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INTACT α-SUBUNIT IS REQUIRED FOR MEMBRANE-BINDING OF HUMAN MITOCHONDRIAL TRIFUNCTIONAL β-OXIDATION PROTEIN, BUT IS NOT NECESSARY FOR CONFERRING 3-KETOACYL-COA THIOLASE ACTIVITY TO THE β-SUBUNIT

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Summary: We have studied the activities of α and β subunit enzymes of the β-oxidation trifunctional protein complex in a patient who does not process the α-subunit. Long-chain 3-ketoacyl-CoA thiolase, the β-subunit enzyme, was transported into the mitochondrial matrix, where it expressed normal levels of activity, but was not translocated to the membrane. Thus, intact α-subunit is required for trifunctional protein membrane translocation, but is not necessary for conferring activity of the β-subunit.

Mitochondrial trifunctional protein (TFP) catalyzes three enzymatic steps in the β-oxidation of fatty acids, including long-chain 2,3-enoyl-CoA hydratase (L-HYD), L-3-hydroxyacyl-CoA dehydrogenase (L-CHAD), and long-chain 3-ketoacyl-CoA thiolase (L-KAT) [1,2]. This heterocomplex, which contains 4 α and 4 β subunits with respective monomer molecular weights of 68 and 380 KDa, binds to the inner mitochondrial membrane [2]. The α-subunit

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encodes the L-HYD and L-CHAD activities, while the β-subunit contains L-KAT activity only [3]. Genetic deficiency of the enzymes of the TFP leads to severe clinical symptoms effecting tissues which require to β-oxidize fatty acids as an energy source [4]. These symptoms include liver disease, both acute with fasting-related hepatic encephalopathy (Reye-like syndrome), or chronic progressive liver disease leading to fulminant hepatic failure. Muscle is also effected with both a cardiomyopathy and skeletal muscle myopathy (with acute episodes of myoglobinuria being reported). Acute episodes, which may result in sudden death, are usually related to periods of fasting or increased energy demand due to fever or excessive muscular activity. Genetic defects described to date fall into two categories. The majority of patients described appear to be homozygous with a specific mutation, a G to C mutation at position 1528 (G1528C) of the α-subunit cDNA which results in a glutamate to glutamine alteration at position 474 of the mature α-subunit. This mutation is located in the L-CHAD domain and reduces L-CHAD activity only [5]. Other patients have been described with defects affecting processing of the whole α-subunit. Recently, two α-subunit splice site mutations that result in failure to process the α-subunit precursor mRNA have been described in a patient with a severe clinical presentation [5]. We present here enzymatic studies on this patient who does not make α-subunit. Both of his mutations cause exon 3 skipping, resulting in undetectable levels of α-subunit protein. However, our results support the concept that the β-subunit may be normally imported into the mitochondria. This patient offers us a unique opportunity to study the interactions of α and β subunits.

MATERIALS AND METHODS

Subject: The patient has been described in detail by Brackett et al. [5]. Essentially, this was a male who presented on the 6th day of life with pallor, lethargy, and tachypnea, with hypoglycemia (25mg/dl, 1.4mmol/L). Urine organic acid analysis revealed a dicarboxylic and 3-hydroxydicarboxylic aciduria consistent with that reported in L-CHAD deficiencies [6]. The L-CHAD activity in intact cultured skin fibroblasts was consistent with a deficiency, (Normals 130.3 - 143.6, positive L-CHAD control (in a patient homozygous for the G1528C mutation) 39.6, this patient 42.4 nmol/min/mg protein). Sequence analysis revealed that the patient was heterozygous for the two different splice site mutations, which cause a universal deletion of exon 3 (71 bp) in the mRNA. These mutations both occur in the 5′-donor splice site following exon 3, a G to A transversion at the invariant position +1 and an A to G mutation at position +3 [5].

Chemicals and Culture Media: All growth media and antibiotics were obtained from Gibco BRL, NY. Coenzyme-A, acetoacetyl-CoA,

NADH, and other chemicals were purchased from Sigma (St. Louis, MO). The 3-ketopalmitoyl-CoA was custom synthesized by Larodon Fine Chemicals (Upsala, Sweden), and was 100% pure on mass spectrometric analysis.

Cell Culture: Fibroblasts were cultured from a skin biopsy using Dulbecco's modified eagles medium containing, fetal bovine serum (10%, vol/vol), penicillin G 100units/ml, streptomycin sulfate 100units/ml, amphotericin B 0.25ug/ml. Fibroblasts lines were cultured in 25cm^2 plastic tissue culture flasks (Corning, NY) in a humidified atmosphere of 5% CO_2 in air at a temperature of 37° C. Fibroblasts between passage 4-15 were used for enzyme assay.

Enzyme Assays: The activity of the short-chain and long-chain L-3-hydroxyacyl-CoA dehydrogenase, (S-CHAD, L-CHAD), in fibroblast and liver homogenate was measured by following the decrease in absorbance of NADH at 340nm, essentially by the method of Venizelos et al. [7]. The assay mixture (final volume 350μl) contained 100mM K-Phosphate buffer, pH 6.3, 0.1 mmol/L dithiothreitol, 0.1% (wt/vol) Triton X-100, 100μmol/L NADH, and fibroblast or liver homogenate (approx. 0.1mg/ml). Reactions were initiated by adding 50μmol/L acetoacetyl-CoA (C4, short-chain), and 40μmol/L 3-ketopalmitoyl-CoA (C16, long-chain). Assays were run on a Cobas-Fara automated analyzer (Roche Instruments, NJ) at 37°C.

The short-chain 3-ketothiolase activity, (S-KAT), was measured at 37°C on a Cobas-Fara, by measuring the decrease in absorbance at 303nm of the 3-keto group, using a reaction mixture (final volume 360μl) containing the following: 100nmol/L Trizma-HCl, pH 8.0, 10nmol/L $MgCl_2$, 50mmol/L KCl, 50μmol/L CoA, and 50μmol/L acetoacetyl-CoA. The reaction was then initiated with the addition of fibroblast or liver homogenate (approx. 0.1mg/ml).

The long-chain 3-ketothiolase (L-KAT) activity was measured using medium (final volume 315μl) of 100mmol/L Tris-HCl, pH 8.3, 50mmol/L KCl, 25mmol/L $MgCl_2$, 0.1% (wt/vol) Triton X-100, 0.2mg/ml bovine serum albumin (essentially fatty acid free), 10μmol/L 3 - ketopalmitoyl-CoA, and fibroblast or liver homogenate (approx. 0.1mg/ml). After a incubation of 2 min. at 37°C, the reaction was started by the addition of 70μmol/L Coenzyme A. The decrease in absorbance was followed at 303nm using the Cobas-Fara.

Mitochondrial Membrane and Matrix Fraction Preparation: Cultured skin fibroblasts were grown as described above. Three 25cm^2 flasks were taken to confluence, trypsinized and washed with Dulbecco's Phosphate Buffered Saline. Cells were then re-suspended in 300μl of 20mmol/L Na-phosphate buffer, pH 7.8, and exposed to ultasonication for 20 seconds, twice, while continually on ice. The cell homogenate was then centrifuged at 100,000xg for 60 minutes at 4°C. The supernatant was aspirated and stored on ice for enzyme analysis (matrix fraction).

After washing the pellet with 200μl of 20mmol/L Na-phosphate buffer pH 7.8, the pellet was homogenized in 200μl of 10 mmol/L Na-phosphate buffer pH 7.8, 0.1% (wt/vol) Na-choleate. The homogenate was then centrifuged at 100,000xg for 20 minutes at 4°C. After aspirating the supernatant, the pellet was homogenized in 100μl of 10mmol/L Na-phosphate buffer pH 7.8, 1% (wt/vol) Na-choleate, and allowed to sit on ice for 15 minutes. The sample was then centrifuged at 100,000xg for 60 minutes at 4°C. The supernatant was then aspirated and stored on ice for immediate enzyme analysis (membrane fraction).

RESULTS

The results of the enzyme assays of the whole cell preparation demonstrated activities consistent with those that we and others

have previously described in patients with L-CHAD deficiency (table 1). The residual activity was comparable to that seen in G1528C homozygous patients, and is probably attributable to the activity of the peroxisomal L-CHAD activity. L-KAT activity in whole cell extracts was within the control range. However, analysis of L-KAT in membrane and matrix fractions of the fibroblasts revealed that the enzyme activity was only demonstrated in the matrix fraction (table 2). Significant L-CHAD activity was not found in either membrane or matrix fractions, as would be anticipated from a patient who could not process the α-subunit.

DISCUSSION

Enzymatic studies of liver and cultured skin fibroblasts from this patient are consistent with many previous reports of L-CHAD deficiency [9,10,11,12,13,14]. Our results clearly demonstrate that there was normal total L-KAT activity in the absence of the α-subunit of human mitochondrial TFP. Normally, following import of the monomeric units into the mitochondria and cleavage of the mitochondrial import signal the subunits are arranged as a heteroctomer of 4 α and 4 β units, bound to the mitochondrial membrane [4]. Our results demonstrate that the β-subunits are processed to the point of becoming normally enzymatically active within the mitochondria. However, our results are consistent with the hypothesis that, in the absence of the α-subunit, no localization of the β-subunit to the membrane occurs. This is consistent with the concept that the α-subunit is essential for membrane binding [4]. It is not known how the localization of L-

Table 1: Whole Cell and Liver Extract L-CHAD and L-KAT Activities

Enzyme Measured		Patient	Controls	
Fibroblasts			Mean ± SD	
	S-CHAD (C4)	117.5	132.9 ± 5.3	(n=3)
	L-CHAD (C16)	42.4	138.2 ± 5.7	(n=3)
	L-KAT (C16)	3.1	3.8 ± 0.4	(n=3)
Liver				
	S-CHAD (C4)	93.1	75.8, 104.2	
	L-CHAD (C16)	74.6	105.9, 131.7	
	L-KAT (C16)	4.1	3.3, 3.5	

activities are in nmol/min/mg protein.

Table 2: Activities of L-CHAD and L-KAT in Matrix and Membrane Fractions of Cultured Skin Fibroblasts

Enzyme Measured	Patient	Controls
Fibroblasts		
Matrix		
S-CHAD	96.2	92.1, 127.5
L-CHAD	24.5	23.2, 50.3
L-KAT	41.3	34.8, 71.6
Membrane		
S-CHAD	151.6	155.5, 198.6
L-CHAD	58.2	167.1, 194.6
L-KAT	0.0	25.1, 46.8

activities are in nmol/min/mg protein.

KAT activity to the matrix effects its structure or activity in vivo, but this is unimportant since, for its physiological action in fatty acid β-oxidation, it requires normal α-subunit enzymes, L-HYD and L-CHAD which perform the precursor enzymatic steps. It is also possible that there exists a second long-chain thiolase enzyme in the mitochondrial matrix, but to date there is no evidence to support this possibility. Another possibility is that the apparent L-KAT activity is conferred by overlapping chain length specificity of the mitochondrial short-chain thiolase (S-KAT) whose activity appears normal in our patient. However, there is little evidence to support this hypothesis as the chain length specificities of S-KAT do not extend to C16.

Therefore, our studies suggest that, at least in vitro, it is possible to obtain normal activity of the β-subunit enzyme L-KAT, in the absence of the α-subunit, but that the α-subunit is required for membrane insertion.

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