normal.

A repeat muscle biopsy was obtained at 24 yr of age and frozen immediately for the studies reported in this manuscript.

*Materials.* Ubiquinone-1 ($UQ_1$)[1] and *n*-decyl coenzyme Q were gifts from Eisai Co., Ltd. (Tokyo, Japan), and Dr. D. Wallace (Emory University, Atlanta, GA), respectively. Antibodies to purified beef heart electron transport chain complexes and related proteins were provided by the following people: to holo complex III and to the Rieske protein of complex III by Dr. Victor Darley-Usmar (Wellcome Research Laboratories, Beckenham, United Kingdom); to subunits VI and XI of complex III by Dr. R. Capaldi (Institute of Molecular Biology, University of Oregon, Eugene, OR); to pig electron transfer flavoprotein:ubiquinone oxidoreductase (ETF:QO) by Dr. Frank Frerman (Department of Pediatrics, University of Colorado Health Sciences Center, Denver, CO); and to beef heart aconitase (aconitate hydratase, EC 4.2.1.3) by Dr. Paul Srere (Dallas Veterans Administration Medical Center, Dallas, TX). Preparation of antisera to beef heart complex I (NADH:ubiquinone oxidoreductase, EC 1.6.5.3) has been described (10).

*Methods.* Control skeletal muscle was obtained with permission from four patients undergoing orthopedic surgery, three individuals who died of trauma, and two patients, one with myoglobinuria and the other with an undiagnosed myopathy, neither of whom had any evidence of mitochondrial dysfunction. Their ages ranged from 11 to 47 yr. The patient sample had been stored at −70°C for 13–18 mo and the control samples at −70°C for 1–24 mo before these studies.

Mitochondria were isolated from frozen muscle by the method of Moreadith et al. (11). Whole homogenate and high speed supernatant were prepared similarly, except BSA was omitted from the final wash and the cytosol fraction was recentrifuged at 12,000 *g* to remove membrane fragments before immunoblotting. Unless otherwise stated all activities were measured at 30°C. Succinate dehydrogenase (succinate:flavin adenine dinucleotide oxidoreductase, EC 1.3.99.1) and complex II (succinate:ubiquinone oxidoreductase, EC 1.3.5.1) were determined by modification of previous procedures (12). Both activities were measured in 50 mM potassium phosphate, pH 7.4, 2 mM KCN, 2 μg/ml rotenone, 2 μg/ml antimycin, and 50 μM dichlorophenol indophenol, after preincubation in 20 mM succinate and buffer containing 0.5% Tween 80 for 20 min. Phenazine ethosulfate (3 mM) and $UQ_1$ (75 μM) were included in the assay of succinate dehydrogenase and complex II, respectively, and the decrease in absorbance recorded at 600 nm. Complex III (ubiquinol:ferricytochrome *c* oxidoreductase, EC 1.10.2.2) was assayed according to Birch-Machin et al. (13) except *n*-decyl coenzyme Q (2,3-dimethoxy-5-methyl-6-*n*-decyl 1,4-benzoquinone; DB) was used as substrate in place of $UQ_1$. Complex IV (ferrocytochrome-*c*:oxygen oxidoreductase, EC 1.9.3.1) was determined according to Wharton and Tzagoloff (14), using 50 mM potassium phosphate, pH 7.0, containing 0.011% laurylmaltoside and 20 μM ferrocytochrome *c*. Citrate synthase (EC 4.1.3.7) was measured by the method of Shepherd and Garland (15) after solubilization of mitochondria in Triton X-100. Rhodanese (thiosulfate:cyanide sulfurtransferase EC 2.8.1.1) was measured by adaptation of the method of Sörbo (16). Samples were incubated in 50 mM $Na_2S_2O_3$, 40 mM $KH_2PO_4$, 50 mM KCN, and 4 mM sodium cholate for 30 min at 20°C in a final vol of 50 μl. The reaction was stopped with 10 μl of 38% formaldehyde and the thiocyanate was determined as described. Measurements were corrected for nonspecific reaction by blanks in which formaldehyde was added before sample. The reaction was linear with time and with sample up to at least 40 μg of protein. Protein was measured by the method of Lowry et al. (17).

For immunoblot analyses, mitochondria were dissociated in 10% glycerol, 3% SDS, 2% β-mercaptoethanol, 0.01% bromophenol blue, 50 mM Tris, pH 6.5 at 100°C for 1 min and were separated by 8, 15, or 10–18% gradient SDS-PAGE, using modifications of the Laemmli method (18). The separated proteins were transferred to nitrocellulose as described by Rosenbaum et al. (19), with the addition of 0.1% SDS to the transfer buffer. Blots were exposed to antibodies for 1–3 h and bound antibodies were visualized using biotinylated protein A followed by avidin-linked alkaline phosphatase reaction (Vectastain ABC Kit; Vector Labs., Inc., Burlingame, CA), and NBT/BCIP (Bio-Rad, Richmond, CA) color development. Each figure represents a single blot.

1. *Abbreviations used in this paper:* ETF:QO, electron transfer flavoprotein:ubiquinone oxidoreductase; $UQ_1$, ubiquinone-1.

## Results

Measurement of respiratory chain activities in mitochondria isolated from frozen muscle (Table I) confirmed the severe deficiency of succinate dehydrogenase and elevated citrate synthase previously demonstrated in freshly isolated mitochondria (1). Complex II activity was reduced to 22% of normal, consistent with the succinate dehydrogenase deficiency. Moreover, measurement of complex III showed that this activity was also low, only 37% of the mean and below the range of controls. These data demonstrate that measurement of the activities of individual complexes, as illustrated here with complex III, may reveal subtle deficiencies that are not detectable by measurement of oxygen consumption or by determination of coupled enzymes such as NADH:cytochrome *c* oxidoreductase (complex I plus III), which was previously shown to be normal in this patient. This suggests that complex III exerts a low degree of control on these activities. Rhodanese activity was also below the control range (48% of the mean), although it was within the 95% confidence limits of the controls. Mixing experiments of control and patient mitochondria gave the expected results, indicating that the reduced activity was not due to the presence of an inhibitor in the patient's sample.

To investigate further the nature of the abnormality in this patient, we carried out immunoblot analyses of the respiratory chain complexes, as well as mitochondrial aconitase and ETF:QO. In previous studies, antibody binding to complex II showed a slight decrease of the 70-kD polypeptide and marked deficiency of the 30- and 13.5-kD polypeptides, especially when compared to the levels of core protein 1 of complex III, a contaminant in the antibody raised against complex II, and in comparison to subunit IV of complex IV (1). Examination of crude homogenate (containing mitochondria) and supernatant (cytosolic fraction) provided no evidence for accumulation of precursors of any of these subunits in the cytosol (data not shown). When complex III was examined in muscle mitochondria (Fig. 2), two major bands that cross-reacted with antibody to the Rieske protein could be identified. These presumably correspond to the precursor (p) and mature (m) forms of this protein (21). Although the precursor form was clearly elevated in the patient, the mature form was markedly reduced. In the controls, the precursor appeared as a doublet, possibly representing two closely related forms, differing perhaps in their oxidation state. An intermediate form of the Rieske protein, tentatively identified in human tissues (21), has not been documented with certainty in mammals. In fact, the precursor lacks the consensus sequence for two-step cleavage (22), suggesting that it may indeed be cleaved to the mature form in a single step. Subunit XI was also very low. Other components of complex III, the core proteins and peptide VI, also appeared to be somewhat reduced whereas cytochrome $c_1$ was present in normal amounts. This contrasts with our findings in another patient with complex III deficiency in which core proteins 1 and 2 were also markedly deficient (20). The individual bands were identified by antibodies raised to the purified subunits (23). The identities of the unlabeled bands in Fig. 2, one of which appears reduced in the patient, have not been determined. When antibody to the Rieske protein was used to probe blots of homogenate and supernatant (Fig. 3), the mature form was markedly reduced in the homogenate from the patient, reflecting the deficiency in mitochondria, whereas the precursor was present in increased amounts in both homogenate and supernatant from the patient.

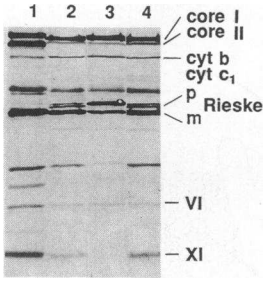


*Figure 2.* Immunoblot of complex III after separation on SDS 10–18% PAGE. Lane *1*, bovine heart mitochondria (40 μg of protein); lanes *2* and *4*, muscle mitochondria from two controls (100 μg of protein); lane *3*, muscle mitochondria from patient (100 μg of protein). The blot was incubated first with an antibody to holo complex III and subsequently with an antibody to the Rieske protein (20) and finally with an antibody to subunit XI. Bands corresponding to subunits of complex III are identified on the right. p and m represent the precursor and mature forms of the Rieske protein.

Immunoblotting of complex I showed deficiency of many bands in the patient's mitochondria. These were tentatively identified according to their relative migration and the reactivity of the complex I antiserum to components of purified iron-protein and flavoprotein fractions of complex I. Deficient bands include those of 39, 24, 13, and 9 kD; the 51- and 18-kD peptides appeared slightly reduced, whereas the 75-kD peptide was present in normal amounts (Fig. 4). In view of the normal oxygen consumption with glutamate plus malate and normal activity of complex I plus III previously reported (1), these results again raise the question of the ability of coupled assays to detect subtle abnormalities in the respiratory chain. Unfortunately, we had insufficient material to measure complex I (NADH:ubiquinone oxidoreductase) activity. The complex I subunit deficiencies in our patient contrast with the normal profile by immunoblot analysis in another patient with mild complex I deficiency associated with the mutation at nucleotide 3243 in the mitochondrially encoded $tRNA^{Leu(UUR)}$ gene

*Table I. Respiratory Chain Activities in Muscle Mitochondria*

| | | Controls ($n$ = 5) | |
|---|---|---|---|
| | Patient | Mean±SD | Range |
| Succinate dehydrogenase* | 34 | 287±60 | 202–343 |
| Complex II* | 83 | 369±88 | 241–464 |
| Complex III‡ | 10 | 27±10 | 16–42 |
| Complex IV‡ | 43 | 78±39 | 36–136 |
| Rhodanese* | 24 | 49±15 | 38–76 |
| Citrate synthase* | 948 | 670±130 | 500–840 |

* Expressed as nanomoles per minute per milligram protein. ‡ Expressed as apparent first order rate constant per minute per milligram protein.

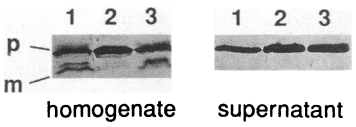


*Figure 3.* Localization of the precursor (p) and mature (m) forms of the Rieske protein in muscle homogenate (containing mitochondria) and supernatant (cytosolic fraction). Proteins were separated on SDS 10–18% PAGE and incubated with antibody to the Rieske protein. Lanes *1* and *3*, control (100 μg protein); lane *2* patient (100 μg protein).

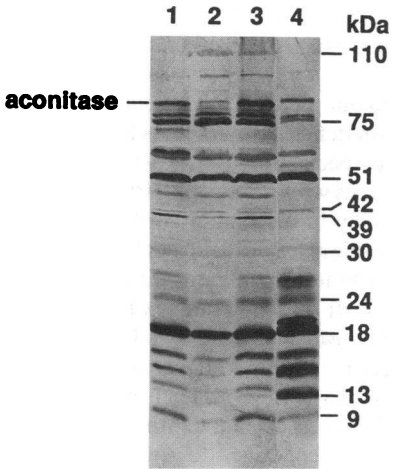


*Figure 4.* Immunoblot of complex I and aconitase after separation on SDS 10–18% PAGE. Lanes *1* and *3*, muscle mitochondria from two controls (100 μg protein); lane *2*, muscle mitochondria from patient (100 μg protein); lane *4*, bovine heart mitochondria (40 μg protein). The blot was incubated first with antisera to bovine heart complex I (10), and subsequently with antiserum to bovine heart aconitase (24).

(25) characteristic of the MELAS (mitochondrial) encephalomyopathy, lactic acidisis, and strokelike episodes syndrome. Reexposure of the complex I blot to antibody raised against mitochondrial aconitase showed that this protein was also deficient in the patient's sample, consistent with the decreased aconitase activity previously demonstrated (1). In other experiments (not shown) a band of ~ 59 kD was detected with this antibody, and was increased in the patient's mitochondria, compared to controls, raising the possibility of an increased susceptibility of the patient's mitochondrial aconitase to degradation. When homogenate and supernatant fractions were examined, there was no evidence for accumulation of a precursor of aconitase in either the patient or controls.

Finally, using an antibody to ETF:QO, another mitochondrial Fe-S protein (26), we found apparently normal amounts of cross-reacting material in the patient's mitochondria (data not shown).

## Discussion

Previous studies of this patient revealed the following abnormalities: (*a*) low oxygen uptake attributable to low systemic arteriovenous $O_2$ difference in peak cycle exercise, consistent with impaired muscle oxidative phosphorylation; (*b*) exaggerated exercise increases in venous lactate and pyruvate relative to oxygen uptake but low lactate/pyruvate ratios consistent with limited carbon flux via the tricarboxylic acid cycle relative to the capacity of the respiratory chain to oxidase NADH; (*c*) low $O_2$ consumption of isolated mitochondria with succinate compared to glutamate as a substrate; and (*d*) severe deficiency of skeletal muscle succinate dehydrogenase and aconitase with mildly deficient NADH dehydrogenase and activity of complex I plus III that was within the normal range (1). Our present results are also consistent with a rate-limiting oxidative defect at the level of complex II and the citric acid cycle in skeletal muscle, but reveal additional respiratory chain abnormalities affecting complexes I and III that suggest a more generalized defect of mitochondrial iron-sulfur proteins. Activity of rhodanese, a mitochondrial enzyme that has been shown to play a role in the formation of the Fe-S clusters of complexes I (27) and II (28) was also below the control range.

The most severe deficiency in our patient involved complex II, the smallest of the respiratory chain complexes, composed of four nuclear encoded polypeptides (29, 30). The 70-kD subunit containing covalently bound flavin adenine dinucleotide and the 30-kD Fe-S protein comprise succinate dehydrogenase. The two smaller peptides, at 15.5 and 13.5 kD, bind coenzyme Q and anchor the enzyme to the mitochondrial inner membrane (31). Severe deficiency of succinate dehydrogenase or succinate:ubiquinone oxidoreductase (complex II), with milder abnormalities of other mitochondrial activities, has been described in only three other patients, two of whom appear to have disorders that differ from our patient. The first had Kearns-Sayre syndrome, with low activity of complex II but normal cross-reacting material, and elevated complex I activity (32). The second had an encephalopathy in addition to progressive muscle weakness (33). Furthermore, in that patient, citrate synthase activity was not elevated in muscle mitochondria and immunoblot analysis of complex II revealed low levels of the 70-kD flavoprotein, in addition to the 30- and 13.5-kD subunits that were reduced in our patient. On the other hand, Linderholm et al. (8) reported a 21-yr-old female of Swedish descent and Schapira et al. (21) reported a 14-yr-old girl of English and Chinese ancestry, both of whom had lifelong weakness, exercise intolerance, and lactic acidosis, and shared many similarities with our patient: the severe myopathy with no evidence of other tissue involvement, similar muscle morphology, including iron-rich granular inclusions within the mitochondria, and very low activities of succinate dehydrogenase. The last patient also had a mild reduction of complex I activity and normal cytochrome levels (21). Furthermore, immunoblot analyses showed severe deficiency of the 30-kD Fe-S subunit (reported by these authors as 27 kD), moderate reduction of the 70-kD flavoprotein of succinate dehydrogenase, and a mild generalized deficiency of complex I subunits. Additionally, the presence of the precursor of the Rieske protein of complex III in muscle homogenate and cytosol, and virtual absence of all forms of this protein in mitochondria led these authors to suggest a defect in the transport of the Rieske protein into mitochondria in addition to the succinate dehydrogenase deficiency.

The data presented here, involving complexes I, II, and III as well as mitochondrial aconitase, suggest a more generalized abnormality of import, processing, or assembly of a group of mitochondrial proteins in our patient. The import of proteins into mitochondria usually involves the recognition of presequences by receptor proteins on the outer membrane, insertion through the inner membrane at translocation contact sites, and cleavage of the presequences by a matrix peptidase (34–37). Addition of prosthetic groups, or cleavage by a second processing peptidase may also be required and many other factors such as cytosolic and mitochondrial heat shock proteins are also essential (38).

Our documentation of low levels of the mature form of the Rieske protein in mitochondria and elevated levels of the precursor form in cytosol are similar to the case reported by Schapira et al. (21), consistent with a mitochondrial import defect. The presence of the precursor form in the mitochondrial fraction in our case could represent protein bound to a receptor on the outer membrane, protein arrested during translocation, or imported protein that had not been further processed. However, a generalized defect of mitochondrial import is excluded on the basis of normal or elevated activities of many imported proteins, such as subunit IV of complex IV and most of the citric acid cycle enzymes. Deficiency of a factor required for

import of all of the affected proteins is also unlikely since we could find no evidence for accumulation of precursors of the 30-kD Fe-S protein of succinate dehydrogenase (39) or of mitochondrial aconitase (40), assuming that the antibodies that cross-react with the mature proteins would also detect the precursor forms. Additionally, an abnormality of the matrix processing peptidase (41) is also unlikely since many other cleaved proteins are unaffected (22).

Several factors point to a primary defect of iron, or Fe-S proteins, in our patient. First, the dense granular inclusions within mitochondria were shown to be rich in iron. Second, mitochondrial aconitase and succinate dehydrogenase are the only citric acid cycle enzymes that were low in activity and are the only ones that contain Fe-S clusters. Third, complexes I and III, which also contain Fe-S proteins, were also affected, whereas complex IV, which does not, was unaffected.

Immunoblot analyses also provide some support for the importance of Fe-S proteins in this disease, for example the severe deficiency of the 30-kD Fe-S protein, but not the 70-kD flavoprotein of succinate dehydrogenase. Complex II may be most severely affected since the Fe-S center of the 30-kD subunit is required to anchor the complex to the inner membrane (42). Disruption of this gene in yeast results in loss of measurable activity and diminished amount of the 70-kD protein, suggesting a lack of stability or inability to assemble properly (43). Deficiency of the 13.5-kD subunit of complex II in our patient may also be secondary to the Fe-S protein deficiency.

The immunoblot of complex I is harder to interpret because of the complexity of this protein, with 39 different subunits, 7 of which are encoded in the mitochondrial genome (44, 45). It also contains approximately eight Fe-S clusters associated with the 75-, 51-, 24-, and 23-kD proteins and possibly two additional subunits (45–47). At least two of these appeared reduced in the patient, namely the 51- and 24-kD proteins. However, one other, the 75-kD subunit, was not reduced and yet other proteins, which appeared low, such as the 18- and 9-kD subunits, are not Fe-S proteins. Deficiency of these last two could reflect interaction with Fe-S proteins. However, it appears that not all Fe-S proteins are uniformly affected.

For complex III, in addition to the abnormalities of the Rieske Fe-S protein discussed above, there was also a marked deficiency of subunit XI. This could be secondary to deficiency of the Rieske protein since bovine heart complex III made deficient in the Rieske protein was also deficient in subunit XI, confirming the requirement of the former for proper assembly of the enzyme (48). Other components of complex III, such as core proteins 1 and 2, cytochrome *b*, and cytochrome $c_1$ were normal or close to normal, emphasizing the selective involvement of Fe-S proteins in this disease.

The import and processing of the Rieske protein in mammals contrasts with that in both *Neurospora* (49) and yeast (50) where cleavage by matrix processing peptidases occurs in two steps before assembly into complex III (34). In yeast mutants that cannot bind the Fe-S cluster, processing to the mature Rieske protein and assembly into complex III still occurs, suggesting that insertion of the Fe-S cluster might normally take place after the second processing step, or even after assembly into the complex (51). The mammalian enzyme, however, lacks the consensus sequence for two-step cleavage (22) and, furthermore, the amino acid sequence of the entire leader peptide corresponds exactly to the complete sequence of subunit IX of complex III (52, 53), suggesting that cleavage of the precursor protein takes place in a single step, perhaps after assembly into the complex. Defective cleavage of the Rieske protein, however, would not explain the other abnormalities seen in this patient.

Several of the findings in our patient are consistent with the profile of mitochondrial iron deficiency. Iron is believed to be taken into mitochondria via specific receptors (54). It is possible that the iron-rich inclusions within mitochondria represent sequestration of iron, possibly in association with abnormal proteins, resulting in a functional iron deficiency. Iron-deficient rats show decreased respiratory function with a more profound effect on complexes I–III than on complex IV, and decreased content of the Fe-S clusters of complexes I, II, and III (55–57). Increased production of lactate and increased activity of noniron citric acid cycle enzymes in skeletal muscle have also been reported (58). However, in contrast to our patient, muscle from iron-deficient rats contained lower levels of cytochrome *b*, $c + c_1$, and $a + a_3$ (59). Additional data in rats suggested a decreased content of fully competent complexes I and II, rather than the presence of impaired enzymes, as suggested by our immunoblotting and spectral data (56). The Fe-S cluster of ETF:QO was also low in iron deficiency (55) whereas, at least by immunoblot, the level of this protein was not reduced in the patient; its activity, however, was not measured.

Although several of the findings in this patient suggest a functional iron deficiency in muscle mitochondria, other features, including lactic acidosis, are reminiscent of acute iron toxicity (60, 61). In hepatocytes of rats given toxic doses of ferrous sulfate, the primary target of intracellular injury appears to be the mitochondria that show electron-dense deposits, presumed to be iron, in the intermembrane space and matrix; they also manifest decreased respiratory control (62). It was proposed that excess ferric ions may shunt electrons away from the respiratory chain via the inner membrane ferric reductase which normally provides ferrous ions for the synthesis of heme (60, 63). Reoxidization of ferrous to ferric ions by molecular oxygen would decrease respiratory chain activity and could precipitate a cellular energy crisis (64). This mechanism could be invoked to explain the recurrent metabolic crises and acute rhabdomyolysis in our patient which would result in temporary reduction of the intracellular iron load.

Involvement of mitochondrial aconitase, in addition to the respiratory chain abnormalities in our patient, first focused our attention on the importance of nonheme Fe-S proteins in this disease (65). Affected proteins contain several different types of Fe-S clusters, for example the [2Fe-2S] Rieske protein (66), the [2Fe-2S], [3Fe-4S], and [4Fe-4S] clusters of the 30-kD iron protein of succinate dehydrogenase (67) and the [2Fe-2S] and [4Fe-4S] clusters of complex I (47, 68). Mitochondrial aconitase is unique in that the inactive enzyme, which contains a [3Fe-4S] cluster, is converted to the active [4Fe-4S] form on addition of an extra iron (69). The decreased level of mature aconitase in our patient, with increased amount of a lower molecular weight protein that cross-reacts with the antibody, may reflect increased degradation of an unstable enzyme, possibly from failure to form the active Fe-S center. It has been proposed that the dynamic Fe-S center of aconitase may respond to changes in mitochondrial iron availability, thus providing an additional level of control of cellular intermediary metabolism. This would be analogous to the structurally related iron regulatory element binding protein in the cytosol,

recently identified as cytosolic aconitase (70), which modulates the levels of ferritin and the transferrin receptor (71, 72). Failure to form the active center of aconitase could also reflect sequestration or inaccessibility of iron.

An alternative explanation for deficiency of a group of proteins, all of which contain Fe-S clusters, would be an abnormality of a protein or factor required for their formation. There is good evidence that rhodanese plays an important role in the formation of the [2Fe-2S] and [4Fe-4S] clusters of mitochondrial proteins (73, 74) including succinate dehydrogenase (28) and complex I (27). However, it seems unlikely that the reduction of rhodanase activity was sufficient to cause the severe deficiency of succinate dehydrogenase in our patient. Further studies are clearly required to elucidate the etiology of the diverse abnormalities of Fe-S proteins in this disease.

## Acknowledgments

We thank Drs. Neil Buist, Caroline Enns, and Brian Ackrell for their helpful discussions, Beth Lee and Philippe Thuillier for excellent technical assistance, and Linda Nay for diligently typing this manuscript. We are also grateful to the staff of the Pacific Northwest Transplant Bank for their help in obtaining tissue samples. Dr. Youssef Hatefi kindly provided purified iron-protein and flavoprotein fractions of complex I and helped characterize our antiserum to complex I.

This work was supported by grants from the Muscular Dystrophy Association (MDA) (N. G. Kennaway, R. G. Haller) and by the Department of Veterans Affairs (R. G. Haller). R. E. Hall is the recipient of a post-doctoral fellowship from the Muscular Dystrophy Association. S. F. Lewis was the recipient of a Research Career Development Award (HL-01581) and National Heart, Lung and Blood Institute grant (HL-06296) from the National Institutes of Health.

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