The Multiple Acyl-Coenzyme A Dehydrogenation Disorders, Glutaric Aciduria Type II and Ethylmalonic-Adipic Aciduria

Mitochondrial Fatty Acid Oxidation, Acyl-Coenzyme A Dehydrogenase, and Electron Transfer Flavoprotein Activities in Fibroblasts

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Abstract

The multiple acyl-coenzyme A (CoA) dehydrogenation disorders (MAD) include severe (S) and mild (M) variants, glutaric aciduria type II (MAD:S) and ethylmalonic-adipic aciduria (MAD:M). Intact MAD:M mitochondria oxidized [1-^{14}C]octanoate, [1-^{14}C]palmityl-CoA, and [1,5-^{14}C]glutarate at 20–46% of control levels; MAD:S mitochondria oxidized these three substrates at 0.4–18% of control levels. In MAD:M mitochondria, acyl-CoA dehydrogenase (ADH) activities were similar to control, whereas MAD:S ADH activities ranged from 38% to 73% of control. Electron transfer flavoprotein (ETF) activities in five MAD:M cell lines ranged from 29 to 51% of control ($P < 0.01$); ETF deficiency was the primary enzymatic defect in two MAD:M lines. In four MAD:S patients, ETF activities ranged from 3% to 6% of control ($P < 0.001$); flavin adenine dinucleotide addition increased residual ETF activity from 4% to 21% of control in a single MAD:S line ($P < 0.01$). Three MAD:S patients had ETF activities ranging from 33 to 53% of control; other investigators found deficient ETF-dehydrogenase activity in these MAD:S and three of our MAD:M cell lines.

Introduction

The multiple acyl-coenzyme A (CoA)[1] dehydrogenation disorders (MAD) include severe (S) and mild (M) variants, termed glutaric aciduria type II and ethylmalonic-adipic aciduria/mild glutaric aciduria type II, respectively. First described in 1976 (1), glutaric aciduria type II (MAD:S) is an inborn error of me-

1. *Abbreviations used in this paper:* ADH, acyl-CoA dehydrogenase; CoA, coenzyme A; CoASH, CoA sulfhydryl; DCIP, dichlorophenol indophenol; ETF, electron transfer flavoprotein; ETF-DH, ETF-dehydrogenase (ETF: coenzyme Q oxidoreductase); FAD, flavin adenine dinucleotide; IVDH, isovaleryl-CoA dehydrogenase; LCADH, long-chain ADH; MAD:M, multiple acyl-CoA dehydrogenation disorder, mild variant; MAD:S, multiple acyl-CoA dehydrogenation disorder, severe variant; MCADH, medium-chain ADH; MS, fibroblast mitochondrial sonic supernatants; NEM, *N*-ethylmaleimide; SCADH, short-chain ADH.

tabolism characterized by hypoglycemia, metabolic acidosis, and excretion of elevated amounts of glutarate, ethylmalonate, isovalerate, α-methylbutyrate, isobutyrate, aliphatic dicarboxylic acids, and their derivatives; several reviews of the MAD are available (2–4). More than 10 cases have been identified to date (5–13). This disease is termed glutaric aciduria type II (GA-II) because glutarate is the predominant urinary metabolite, differing from glutaric aciduria type I in which glutarate and its metabolites (glutaconate and 3-hydroxyglutarate) accumulate (14).

Ethylmalonic-adipic aciduria/mild GA-II (MAD:M) is associated with episodic hypoglycemia, acidosis, prolonged survival, and excretion of elevated amounts of ethylmalonate, adipate, and hexanoylglycine (15–17). MAD:S and MAD:M are clinically and biochemically related, with MAD:S usually being fatal in the neonatal period, while MAD:M has a milder and more variable clinical course. MAD:M patients, when stressed with serious illness, catabolic states, and/or precursor loading, excrete metabolites similar to those found in MAD:S, underlining the similarity between the two variants (15, 16). Riboflavin-responsive MAD:M variants have been identified clinically and confirmed biochemically; more than five such cases are known to the authors (18–21). These two organic acidurias have been identified as multiple acyl-CoA dehydrogenation disorders because their urinary metabolites share two common characteristics (2–4). First, they are all dethiolated acyl-CoA's or direct derivatives of these acyl-CoA's. Second, the first step in the catabolism of all these acyl-CoA's is dehydrogenation at carbons 2 and 3 in the mitochondrial matrix. These two characteristics, coupled with the results of radiolabeled substrate oxidation studies in vitro (1, 8, 15, 16, 22, 23), suggest that enzymatic dehydrogenation of multiple acyl CoA's is functionally deficient in these disorders.

It has been postulated that the MAD are caused by deficient electron transfer from the flavin adenine dinucleotide (FAD)-requiring acyl-CoA dehydrogenases (ADH) to the ubiquinone (CoQ$_{10}$) of the electron transport chain (2–4, 22, 24). The electron transfer flavoprotein (ETF) and the iron-sulfur flavoprotein, ETF dehydrogenase (ETF-DH; ETF:ubiquinone oxidoreductase) mediate this sequential transfer of electrons (Fig. 1). Defects in either enzyme would explain the impaired oxidation of ^{14}C-labeled fatty and amino acids by intact MAD fibroblasts, and the observed normal or near normal ADH activities (10, 22, 25). Inasmuch as most other biochemical studies of the MAD employed intact cells or crude homogenates, we assayed mitochondrial acyl-CoA oxidation, ADHs, and ETF in MAD fibroblasts. MAD:S cell lines have moderately decreased ADH activities; we found severe and mild ETF deficiencies in four MAD:S and five MAD:M patients, respectively. We studied three other MAD patients with severe clinical and biochemical phenotypes who demonstrated only moderately decreased ETF activities. Other investigators found deficient ETF-DH in these latter three
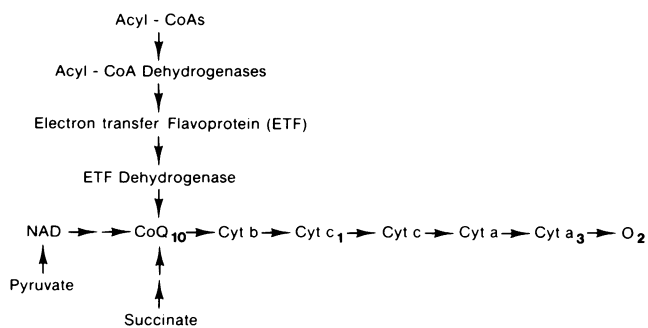
Acyl - CoAs

↓

Acyl - CoA Dehydrogenases

↓

Electron transfer Flavoprotein (ETF)

↓

ETF Dehydrogenase

↓

NAD → → CoQ$_{10}$→ Cyt b → Cyt c$_1$ → Cyt c → Cyt a → Cyt a$_3$ → O$_2$

↑   ↑
Pyruvate   ↑
                  Succinate

Figure 1. Schema depicting the pathway of electron flow from the ADHs to molecular oxygen.

MAD:S patients and three of the five MAD:M patients, confirming that either ETF or ETF-DH deficiency produces both MAD phenotypes (24, 25).

Methods

Chemicals. Substrates for mitochondrial oxidation studies were [1,5-^{14}C]glutaric acid (2.5 mCi/mmol), [1-^{14}C]octanoic acid (4.7 mCi/mmol), [1-^{14}C]palmityl-CoA (1.0 mCi/mmol), and [1,4-^{14}C]succinic acid (1.0 mCi/mmol) obtained from New England Nuclear (Boston, MA) and Research Products International Corp. (Mt. Prospect, IL). [2,3-^{3}H]butyric acid (10 mCi/mmol), [2,3-^{3}H]octanoic acid (5.0 mCi/mmol), and [2,3-^{3}H]isovaleric acid (10 mCi/mmol) were synthesized by New England Nuclear. The corresponding acyl-CoA esters were synthesized by the mixed anhydride method, as we described earlier (26). Pure pig liver medium-chain ADH (MCADH) and ETF were gifts from Dr. Carole L. Hall, Georgia Institute of Technology, Atlanta, GA. FAD, phenazine methosulfate, N-ethylmaleimide (NEM), and dichlorophenol indophenol (DCIP) were purchased from Sigma Chemical Co. (St. Louis, MO).

Culture of fibroblasts. Skin fibroblasts were obtained from seven MAD:S patients who presented in the neonatal period, three female (1540, 6; 1441, 7; 1520, 3) and four males (1400, 1; 1430, 13; 1515, 5; 1401, courtesy of Dr. Peggy Falace, University of Kentucky, Lexington). The five MAD:M patients included one female (493, 15), and three males (741, patient B.K. in 17; 840 and 930, courtesy of Dr. Larry Sweetman, University of California, San Diego) who all presented in infancy, as well as one female (632) who presented with mild GA-II in adulthood (16). Patient 1401 is a male neonate who died at 3 d of age with severe metabolic acidosis, hypoglycemia, and a characteristic MAD:S organic aciduria. Patient 840 is a 6-yr-old male who is neurologically devastated after a prolonged episode of seizures and acidotic coma; he excretes ethylmalonate and small amounts of glutarate and adipate. Patient 930 is a 4-yr-old male with growth failure and developmental delay whose urinary metabolite profile is similar to that of patient 840. Control fibroblast cultures were obtained from 11 normal individuals. Full clinical and biochemical descriptions of patients 1401, 840, and 930 will be published separately.

Fibroblasts were cultured in Eagle's minimal essential medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 140 μM penicillin, 86 μM streptomycin, and 162 μM neomycin. Fibroblasts were subcultured 1:4 every 2 wk and the media were changed weekly. Fibroblasts of passage 6-20 were used in all experiments.

Isolation of mitochondria. Fibroblast mitochondria were isolated from three 750-cm^2 glass roller bottles of each cell line by a method previously described (27), except that digitonin treatment of the mitochondria was omitted. In all experiments, mitochondria from two control cell lines were assayed simultaneously with mitochondria from three to five MAD lines. Intact mitochondria were resuspended in a buffer consisting of KCl 100 mM, Tris-HCl 50 mM, pH 7.5, MgCl$_2$ 5 mM, ATP 1 mM, and EGTA 1 mM for ^{14}C-substrate oxidation experiments. The oxidation of

[1-^{14}C]octanoate and -palmityl-CoA by intact mitochondria was performed in triplicate as described earlier (28). For the oxidation of [1,5-^{14}C]glutarate (0.5 mM), 0.2 mM CoA, and 2.5 mM ATP were added to the incubation medium; with [1,4-^{14}C]succinate (2 mM), 2.5 mM ATP was added. These combinations of cofactors were selected to optimize oxidation of each substrate (data not shown).

For ADH and ETF assays, mitochondria were sonicated at 0°C for 30 s in 0.4-0.8 ml of 20 mM KP$_i$, pH 7.6, using a Fisher sonic disrupter model 300 with a microtip (Fisher Scientific Co., Pittsburgh, PA); the sonicates were then centrifuged at 100,000 g for 1 h. Matrix glutamate dehydrogenase activity was assayed as described earlier (27). The mitochondrial sonic supernatants (MS) were assayed for ADH and ETF as described below.

ADH assays. ADH activities were determined by both dye reduction and tritium-release assays. The dye reduction method follows the reduction of DCIP by endogenous ADH reduced by appropriate acyl-CoA substrates. The reaction is initiated by 440 pmol pig liver ETF (28). The tritium-release assays for ADH activities were performed in duplicate as detailed previously using [2,3-^{3}H]acyl-CoA's as substrates (29). Both methods use 0.1-0.5 mg of MS protein per assay.

Whole-cell sonic supernatants were obtained from two confluent 150-cm^2 culture flasks for each cell line; cells were sonicated in 1.0-2.0 ml of 10 mM KP$_i$, pH 7.6, containing 20 μM FAD, using a Fisher sonic disruptor, model 300, with a microtip at 0°C for 1 min, and centrifuged at 50,000 g for 20 min. The cell supernatant was used in tritium-release assays and the ^{3}H$_2$O formed from the substrates was quantitated by lyophilization, as previously described (29).

ETF assays. Mitochondrial ETF activity was assayed by a modification of the dye reduction method used in previous assays for this enzyme. NEM, pure pig liver MCADH, and octanoyl-CoA are added to a reaction mixture containing DCIP as a terminal electron acceptor and 300-600 μg of MS protein. NEM eliminates artefactual dye reduction by free CoA sulfhydryl (CoASH) derived from thioesterase-catalyzed hydrolysis of octanoyl-CoA (30, 31). The total volume of the assay mixture is 560 μl. Final concentrations of all reagents were: octanoyl-CoA 50 μM; MCADH 165 pmol flavin/assay; KP$_i$ 20 mM pH 7.6; DCIP 70 μM, and NEM 0.2 mM. MS ETF activity was calculated using the initial rate of DCIP reduction after MCADH addition (OD 600 nm). 165 pmol pig liver MCADH and 50 μM octanoyl-CoA reduced DCIP at 72±15 pmol/min, a rate equal to 8±2% of that found in control MS ($n = 12$); this background rate was invariant over 10 min of assay. The Student t test was used for statistical analysis, and variation is expressed as the standard error of the mean. Unless otherwise stated, all P values are two-tailed and refer to comparisons between the individual MAD and control values cited. The number of determinations indicates the number of individual mitochondrial preparations assayed.

Results

Mitochondrial oxidation of ^{14}C-labeled substrates. Intact MAD fibroblasts demonstrate blocked ^{14}C-labeled fatty acid, branched chain amino acid, and lysine catabolism (1-4). We have also demonstrated defective ^{14}C-labeled substrate oxidation in isolated fibroblast mitochondria (Table I). These mitochondrial preparations have been purified 10-15-fold during isolation (27). Glutamate dehydrogenase activities were similar in control and MAD MS, confirming that all mitochondrial preparations were of comparable purity ($n \geq 29$; $P > 0.15$; data not shown). Mitochondria from MAD:M cell lines 493, 632, and 741 oxidized [1-^{14}C]octanoate at 24-40% ($P < 0.05$), [1-^{14}C]palmityl-CoA at 24-31% ($P > 0.1$), and [1,5-^{14}C]glutarate at 20-46% ($P < 0.05$) of control values, respectively; for the MAD:M cell lines as a group, [1-^{14}C]palmityl-CoA oxidation was 27% of control ($P = 0.01$). Mitochondria from MAD:S cell lines 1441 and 1401 oxidized [1-^{14}C]octanoate at 3% and 0.4%, [1-^{14}C]palmityl-CoA at 11% and 8%, and [1,5-^{14}C]glutarate at 11% and 18% of control

Table I. ^{14}C-labeled Substrate Oxidation by Intact Fibroblast Mitochondria

	[1-^{14}C]Octanoate	[1-^{14}C]Palmityl-CoA	[1,5-^{14}C]Glutarate	[1,4-^{14}C]Succinate
	nmol [14]CO$_2$/mg protein/h±SEM			
Normal controls	16.1±2.4	3.23±0.73	4.69±0.56	161±20.8
MAD:M				
493	4.22±1.10*	0.86±0.12	0.94±0.56*	201±44.8
632	3.83±0.32*	0.77±0.54	2.15±0.75*	122±12.2
741	6.43±2.69*	1.00±0.22	2.15±0.55*	130±24.0
MAD:S				
1441	0.42±0.14‡	0.35±0.24*	0.53±0.22‡	121±31.3
1401	0.06±0.03‡	0.27±0.13*	0.83±0.45‡	138±39.4

Culture of diploid skin fibroblasts, mitochondrial isolation, and ^{14}C-substrate oxidation studies were performed in triplicate as described earlier (22, 28). The final concentration and specific activity of each substrate, and concentration of cofactors were as follows: [1,5-^{14}C]glutarate, 0.5 mM, 2.5 mCi/mol, CoA 0.1 mM, ATP 2.5 mM; [1-^{14}C]octanoate 1.1 mM, 4.7 mCi/mmol, L-(−)-carnitine 0.5 mM, CoA 0.1 mM, ATP 2.5 mM; [1-^{14}C]palmityl-CoA, 0.1 mM, 1.0 mCi/mmol, L-(−)-carnitine 0.5 mM and ATP 2.5 mM, [1-^{14}C]succinate, 2.0 mM, 1.0 mCi/mmol, ATP 2.5 mM. The number of determinations ranged from 5 to 11 for the control lines and 2 to 4 for each MAD line. * $P < 0.05$. ‡ $P < 0.01$.

values, respectively ($P < 0.05$). [1,4-^{14}C]succinate oxidation was normal ($P > 0.3$) in mitochondrial preparations from all MAD cell lines.

Mitochondrial ADH activities. We have developed tritium-release assays for ADH activities in fibroblast preparations using [2,3-^{3}H]acyl-CoA's as substrates (27, 29). Enzymatic removal of tritium from carbons 2 and 3 of the substrate forms [3]H$_2$O, which is separated quantitatively from unreacted substrate by anion-exchange chromatography and lyophilization. Short-chain ADH (SCADH), MCADH, and isovaleryl-CoA dehydrogenase (IVDH) activities in MAD cell sonicates were measured using [2,3-^{3}H]butyryl-, -octanoyl-, and -isovaleryl-CoA's as substrates and ranged from 63% to 128% of control ($P > 0.2$; data not shown). However, in MS, MCADH activities in three MAD:M lines were 70–74% and in the MAD:S cell lines as a group, 50% of control ($P = 0.01$; data not shown). MS IVDH activities were 66–82% and 61–73% of control in MAD:M and MAD:S lines, respectively ($P > 0.1$; Table II). We also assayed MS ADH activities using a dye-reduction method we have described previously (27, 29). SCADH activities were 58% and 80% of control in two MAD:M lines ($P > 0.1$) and averaged 52% for five MAD:S lines (Table II; $P < 0.05$). MCADH activity was 72–105% of control in four MAD:M cell lines ($P > 0.2$), whereas cell line 741 had activity 42% of control ($P = 0.03$). MCADH activity in six MAD:S cell lines averaged 60% of control ($P < 0.01$). Addition of 20 μM FAD did not significantly change MCADH activity in control or MAD cell lines. Long-chain ADH (LCADH) activities were 40–111% of control in two MAD:M lines ($P > 0.1$), but averaged 52% of control in the five MAD:S lines ($P = 0.02$).

Because the proposed enzymatic defects in the MAD involve either ETF or ETF-DH, these lowered MAD:S ADH activities were not anticipated. Inasmuch as ETF or ETF-DH deficiency would block normal reoxidation of ADHs reduced by their substrates, increased turnover or relative instability of the reduced enzymes might explain these decreases. However, fibroblast MCADH reduced by octanoyl-CoA lost no activity after incubation at 37°C for 60 min, whereas enzyme incubated without substrate lost >70% of its initial activity over the same interval (Table III); incubating oxidized and reduced pig liver MCADH at both 37°C and 50°C gave qualitatively identical results (data not shown). Thus, interaction with the substrate appears to sta-

bilize rather than destabilize this enzyme against thermal inactivation, possibly related to the protection by substrate of an essential cysteine residue located near the FAD-binding site (32).

Mitochondrial ETF activities. Because ETF catalyzes the electron transfer from the reduced ADHs to ETF-DH in vivo, we reacted fibroblast MS ETF with pure pig liver MCADH and 50 μM octanoyl-CoA. The reduced ETF then transfers its electrons to the electron acceptor dye DCIP, the rate of DCIP reduction reflecting ETF activity. The nonspecific reduction of DCIP by free CoASH derived from thioesterase-catalyzed acyl-CoA hydrolysis was inhibited by the sulfhydryl-alkylating agent NEM (30, 31). To ensure maximal recovery of ETF activity, we assayed 0.88 and 2.5 pmol pig ETF with increasing amounts of pig liver MCADH; 2.5 pmol pig liver ETF produced rates of DCIP reduction similar to those in control MS. 165 pmol MCADH gave near maximal rates of DCIP reduction (Table IV). Because 495 pmol pig MCADH increased background DCIP reduction proportionately and did not greatly increase apparent ETF activity, we used 165 pmol MCADH in all ETF assays reported here.

Our assay system measured ETF activity accurately, inasmuch as we observed a linear increase in enzymatic activity with increasing concentrations of pure pig liver ETF (Fig. 2). Additions of MAD:S MS (500 μg of protein per assay) and 0.2 mM NEM to the same reaction mixture did not significantly alter recovery of pig liver ETF activity. Thus, NEM does not inhibit ETF activity in fibroblast MS, and MAD:S mitochondria contain no inhibitor of pig liver ETF. There is a linear relationship between ETF activity and MS protein up to 650 μg of protein per assay, representing the range of protein concentrations typically used in our experiments (Fig. 3). Heat treatment (60°C for 10 min) completely eliminated control ETF activity, as did rabbit antiserum to pig liver ETF; nonimmune rabbit serum did not inhibit the reaction (data not shown).

ETF activities in the MAD:M cell lines 493, 741, 840, and 930 ranged from 29% to 38% of control ($P < 0.01$; Table V). Cell line 632 from an adult female with mild GA-II/MAD:M had ETF activity 51% of control ($P < 0.01$), significantly greater than that found in the other MAD:M cell lines (one-tailed $P < 0.04$). ETF activities in the MAD:S cell lines, 1400, 1401, 1430, and 1441 ranged from 3% to 6% of control ($P < 0.001$);

Table II. ADH Activities in Mitochondrial Sonic Supernatants

		Substrates			
		Octanoyl-CoA			
Cell lines	Butyryl-CoA	−FAD	+FAD	Palmityl-CoA	Isovaleryl-CoA
	pmol/min/mg protein±SEM				
Normal controls	900±99	1,621±121	1,289±127	862±127	208±38
MAD:M					
493	*	1,231±233	809±101	*	171±53
632	*	1,598±363	1,634±555	*	139±17
741	*	679±160‡	*	*	138±14
840	718±152	1,702±478	*	518±71	*
930	519±62‡	1,161±104	1,241±97	878±31	*
MAD:S					
1441	*	763±239‡	754±158‡	*	127±29
1401	*	970±305	977±215	*	149±17
1400	589±117	1,166±201	893±142	461	*
1430	365±57‡	782±233‡	*	343±92	*
1520	463±126‡	828±238‡	487	427±169	152
1515	485±177	641±92‡	754±152‡	571±71	*
1540	446±100	*	*	444	*

Butyryl-, octanoyl-, and palmityl-CoA dehydrogenase activities were measured as previously described (28); 440 pmol of pig liver ETF were added to initiate the reaction. Concentration of all acyl-CoA's were 50 μM, except butyryl-CoA (100 μM). The reduction rate of DCIP was calculated from the change in absorbance (600 nm) during the first minute of reaction in the presence of 0.2 mM NEM. 0.05–0.20 mg of protein of mitochondrial protein was used per assay; 20 μM FAD was added in the indicated experiments. Tritium release assay for IVDH was performed in duplicate as described (27, 29). Concentration and specific activity of [2,3-^{3}H]isovaleryl-CoA were 100 μM and 10.0 mCi/mmol. Phenazine methosulfate and FAD concentrations were 10 mM and 20 μM, respectively. The number of determinations ranged from 7 to 29 for control lines and 1 to 9 for each MAD line. * Not determined. ‡ $P < 0.05$.

activities in these four lines did not differ significantly ($P > 0.15$). However, three MAD:S cell lines, 1515, 1520, and 1540, had ETF activities 33% to 37% of control. Addition of 20 μM FAD, the ionically bound cofactor of ETF, did not significantly increase

Table III. Effect of Octanoyl-CoA on the Thermal Stability of Fibroblast MCADH Activity

	[Octanoyl-CoA]	
Incubation time	0	50 μM
min	*pmol DCIP reduced/mg protein/min*	
0	1,580	1,320
10	1,510	1,510
20	898	1,220
30	720	1,290
40	552	1,340
60	408	1,440

Control fibroblast mitochondrial supernatant was used as the source of MCADH activity. One half of the sample was reduced with 50 μM octanoyl-CoA; both oxidized and reduced samples were incubated at 37°C in 20 mM KPi, pH 7.6, and assayed at 10-min intervals. The activity was determined as described (28) with 440 pmol of pig liver ETF added to initiate the reaction. Final concentrations of octanoyl-CoA and NEM were 100 μM and 0.2 mM, respectively. The rate of reduction of DCIP was calculated from the change in absorbance (600 nm) during the first minute of reaction at 30°C.

control ETF activities. After FAD addition, MAD:M ETF activities ranged from 30% to 57% of control ($P < 0.03$); activities in MAD:S lines 1400, 1401, and 1441 ranged from 2% to 10% of control ($P < 0.001$) and from 36% to 53% of control in MAD:S lines 1515, 1520, and 1540 ($P < 0.04$). Although FAD did not change ETF activities in the MAD:M and these six MAD:S lines ($P > 0.2$), in MAD:S line 1430, derived from a patient surviving to 7 mo (13), FAD raised ETF activity from 4% to 21% of control ($P = 0.01$). When the results of those experiments assaying ETF

Table IV. Dependence of Pig Liver ETF Activity on Pig Liver MCADH

	ETF	
MCADH	0.88	2.5
pmol	*pmol DCIP reduced/min*	
41.2	18	63
82.5	30	78
165	46	98
495	54	108

Pig liver ETF was assayed with increasing amounts of pig liver MCADH and 50 μM octanoyl-CoA in 20 mM KP, 7.6. The amounts of ETF and MCADH are expressed in picomoles of flavin. The rate of DCIP reduction was calculated from the change in absorbance (600 nm) during the first minute of the reaction at 30°C.
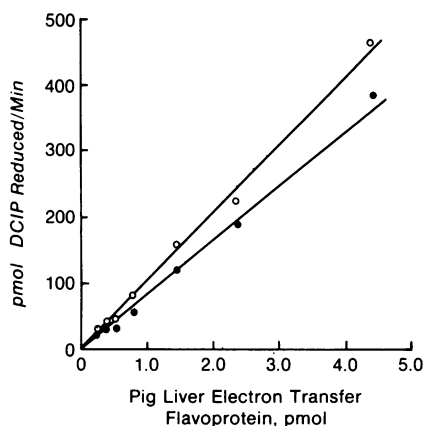
Figure 2. The dependence of apparent ETF activity on added pig liver ETF. Varying amounts of pure ETF were added to buffer alone (20 mM KP$_i$, pH 7.6, ●) or buffer containing 500 μg of MAD:S MS protein/assay and 0.2 mM NEM (○). ETF assays are described in Methods.

with and without FAD in the same MS preparation were analyzed separately, activity in line 1430 still increased significantly (one-tailed $P = 0.04$).

To determine whether an ETF inhibitor was present in MAD mitochondria, equal volumes of control, MAD:S and/or MAD:M MS, were mixed pairwise, incubated at 30°C for 3 min, and assayed for ETF activity. In nine different combinations, observed ETF activities ranged from 82% to 112% of the predicted values, thereby excluding the existence of an ETF inhibitor in MAD MS (data not shown).

ETF activities in cell lines 1515, 1520, and 1540 are significantly greater than the other four MAD:S patients under all conditions of assay ($P < 0.01$). Recently, Frerman and Goodman (25) found absent ETF-DH activity in these cell lines. Analogous to the lowered ADH activities found in all MAD:S cell lines (Table II), the decreased ETF activities in these ETF-DH deficient lines could result from increased turnover and/or instability of ETF reduced by the ADH in vivo. However, both pig liver and control fibroblast MS ETF resisted thermal denaturation at 37°C in the reduced state for 60 min, whereas oxidized ETF lost up
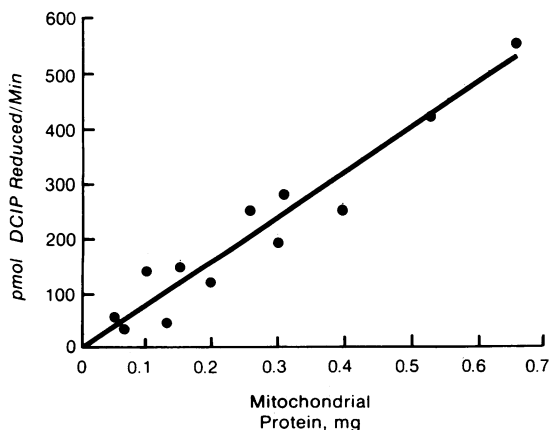

Figure 3. Dependence of apparent ETF activity on added fibroblast mitochondrial protein. Varying amounts of control MS were used as the enzyme source; the results of two separate experiments are combined. ETF assays are described in Methods.

Table V. ETF Activities in MAD Fibroblast Mitochondrial Supernatants

	Additions	
Cell lines	None	FAD, 20 μM
	pmol DCIP reduced/min/mg protein±SEM	
Normal controls	886±57	859±67
MAD:M		
493	307±56‡	425±153‡
632	454±44‡	322±60‡
741	254±39‡	418±93‡
840	257±28‡	254±39
930	338±35‡	488±100*
MAD:S		
1400	26±13‡	13±4‡
1401	53±12‡	86±42‡
1430	29±12‡	184±67‡
1441	46±9‡	40±28‡
1515	288±61‡	434±122*
1520	310±40‡	312±94‡
1540	324±49‡	456±97*

Mitochondria were isolated as described (27) and ETF activity was measured by the dye reduction assay. Pig liver MCADH (165 pmol) initiated the reaction, with 0.1–0.5 mg of mitochondrial protein as the ETF source. The concentration of octanoyl-CoA was 50 μM and of NEM, 0.2 mM. The rate of reduction of DCIP was calculated from the change in absorbance (600 nm) during the first minute of the reaction at 30°C. The number of determinations ranged from 1 to 6 for each of nine control lines and 5 to 15 for each MAD line. 20 μM FAD was added in the indicated experiments, with the number of determinations ranging from one to three for each of seven control lines and two to five for each MAD line.
* $P < 0.05$.
‡ $P < 0.01$.

to 22% of its initial activity under the same conditions (Table VI; other data not shown). The origin of ETF activity reductions in the ETF-DH-deficient lines remains unclear.

Table VI. Effect of Octanoyl-CoA and MCADH on the Thermal Stability of Fibroblast ETF Activity

Incubation time	Octanoyl-CoA.........0 MCADH.............0	10 μM 4.0 pmol
min	pmols DCIP reduced/mg protein/min	
0	874	861
10	860	884
20	845	873
30	723	879
40	676	875
60	682	884

Control fibroblast mitochondrial supernatant was used as the source of ETF activity. One half of the sample was reduced with octanoyl-CoA and pig liver MCADH; both oxidized and reduced samples were incubated at 37°C and assayed at 10-min intervals. The activity was determined as described in Table V. Final concentrations of octanoyl-CoA and NEM were 50 μm and 0.2 mM, respectively.

Discussion

We and others have demonstrated that the oxidation of ^{14}C-labeled precursors of many acyl-CoA's was impaired in intact MAD fibroblasts. These oxidative defects are generally severe in MAD:S cell lines and milder in MAD:M cell lines. Consistent with these results, we found severely depressed oxidation of ^{14}C-octanoate, -palmityl-CoA, and -glutarate in isolated MAD:S mitochondria (<18% of control) and more mild oxidative defects in MAD:M mitochondria (20–46% of control). Our findings are similar to Christensen's results in lines 1441, 1515, and 1540 (23); he also concluded that either ETF or ETF-DH deficiency caused MAD:S. However, we demonstrated an oxidative defect in MAD:M line 632, which was indistinguishable from control in his fibroblast homogenate system. Our results clearly separate the MAD into two variants with differing biochemical severity at the mitochondrial level.

All the acyl-CoA's accumulated in the MAD are subsequently dehydrogenated in the mitochondrial matrix. For straight-chain fatty acids, this first reaction of the β-oxidation spiral is catalyzed by the three flavoprotein ADHs, SCADH, MCADH, and LCADH, that oxidize short-, medium-, and long-chain fatty acyl-CoA's, respectively (30, 31, 33). Other mitochondrial flavoprotein dehydrogenases include isovaleryl-CoA, 2-methyl branched-chain acyl-CoA, glutaryl-CoA, sarcosine, and dimethylglycine dehydrogenases; none of these eight enzymes share a common subunit (14, 34–36). The first six ADHs are homotetramers (α_4) containing four ionically bound FAD/mole (33–35; S. Goodman, personal communication), while sarcosine and dimethylglycine DH are monomers containing one covalently bound FAD/mole (36). The reduced dehydrogenases are reoxidized by a second mitochondrial matrix enzyme, ETF (31, 37–39). ETF then transfers electrons via ETF-DH to ubiquinone in the electron transport chain (40–42).

We have assayed four ADHs in MAD cell and mitochondrial supernatants using both dye-reduction and tritium-release assays. Both methods assay the ADHs accurately and the observed activities are not dependent on endogenous ETF or ETF-DH activities. In MAD cell supernatants, ADH activities were normal (>63% of control; $P > 0.1$) as measured by the tritium-release assay. In MAD:M mitochondrial supernatants, we generally found normal or near normal ADH activities. However, MAD:S ADH activities were moderately but significantly decreased and ranged from 38% to 73% of control depending on the cell line and activity considered. ADH activities in the ETF-deficient cell lines (1400, 1401, 1430, 1441) were identical to those in the ETF-DH deficient MAD:S lines (1515, 1520, and 1540; $P > 0.2$). These decreases are not explained by lability of substrate-reduced ADH, in that octanoyl CoA reduction clearly stabilized, rather than destabilized, MCADH against thermal denaturation. Because no specific inhibitors of either ETF or ETF-DH have been identified that are compatible with long-term cell survival, we cannot model these ADH activity decreases in cultured fibroblasts.

Inasmuch as we alone have assayed the ADH in MAD:S fibroblast mitochondria, direct comparisons with the data of others remain difficult. However, some ADH assay results in whole cell homogenates substantiate our present observations. Frerman and Goodman (25) found that SCADH, MCADH, LCADH, and IVDH activities averaged 60–88% of control in cell homogenates from five MAD:S cell lines, four of which we also studied. Bennett et al. (11) found 51% of control SCADH

activity in liver homogenates from a single MAD:S patient. Our results might be explained by our recovering more effectively a relatively labile ADH fraction from control mitochondria that is lost in control whole cell homogenates.

Catalytic turnover of reduced ADHs requires their reoxidation by another mitochondrial matrix enzyme, ETF. ETFs isolated from many mammalian sources are heterodimeric proteins with molecular weights of ~60,000 containing one ionically bound FAD/mole (38, 39). Peptide mapping and amino acid analysis indicate that the two subunits are of different primary structure (38). The mutual affinities of ETF, the ADH and ETF-DH for one another are extremely high (<1 μM) and ETF and ETF-DH may form an ionic complex in vivo (41). Inasmuch as our attempts to quantitate fibroblast mitochondria ETF spectrofluorometrically were unsuccessful owing to high background fluorescence (data not shown), we assayed ETF activity by modifying the classical dye-reduction method. The quantitative recovery of pig liver ETF added to MAD:S MS, linear response to MS protein, use of saturating amounts of MCADH, and inhibition by ETF antisera all confirm that we are accurately measuring ETF activity. Our results also demonstrate that apparent ETF activity is markedly less than the sum of apparent SCADH, MCADH, LCADH, and IVDH activities in control fibroblast MS when assayed under analogous conditions (Tables II and V). If α-methyl ADH and glutaryl-CoA DH activities are roughly comparable to those of SCADH, LCADH, and IVDH (14, 43), then ETF activity is at least five- to seven-fold lower than the sum of those six ADHs from which it accepts electrons. Analogously, Sakurai et al. (44) found ETF activity to be 20-fold lower than ADH activities in rat liver mitochondria. In that any significant reductions in ETF activity should decrease substantially substrate flux through the ADHs, intermediate ETF activities, as in MAD:M, may produce acyl-CoA accumulation with the resulting organic aciduria and clinical symptoms.

With this assay, we found reduced ETF activity in all five MAD:M lines, and we have demonstrated profoundly deficient ETF activity (<6% of control) in four MAD:S lines. Combined with their clinical findings, whole cell and intact mitochondrial oxidations, and ADH activities, the biochemical etiology of MAD:S in these four patients is clearly ETF deficiency. In cell line 1400, derived from the first MAD:S patient (P.K.; 1), ETF activity was 3% of control. In earlier preliminary studies with this cell line, Rhead et al. (22) found normal ETF activity (159% of control). However, in those experiments, NEM was not used to sequester free CoASH produced by thioesterase-mediated octanoyl-CoA hydrolysis. This free CoASH in turn artefactually reduced DCIP, as evidenced by apparent ETF activities in control MS fourfold greater (22) than those found in this study (Table V). Using higher amounts of pig liver MCADH in these experiments (165 vs. 102 pmol flavin) also increased assay sensitivity.

Although deficient cellular riboflavin uptake, FAD synthesis, and/or mitochondrial FAD uptake could explain the ADH, ETF, and/or ETF-DH deficiencies of the MAD, incorporation of ^{14}C-labeled riboflavin into FMN and FAD has been normal in kidney and/or fibroblast homogenates from several MAD:S patients (45, 46). FAD addition did not increase ETF activities in control, MAD:M, or six of the seven MAD:S cell lines, all of the latter group dying before 2 mo of age. However, in cell line 1430, derived from a patient who survived to 7 mo, 20 μM FAD increased residual ETF activity fivefold. Two other observations are important regarding this patient. First, a female sibling with a similar clinical presentation survived to 5 mo, consistent with

the milder clinical course observed in other vitamin-responsive inborn errors of metabolism (47). Second, the fibroblasts of line 1430 oxidize [1-^{14}C]butyrate at 16% of control after culture for 2 wk in high riboflavin concentrations (2 mg/ml) and at 3% of control after culture in riboflavin-deficient medium (1.4 μg/liter); control fibroblasts oxidize [^{14}C]butyrate equally well under both conditions (data not shown). The ETF activity increase in line 1430 may result from FAD binding to an ETF apoenzyme with a lowered affinity for the cofactor and partially restoring enzymatic activity. FAD addition produces qualitatively similar ETF activity increases in riboflavin-depleted normal fibroblasts (data not shown), confirming that ETF holoenzyme can be reconstituted from the apoenzyme under other conditions. In sum, these data suggest that line 1430 is a ETF-deficient MAD:S patient responsive to FAD in vitro.

Although 2,3-enoyl-CoA's, 3-keto-acyl-CoA's, and ETF-semiquinone inhibit the ADH (33, 48), inhibition of ETF or ETF-DH by β-oxidation intermediates has not been studied. We found no evidence of ETF inhibitors in the MAD; MS from the ETF-deficient MAD:S cell lines did not inhibit the reaction of pure pig liver MCADH and ETF (Fig. 2; other data not shown). Mixing experiments among MAD:M, MAD:S, and control MS yielded ETF activities close to the predicted values, giving no evidence of an unknown accumulated metabolite causing secondary ETF deficiencies in the MAD.

In MAD:S patients 1515, 1520, and 1540, whose organic acidurias, whole cell oxidation studies, and ADH activities are virtually identical to those of ETF-deficient patients, ETF activities ranged from 36% to 41% of control and were not significantly changed by FAD addition. These three patients were also distinguished by a characteristic facial appearance, polycystic kidneys, and other congenital anomalies not present in any of the ETF-deficient MAD:S patients (3, 5, 6, 9, 12). Frerman and Goodman (25) have recently shown that these three MAD:S cell lines are severely deficient (≤0.6% of control) in ETF-DH activity. In lines 1515 and 1540 (their 1730 and KH), ETF-DH polypeptide antigen was absent using immunoblots, whereas in line 1520 (their 1691), small amounts of ETF-DH antigen were detected. They also found normal ETF-DH activity and antigen in cell lines 1400 and 1441 (their 1196 and 1441). We were unable to detect the characteristic (g_z = 2.08; 40, 41) electron paramagnetic resonance signal of reduced ETF-DH in control or MAD fibroblast mitochondrial inner membranes at 11–13°K (data not shown). In collaboration with us, Beckman and Frerman (42) have assayed ETF-DH activities in these five MAD:M cell lines using the same comproportionation method. In cell lines 493, 632, and 741, ETF-DH activities were 16%, 21%, and 50% of control, respectively, and were normal (>80% of control) in lines 840 and 930 (F. Frerman, personal communication). Although it is impossible to directly compare absolute ETF and ETF-DH specific activities obtained in our respective enzymatic assays owing to marked differences in cellular subfractions and assay conditions used, these observations suggest that intermediate deficiencies of either ETF-DH (lines 493, 632, and 741) or ETF (lines 840 and 930) can produce a MAD:M phenotype.

Frerman and Goodman (25) found decreased amounts of both ETF α- and β-subunits in line 1441 and abnormal mobility of the α-subunit; in line 1400, only small amounts of a α-subunit with abnormal electrophoretic mobility were detected. Tanaka and co-workers (49) have extended these results by radiolabeling these MAD:S lines with [^{53}S]methionine, followed by immunoprecipitation of labeled fibroblast ETF and analysis by elec-

trophoresis. In lines 1400, 1430, and 1441 (their 605, 1313, and 1391, respectively), the ETF β-subunit was synthesized and migrated normally, in contrast to the results of Frerman and Goodman (25). In line 1400, no detectable α-subunit was synthesized, and in line 1430, small amounts of an unstable α-subunit of normal mobility were detected and the α-subunit in line 1441 had a mol wt 1,000 less than normal. They concluded that defective synthesis of the ETF α-subunit was the primary lesion in these three lines. No abnormalities of either ETF subunit were found in MAD lines 493, 632, 741, 1515, and 1520. These studies have extended our understanding of the molecular heterogeneity underlying the MAD and are consistent with the enzymatic assays reported here, inasmuch as ETF activity in line 1400 was not above background in the majority (10/15) of the assays performed. The 5% of control activity noted in line 1441 might reflect true residual activity catalyzed by an abnormal ETF heterodimer. In line 1430, FAD may stabilize a labile apoenzyme and partially restore ETF activity. Further biochemical and immunochemical analyses are necessary to confirm these suggestions.

Other important questions remain unanswered regarding the MAD. Because ETF-DH-deficient MAD:S patients are one of the few disorders of intermediary metabolism associated with disturbed morphogenesis, they represent a promising area for future investigations into the relationship of cellular energy metabolism and malformation syndromes. Although presumably secondary to the primary enzymatic defects, the moderately decreased ADH and ADH/ETF activities found in the ETF- and ETF-DH-deficient MAD:S cell lines, respectively, remain unexplained. We have shown that these decreases do not reflect short-term instability of either ADH or ETF reduced by substrate. Unfortunately, there are no known specific in vivo inhibitors of ETF or ETF-DH to permit our studying these phenomena in intact normal fibroblasts. The available data confirm both the biochemical and phenotypic similarities between MAD:M and MAD:S and emphasize the enzymatic and molecular heterogeneity underlying defective electron transfer from the β-oxidation spiral to the electron-transport chain.

Acknowledgments

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