Impaired Mitochondrial β-Oxidation in a Patient with an Abnormality of the Respiratory Chain

Studies in Skeletal Muscle Mitochondria

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Abstract

Defects of complex I of the mitochondrial respiratory chain are important causes of neurological disease. We report studies that demonstrate a severe deficiency of complex I activity with less severe abnormalities of complexes III and IV (< 5, 63, and 30% of control values, respectively) in a skeletal muscle mitochondrial fraction from a 22-yr-old female with weakness, lactic acidemia, and the deposition of intramuscular neutral lipid. The observation that lipid accumulates in this and other patients with complex I deficiency suggests impaired mitochondrial fatty acid oxidation. To investigate this mechanism we have shown impaired flux through β-oxidation ([U-^{14}C]hexadecanoate oxidation was 66% of control rate) and accumulation of specific acyl-CoA ester intermediates. The changes in fatty acid metabolism in complex I deficiency are secondary to the reduced state within the mitochondrial matrix with low NAD^+/NADH ratios. (*J. Clin. Invest.* 1990. 85:177–184.) β-oxidation • complex I • mitochondria

Introduction

Inborn errors of one or more of the complexes of the respiratory chain are increasingly being recognized as important causes of disease. Patients with these defects present in a variety of ways including fatal lactic acidosis in infancy, muscle disease, and encephalopathy (1). The electron transport or respiratory chain consists of four complexes: complex I (NADH/ubiquinone oxidoreductase), complex II (succinate/ubiquinone oxidoreductase), complex III (ubiquinol/cytochrome *c* oxidoreductase), and complex IV (cytochrome *c* oxidase). Complex I consists of at least 25 subunits and contains 1 mol of flavin and several iron–sulphur centers (2). Seven subunits are coded for by the mitochondrial genome (3, 4) and those subunits that are nuclearly coded have to be translocated into the mitochondrial matrix for correct assembly of the complex (5).

Disorders of complex I have been described in about 40 patients (6). Some patients presented with a multisystem disease that was fatal in infancy (7, 8, 9), some with predominantly muscular symptoms, with or without chronic progressive external ophthalmoplegia (10), whereas in a third group, the disease appears mainly to involve the central nervous system (11, 12).

Investigation of complex I deficiency has concentrated on identifying the defective subunit or subunits by electron paramagnetic resonance spectroscopy (7), Western blotting (13, 14, 15), and analysis of the mitochondrial genome (16). There have, however, been no investigations of the secondary effects of complex I deficiency on other metabolic pathways. A defect of complex I would impair oxidation of NADH, whether formed during pyruvate oxidation, the NAD^+-linked reactions of the citrate cycle, or mitochondrial β-oxidation. We were particularly interested in the effect on β-oxidation since we have observed lipid accumulation in skeletal muscle from several patients with complex I deficiency.

Mitochondrial β-oxidation of saturated acyl-CoA esters proceeds by a repeated cycle of four concerted reactions: flavoprotein-linked dehydrogenation, hydration, NAD^+-linked dehydrogenation, and thiolysis. The three chain-length–specific acyl-CoA dehydrogenases catalyze the first dehydrogenation step and are linked to complex III of the respiratory chain by electron transfer flavoprotein (ETF)[1] and ETF/ubiquinone oxidoreductase (17). The second dehydrogenation step is catalyzed by two chain-length–specific NAD^+-dependent 3-hydroxyacyl-CoA dehydrogenases (18) that transfer electrons to complex I. The control of β-oxidation in the mitochondrial matrix occurs at several sites and is partly dependent on the redox state (19). The rate of oxidation is slowed in reduced states since low NAD^+/NADH ratios impair the activity of the hydroxyacyl-CoA dehydrogenases (19, 20) and increase the formation of ETF semiquinone, a potent inhibitor of the acyl-CoA dehydrogenases (21). These changes affect the steady-state concentrations of acyl-CoA intermediates which, in turn, may change the control strength of other enzymes of the pathway (22). It is clear, therefore, that a defect of complex I could lead to secondary inhibition of mitochondrial β-oxidation.

Methods

Case history

The patient (L.H.), a 22-yr-old woman, presented aged 18 with a short history of limb weakness, exertional muscle pain, and a progressive tendency to walk on her toes. Her previous medical history was unremarkable apart from repeated minor respiratory tract infections. There

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1. *Abbreviation used in this paper:* ETF, electron transfer flavoprotein.

was a family history of muscle disease: Her mother had died aged 35 from cardiorespiratory failure and investigation had shown accumulation of neutral lipid and subsarcolemmal aggregation of mitochondria in her skeletal muscle. A sister, aged 15, and brother, aged 10, had also died of cardiorespiratory failure secondary to undiagnosed muscle disease. Both were studied elsewhere and neither was investigated for mitochondrial dysfunction.

L.H. had mild bilateral facial weakness, proximal weakness of both upper and lower limbs, and marked bilateral tendo-achilles contractures, which caused her toe-walking. There was a mild persistent metabolic acidosis (serum bicarbonate 19 mM), a high fasting serum lactate concentration (2.83 mM; normal range < 1.7 mM), and a high serum creatine kinase activity ranging from 178–574 U/liter (normal range < 140 U/liter). The plasma concentration of free carnitine was low (21 μM; normal range for 30 control subjects 28.7–45.8 μM) and the concentration of short-chain acylated carnitine was high (19.1 μM; normal range for 30 control subjects 2.3–9.3 μM). Changes compatible with a myopathy were detected by electromyography.

She has been treated with riboflavin (25 mg three times a day), then flavin mononucleotide (25 mg three times a day), and then ubiquinone$_{10}$ (50 mg three times a day) with no symptomatic or objective improvement. Over the last four years there has been gradual deterioration with increasing weakness, especially of respiratory muscles, which have led to several admissions with serious chest infections.

Experimental

Preparation of mitochondrial fractions. Muscle was obtained by open biopsy (vastus lateralis) under local anesthesia. A portion was quickly frozen in dichlorodifluoromethane (Arcton 12, Imperial Chemical Industries) cooled to −150°C in liquid nitrogen and this was used for histochemistry (23). The remainder was used to prepare a mitochondrial fraction (24). Control fractions were prepared from muscle obtained by biopsy from patients in whom no neuromuscular disease was found.

Measurement of succinate, pyruvate, and oxoglutarate oxidation by mitochondria. Substrate oxidations by mitochondrial fractions (0.2–0.4 mg · ml^{-1} final protein concentration) were recorded spectrophotometrically using a dual-wavelength spectrophotometer (model 557; Hitachi Ltd., Tokyo), by following the reduction of ferricyanide at 420 nm with 475 nm as a reference wavelength (25).

Spectrophotometric assay of individual respiratory chain complexes. The activities of complexes I–IV were determined as previously described (26) except that 15 μM cytochrome *c*(II) was used to determine the activity of complex IV.

Determination of cytochrome concentrations. Low temperature reduced-minus oxidized spectra of mitochondrial fractions were recorded after reduction with dithionite (27). The wavelengths, extinction coefficients, and equations quoted by Tervoort et al. (28) and the intensification factors quoted by Wilson (29) were used to calculate the cytochrome concentrations.

Immunoblot analysis of peptide subunits of complex I. Mitochondrial proteins were separated by SDS-polyacrylamide gel electrophoresis (30) using a 5% stacking gel and a 15% separating gel. All samples contained 3 mM *p*-aminobenzamidine to minimize proteolysis. Proteins were transferred to nitrocellulose (0.45 μm pore size) (31) with the addition of 0.1% SDS to the transfer buffer. Antisera to holo-complex I was raised in rabbits against purified beef-heart complex I. Immunoreactive peptides were detected by the immunoperoxidase method with 4-chloro-1-naphthol as substrate (32).

Restriction mapping of mtDNA. DNA was extracted from the pellet obtained after the first low speed centrifugation steps of the mitochondrial preparation (33). The DNA was digested with Ava II, Pvu II, Pst I, Hind III, and Eco RI. To exclude small deletions (or duplications), short sections of the mitochondrial genome were amplified by the polymerase chain reaction (34) using heat stable DNA polymerase and conditions previously described (35). 23 oligonucleotide primers, each 20 bases long, were used to amplify DNA segments between position 6005 and 345 relative to the reference sequence. The DNA digests and the amplified DNA were electrophoresed on 0.9% agarose gels, transferred to nylon membranes by Southern blotting (36), and hybridized with a hexonucleotide-labeled purified mitochondrial DNA (37).

Measurement of flux and acyl-CoA intermediates of β-oxidation. Incubations were made at 30°C in a final volume of 1.0 ml containing 110 mM KCl, 10 mM Hepes, 5 mM $MgCl_2$, 2.5 mM potassium phosphate, 1 mM EGTA, 0.2 mg cytochrome *c*, 5 mM ATP, 1 mM carnitine, 100 μM CoA, pH 7.4, and 1 mg mitochondrial protein (24). Mitochondrial fractions were preincubated for 5 min and the reaction started with 36 nmol [U-^{14}C]hexadecanoate (complexed in a molar ratio of 5:1 with fatty acid–free BSA). The samples were quenched with 200 μl glacial acetic acid and 30 nmol of heptadecanoyl-CoA added as internal standard. A 50 μl sample was withdrawn and mixed with 150 μl 5 M-$HClO_4$, centrifuged (9,000 g_{av} for 10 min) to remove precipitated protein and unchanged substrate. Then 150 μl of the supernatant was added to 8 ml of scintillation cocktail and counted to determine total acid-soluble metabolites. The rest of the sample was used to prepare an acyl-CoA fraction that was free of acyl-carnitines and this was analyzed by reverse phase HPLC with on-line photodiode array and radiochemical detection (38).

Synthesis of 3-oxohexadecanoyl-CoA. Hexadec-2-ynoic acid synthesized by the method of Wood and Lee (39) was used to prepare the corresponding CoA ester from its mixed anhydride (40). This was converted to 3-oxohexadecanoyl-CoA with crotonase (41) and purified by HPLC.

Measurement of 3-hydoxyacyl-CoA dehydrogenase activity. The activities of the 3-hydroxyacyl-CoA dehydrogenases were determined in muscle mitochondria that had been freeze-thawed three times in hypotonic buffer followed by addition of 2 mg Triton X-100/mg mitochondrial protein. The activities were measured in assay medium containing 100 mM potassium phosphate, 0.1 mg^{-1} · ml^{-1} BSA, 0.1 mM NADH, 2.5–5 μg mitochondrial protein, pH 7.2 and 30°C. The reaction was started by adding 40 μM 3-oxohexadecanoyl-CoA or acetoacetyl-CoA.

Results

Cytochemistry. There was widespread atrophy and vacuolation. The vacuoles contained neutral lipid (Fig. 1 *A*). Subsarcolemmal aggregation of mitochondria compatible with a mitochondrial myopathy were seen in sections stained for succinate dehydrogenase activity (Fig. 1 *B*).

Biochemistry. The rates of oxidation of NAD^+-linked substrates by mitochondrial fractions were slow, but the rate of succinate oxidation was normal (Table I). The activity of complex I activity was < 5% of control values (Table II). In addition, there were low activities of complex III (63% of control values) and of complex IV (30% of control values). The concentration of cytochrome aa_3 and cytochrome *b* were low when compared with the concentration of cytochrome *c* (Fig. 2, Table III).

Immunoblot analysis of mitochondrial proteins from the patient and controls, using monospecific antisera against holo-complex I, showed that apart from the 51-kD subunit, all the other detectable peptides were present in smaller amounts than controls (Fig. 3). There was a low concentration of all complex IV subunits in the mitochondrial fraction from the patient using monospecific antisera against holo-complex IV (results not shown).

The restriction mapping of mitochondrial DNA from whole blood from this patient showed no unusual features and has been reported elsewhere (42). The polymerase chain reaction was used to exclude deletions, duplications, or insertions in excess of 100 bp in muscle mitochondrial DNA over the two-thirds of the genome where recombination occurs most

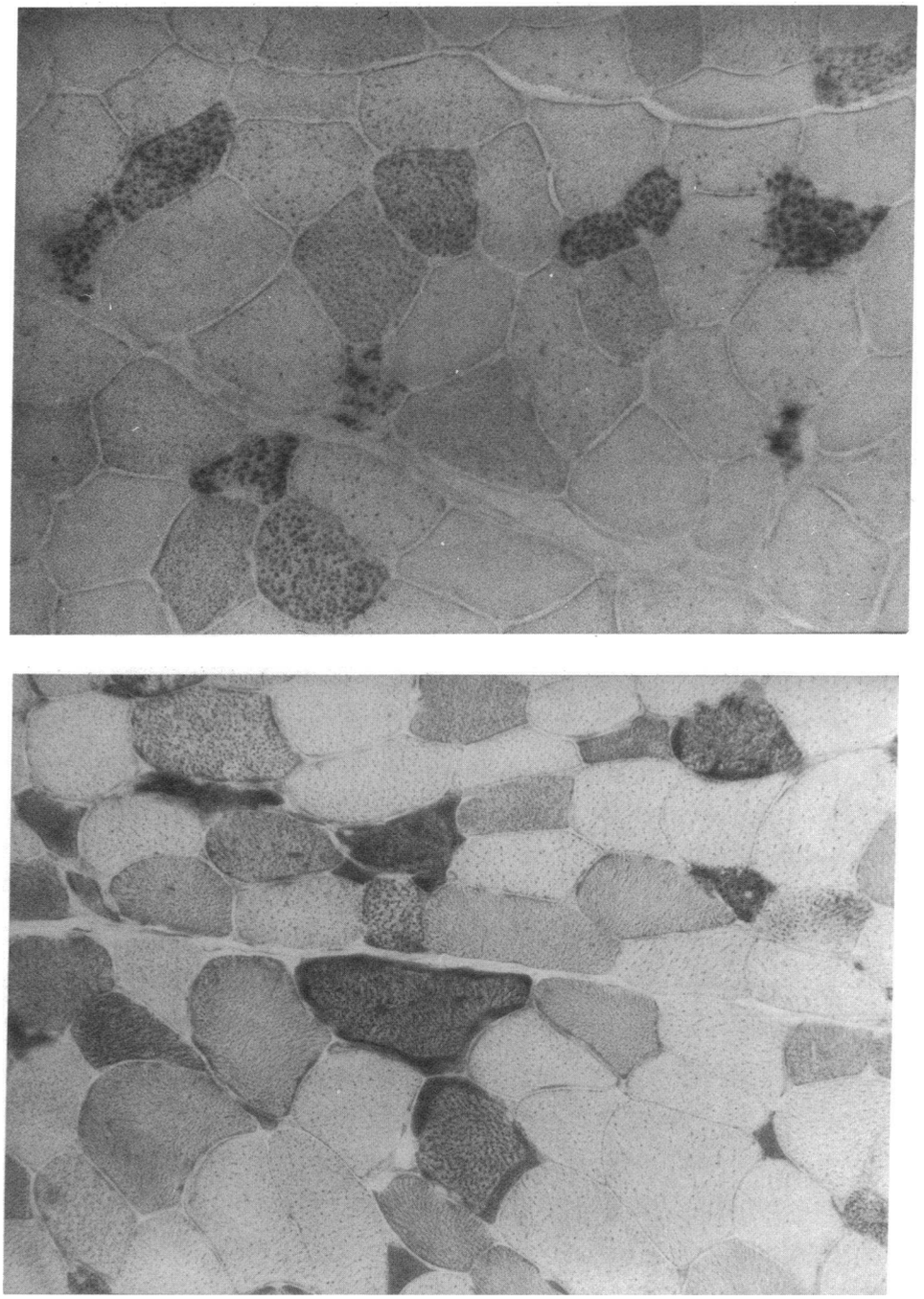

Figure 1. Skeletal muscle morphology. Skeletal muscle sections were stained for: (*top*) Oil Red O demonstrating accumulation of neutral lipid; (*bottom*) succinate dehydrogenase activity to show subsarcolemmal accumulation of mitochondria.

Table I. Rates of Oxidation of NAD$^+$-linked Substrates and Succinate in Skeletal Muscle Mitochondrial Fractions

Skeletal muscle	Patient	Controls
10 mM succinate	319	278±37
10 mM pyruvate + 1 mM malate	35	207±52
10 mM oxoglutarate	38	147±33

Rates are expressed as nanomoles ferricyanide-reduced (in the presence of 10 mM ADP) $min^{-1} \cdot mg\ protein^{-1}$. The figures shown for controls are mean±SD and represent the values for 14 subjects. The rate of oxidation of NAD$^+$-linked substrates is slow, but the oxidation of succinate is normal.

frequently. Restriction mapping excluded heteroplasmy due to duplications, deletions, and insertions in excess of 2 kb over the remainder.

The maximum flux through β-oxidation, in vitro, was ~ 66% of that in control mitochondria (Fig. 4). Radio-HPLC analysis of the acyl-CoA esters isolated from the patient's mitochondria after incubation with [U-^{14}C]hexadecanoate demonstrated the presence of hexadec-2-enoyl-CoA, 3-hydroxyhexadecanoyl-CoA, tetradecanoyl-CoA, and tetradec-2-enoyl-CoA (Fig. 5 *A*). These intermediates of β-oxidation were not detected in incubations of control mitochondrial fractions (Fig. 5 *B*). In addition, there was a low concentration of acetyl-CoA in the mitochondrial fraction from the patient consistent with the slow flux observed.

Table II. Activity of Respiratory Chain Complexes in Skeletal Muscle Mitochondrial Fractions

Skeletal muscle	Patient	Controls
Complex I	0.5	216±40 (n = 9)
Complex II	412.6	316±83 (n = 10)
Complex III	0.50	0.8±0.1 (n = 11)
Complex IV	0.62	2.10±0.28 (n = 10)

Results are expressed as nanomoles of NADH-oxidized $min^{-1} \cdot mg\ protein^{-1}$ (complex I), nanomoles of ubiquinone-reduced $min^{-1} \cdot mg\ protein^{-1}$ (complex II), or as apparent first-order rate constants ($s^{-1} \cdot mg\ protein^{-1}$) (complexes III and IV). The figures shown for the controls are mean±SD. There is a marked deficiency of complex I activity, with low activity of complexes III and IV.

The activity of 3-hydroxyacyl-CoA dehydrogenase was normal with acetoacetyl-CoA as substrate (1.58 $\mu mol \cdot min^{-1} \cdot mg\ protein^{-1}$; controls [$n$ = 3] 1.38±0.28 [mean±SD]) and slightly high with 3-oxohexadecanoyl-CoA as substrate (0.91 $\mu mol \cdot min^{-1} \cdot mg\ protein^{-1}$; controls [$n$ = 3] 0.83±0.03).

Discussion

The family history of muscle disease and the histochemical findings in the mother strongly suggest that the patient has an inherited disorder causing mitochondrial dysfunction consistent with either Mendelian or maternal (mitochondrial) inheritance. It appears that the defect is confined to skeletal muscle, as there was only mild lactic acidosis, even after exercise (8.0 mM lactate); she became ketonemic during a 36-h fast

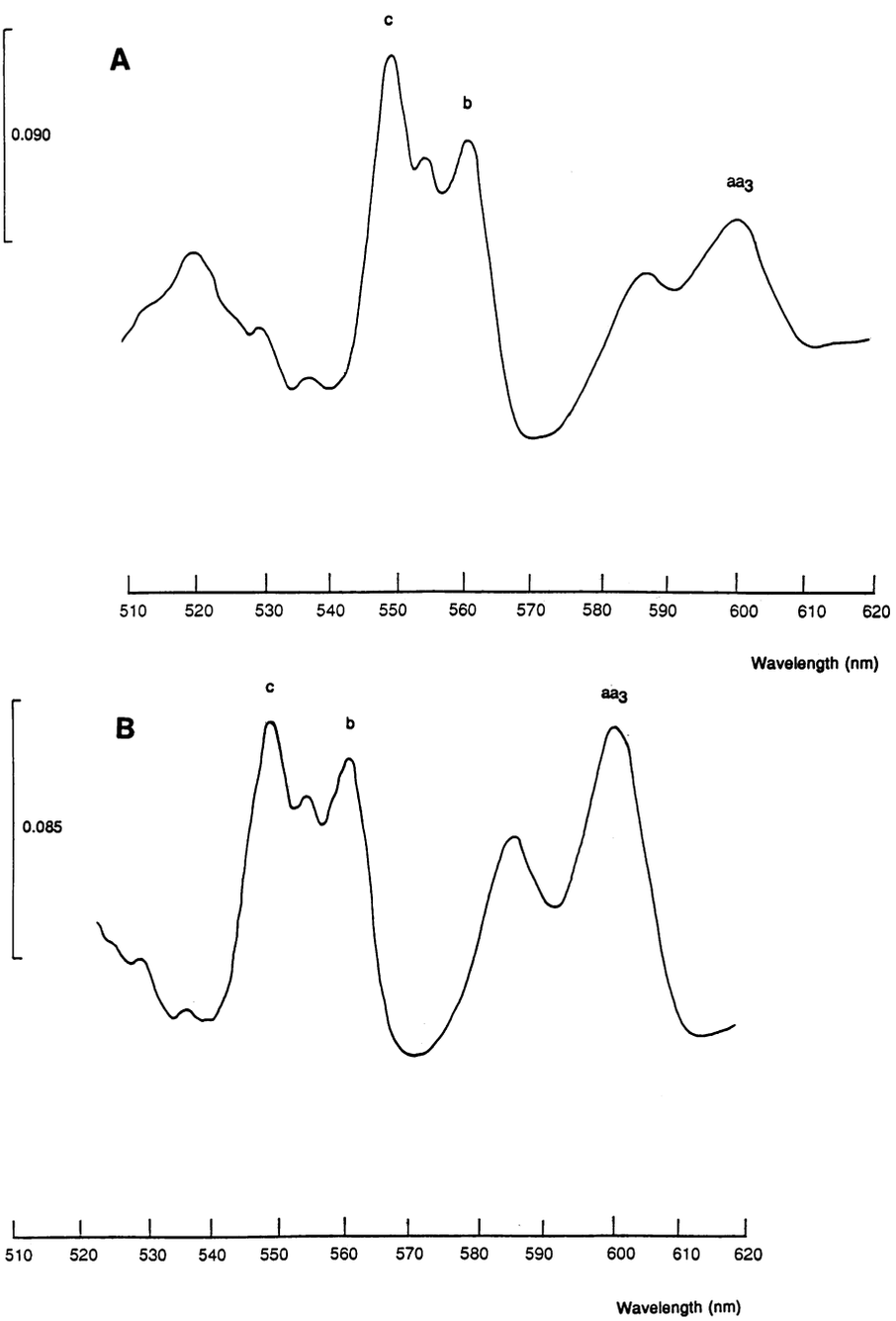

Figure 2. Cytochrome spectra of skeletal muscle mitochondrial fractions. (*A*) Patient. (*B*) Control. The cytochrome spectra were recorded at −190°C; the sample cell containing 1.65 mg (control) and 2.25 mg (patient) of mitochondrial protein was reduced with dithionite. The concentrations of the cytochromes are given in Table III.

Table III. Concentrations of Cytochromes in Skeletal Muscle Mitochondria

	Patient	Controls
Cytochrome aa_3	0.109	0.24±0.07
Cytochrome *b*	0.087	0.151±0.038
Cytochrome *c*	0.278	0.296±0.069
Cytochrome aa_3/c	0.391	0.836±0.178
Cytochrome *b/c*	0.322	0.505±0.081

Results are expressed as $\mu mol^{-1} \cdot mg\ protein^{-1}$. The figures shown for controls are mean±SD and represent the values for 10 subjects. The concentration of cytochromes aa_3 and *b* are low when compared with the concentration of cytochrome *c*.

(total ketone bodies 4 mM) and remained euglycemic (glucose 4.2 mM), indicating that her liver is unaffected; electroencephalogram and psychometric testing were normal.

Our results clearly show that this patient has defective function of the mitochondrial respiratory chain. A mitochondrial fraction prepared from skeletal muscle oxidised NAD^+-linked substrates slowly, although the rate of oxidation of succinate was normal, suggesting that the lesion involves complex I of the respiratory chain. This was confirmed by the very low NADH/ubiquinone oxidoreductase activity. Complex II activity was normal, but the activities of complexes III and IV were low and associated with low concentrations of cytochrome *b* and aa_3, respectively (Table II). Whereas the activity of complexes III and IV are low, the most severe deficiency involves complex I activity.

Apart from the 51-kD subunit (nuclear coded), there were low amounts of all the detectable peptides of complex I. Immunoblots of complex IV in the mitochondrial fraction from the patient also revealed low concentrations of all subunits rather than the absence of a specific subunit. Low concentrations of all immunoreactive peptides of complex I have been found in other cases of complex I deficiency (14, 15). However, several cases have been reported in which one or more individual subunit could not be detected; Moreadith et al. (13) demonstrated lack of the 75-kD (nuclear coded) and 13-kD (nuclear coded) subunits, and Schapira et al. (15) found a deficiency of the 24 kD FeS-protein (nuclear coded).

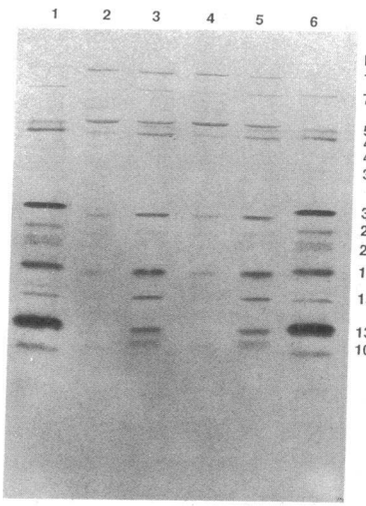

Figure 3. Immunoblot analysis of complex I in human skeletal muscle mitochondria. Mitochondrial proteins were separated by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and reacted with anti-holocomplex I antibodies. Lanes *1* and *6*, purified bovine complex I; lanes *3* and *5*, control mitochondria, loaded with 100 μg protein; lane *2*, patient, 100 μg loaded; lane *4*, patient, 150 μg loaded. The molecular weight of the individual subunits is marked on the right. This immunoblot demonstrates that the amounts of the detectable subunits of complex I are low apart from the 51-kD subunit. The 110-kD band represents the pyridine nucleotide transhydrogenase that copurifies with complex I.

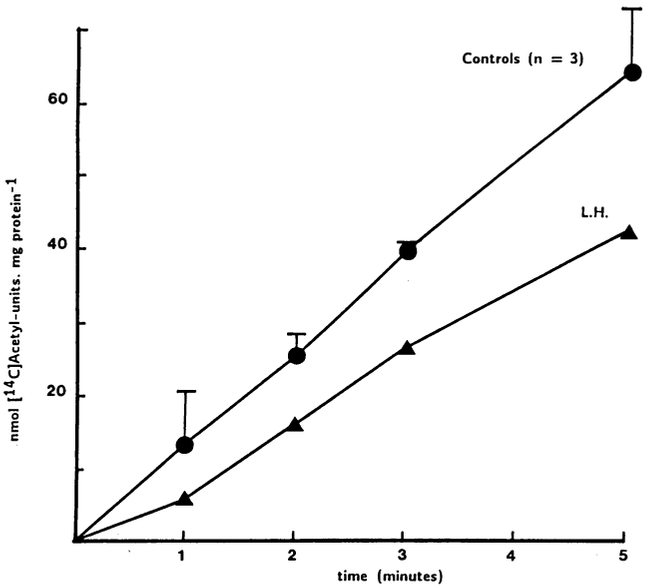

Figure 4. Oxidation of [U-^{14}C]hexadecanoate in skeletal muscle mitochondrial fractions from the patient and three controls. Mitochondrial fractions (1–3 mg) were incubated with 36 nmol [U-^{14}C]hexadecanoate. The samples were quenched and the acid-soluble material counted. The oxidation of [U-^{14}C]hexadecanoate in the mitochondrial fraction from the patient was 10 nmol [^{14}C]acetyl units formed $\cdot min^{-1} \cdot mg^{-1}$ and the rate for the mitochondrial fractions from controls (mean±SD) was 15±1.9 nmol [^{14}C]acetyl units formed $\cdot min^{-1} \cdot mg^{-1}$.

There are several possible explanations for the severe deficiency of complex I and the less severe deficiencies of complexes III and IV. Mutation of either nuclear or mitochondrial genes might be involved. A defect in either a nuclear or mitochondrially coded subunit of complex I may affect the synthesis, processing, or assembly of the other subunits of complex I. We have excluded a large deletion of mitochondrial DNA by restriction mapping and amplification, but it is possible that there may be a small deletion or point mutation of DNA coding for a subunit of complex I. Alternatively, there may be an abnormality that has a general effect on the synthesis, import, or transport of mitochondrial protein. If involving the mitochondrial DNA, a mutation involving the control regions for heavy- and light-stranded promoter would impair protein synthesis. A nuclear mutation, affecting a protein involved in mitochondrial function such as RNA polymerase (43) or chaperonins (44) might explain the widespread biochemical defect. In either case it is difficult to explain why such a mechanism should affect complex I much more severely than complexes III and IV.

The accumulation of intramuscular lipid suggesting impaired fatty acid oxidation has been observed in other patients with apparent complex I deficiency (45; Bindoff, L. A., unpublished observations). In vitro, the flux through β-oxidation measured under optimum conditions (Fig. 4) was only 34%

slower in skeletal muscle mitochondria from our patient oxidizing [U-^{14}C]hexadecanoate than in those from controls, even though the complex I activity was very low (Table II). It might appear surprising, therefore, that such a relatively small difference would cause a major disturbance of the pattern of acyl-CoA intermediates of β-oxidation, and that this would be associated with lipid deposition in vivo. The pattern of intermediates is similar to that found in rat liver mitochondria oxidizing [U-^{14}C]hexadecanoate in the presence of rotenone, which inhibits complex I by ~ 95% and causes 75% decrease in the flux (38). This suggests that the pattern of intermediates does not depend simply on the rate of β-oxidation. Electrons from both NADH and $ETFH_2$ feed into the respiratory chain at complex III via complexes I and ETF dehydrogenase, respectively. If complex I activity is low, the NAD^+/NADH pool will be more reduced in the steady state than the $ETF/ETFH_2$ pool. If the control strength (22) of the 3-hydoxyacyl-CoA dehydrogenases is low, the flux through β-oxidation may not be a simple function of the redox states of these pools, although the redox states will partly determine the steady state concentrations of the acyl-CoA intermediates. In vivo, electrons derived from the oxidation of citrate cycle and other substrates compete with those derived from β-oxidation (20). In patients with defects of complex I, the oxidation of fatty acids would be impaired to a greater extent, in vivo than in vitro due to the absence of other substrates. It is therefore reasonable to suggest that lipid accumulation in the muscles of patients with deficiency of complex I is due to impaired fatty acid oxidation.

The mitochondrial [CoA]/[acyl-CoA] ratio is buffered by

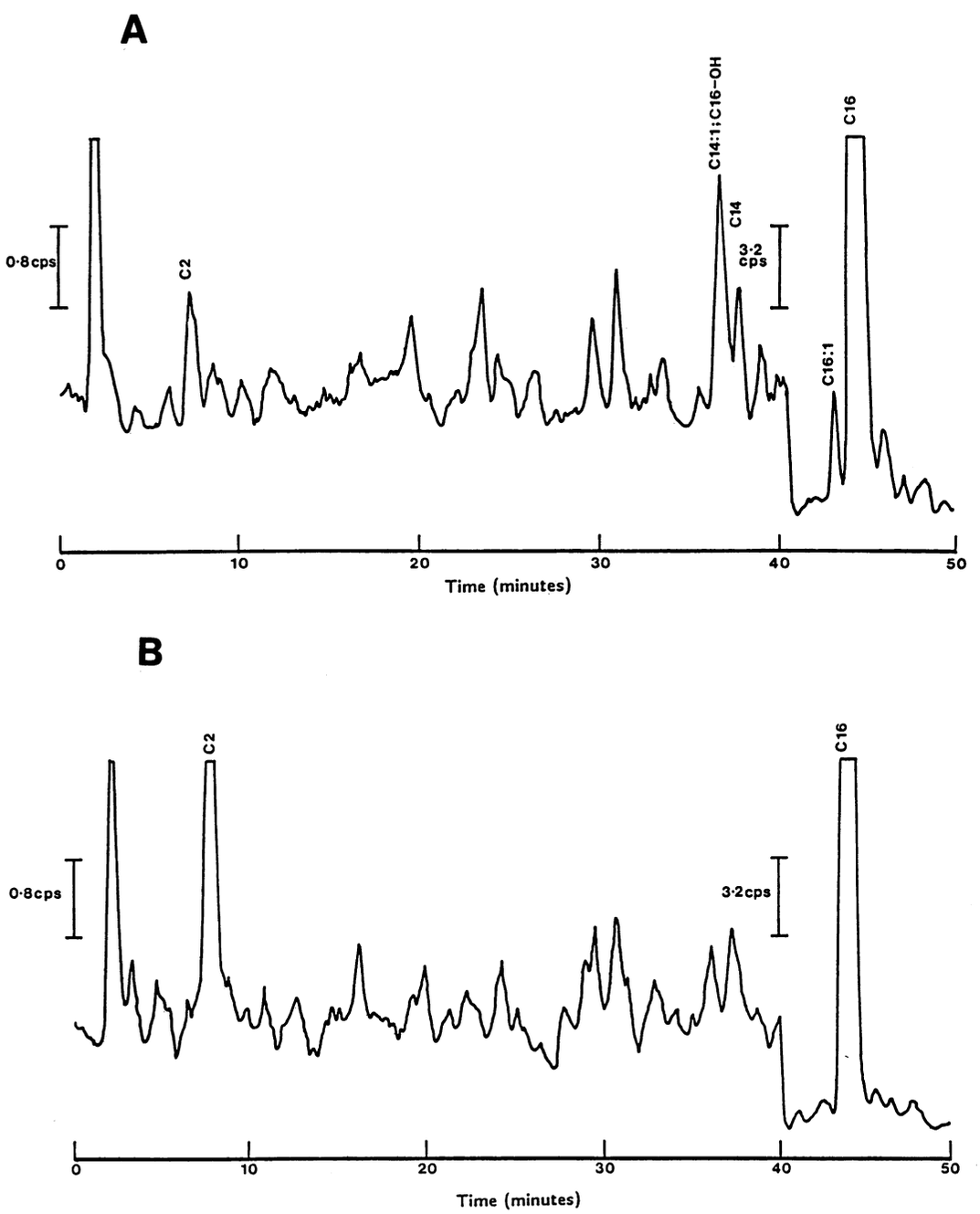

Figure 5. Radiochromatograms of ^{14}C acyl-CoA ester intermediates in human skeletal muscle mitochondria from (*A*) patient and (*B*) control. The acyl-CoA fraction, after incubation for 3 min as described in Fig. 4, was analyzed by reverse-phase HPLC with on-line photodiode array and radiochemical detection. Detectable concentrations of hexadec-2-enoyl-CoA, 3-hydroxyhexadececanoyl-CoA, tetradecanoyl-CoA, and tetradec-2-enoyl-CoA were found in the patient but not control fractions, and the concentration of acetyl-CoA was lower in the patient compared with control, confirming slowed flux through fatty acid oxidation. The identification of the hexadec-2-enoyl-CoA was confirmed spectroscopically (see reference 38). The 3-hydroxyhexadecanoyl-CoA and the tetradec-2-enoyl-CoA co-elute (see reference 38). The identification of the compounds is C16, hexadecanoyl-CoA, C16:1, hexadec-2-enoyl-CoA, C16-OH, 3-hydroxyhexadecanoyl-CoA, C14, tetradecanoyl-CoA, C14:1, tetradec-2-enoyl-CoA, and C2, acetyl-CoA.

carnitine. The low free carnitine concentration with a high concentration of acylated carnitine in the plasma from the patient is probably secondary to impaired fatty acid oxidation (46). Both 3-hydroxyacyl-CoA esters and 3-hydroxyacylcarnitine esters are substrates for carnitine palmitoyltransferase (47). Further, rat liver mitochondria fractions oxidizing hexadecanoyl-carnitine in the presence of rotenone form 3-hydroxyhexadecanoyl-carnitine (19). The mechanism by which low [carnitine]/[acyl-carnitine] ratio cause secondary carnitine deficiency is not known.

Lipid storage myopathy was first described by Bradley et al. (48). It has often been associated with carnitine deficiency, and both these phenomena are usually secondary to a defect of mitochondrial β-oxidation (49, 50). However, it is clear from our results that such a presentation may also be due to a defect of the respiratory chain. This means that careful and complete investigation of both β-oxidation and the respiratory chain are essential in a patient presenting with a lipid storage myopathy.

Acknowledgments

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