Catalytic Defect of Medium-chain Acyl-Coenzyme A Dehydrogenase Deficiency

Lack of Both Cofactor Responsiveness and Biochemical Heterogeneity in Eight Patients

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Abstract

Medium-chain acyl-coenzyme A (CoA) dehydrogenase (MCADH; EC 1.3.99.3) deficiency (MCD) is an inborn error of β-oxidation. We measured ^3H_2O formed by the dehydrogenation of [2,3-^{3}H]acyl-CoAs in a ^{3}H-release assay. Short-chain acyl-CoA dehydrogenase (SCADH; EC 1.3.99.2), MCADH, and isovaleryl-CoA dehydrogenase (IVDH; EC 1.3.99.10) activities were assayed with 100 μM [2,3-^{3}H]butyryl-, -octanoyl-, and -isovaleryl-CoAs, respectively, in fibroblasts cultured from normal controls and MCD patients. Without the artificial electron acceptor phenazine methosulfate (PMS), MCADH activity in fibroblast mitochondrial sonic supernatants (MS) was 54% of control in two MCD cell lines ($P < 0.05$). Addition of 10 mM PMS raised control acyl-CoA dehydrogenase activities 16-fold and revealed MCADH and SCADH activities to be 5 ($P < 0.01$) and 73% ($P > 0.1$) of control, respectively. Thus, the catalytic defect in MCD involves substrate binding and/or dehydrogenation by MCADH and not the subsequent reoxidation of reduced MCADH by electron acceptors. 20 μM flavin adenine dinucleotide (FAD) did not stimulate MCD MCADH activity in either the ^{3}H-release or electron-transfer(ring) flavoprotein-linked dye-reduction assays. Mixing experiments revealed no MCADH inhibitor in MCD MS; IVDH activities were identical in both control and MCD MS. In postmortem liver MS from another MCD patient, ^3H_2O formation from [2,3-^{3}H]octanoyl-CoA was 15% of control. When ^3H_2O formation was assayed with 200 μM [2,3-^{3}H]acyl-CoAs, 15 mM PMS, and 20 μM FAD in fibroblast sonic supernatants from seven MCD cell lines, SCADH, MCADH, and IVDH activities were 72–112% ($P > 0.1$), 4–9% ($P < 0.01$), and 86–135% ($P > 0.1$) of control, respectively, revealing no significant biochemical heterogeneity among these patients.

Introduction

Medium-chain acyl-coenzyme A (CoA)[1] dehydrogenase (MCADH) deficiency (MCD), first described clinically in 1976, is an inborn error of fatty acid metabolism due to a deficiency of the acyl-CoA dehydrogenase (ADH) catalyzing the first step in the β-oxidation of medium-chain-length fatty acids (1–5). This disorder generally presents with nonketotic hypoglycemia, acidosis, and elevated urinary excretion of straight-chain C_6-C_{10}-ω-dicarboxylic acids, although some patients in remission excrete few or no abnormal metabolites (1, 4–6). These dicarboxylic acids are formed via ω-oxidation of accumulated C_{10}-C_{12}-monocarboxylic acids, which are then shortened by β-oxidation to medium-chain lengths (7, 8). Although a dicarboxylic aciduria has been noted in patients with glycogen storage disease, congenital lactic acidosis, severe ketoacidosis, multiple acyl-CoA dehydrogenation deficiency (glutaric aciduria type II), and systemic carnitine deficiency (9–13), the isolated excretion of straight-chain C_6-C_{10}-dicarboxylic acids without associated ketosis in MCD is consistent with the defective mitochondrial β-oxidation produced by MCADH deficiency.

We first demonstrated deficient MCADH activity in MCD fibroblast mitochondrial sonic supernatants (MS) with an electron-transfer(ring) flavoprotein (ETF)-linked dye-reduction assay (2). We describe here a tritium-release ADH assay employing [2,3-^{3}H]acyl-CoAs as substrates. In MS and cell sonic supernatants (CS) from two MCD cell lines, this assay demonstrates that MCADH deficiency is secondary to defective acyl-CoA binding and/or dehydrogenation. We also show MCADH deficiency in CS from the patient first studied by Gregersen et al. (1) and Kolvraa et al. (3) and in CS or liver MS from five previously unstudied patients with the clinical phenotype and organic aciduria of MCD.

Methods

[2,3-^{3}H]Butyric acid (*n*-butanoic acid; 10 mCi/mmol), [2,3-^{3}H]octanoic acid (5 mCi/mmol), and [2,3-^{3}H]isovaleric acid (3-methyl-butanoic acid; 10 mCi/mmol) were obtained from New England Nuclear, Boston, MA. Phenazine methosulfate (5-methyl-phenazinium methyl sulfate; PMS) and flavin adenine dinucleotide (riboflavin 5′-adenosine diphosphate; FAD) were obtained from Sigma Chemical Co., St. Louis, MO. [2,3-^{3}H]Acyl-CoA esters were synthesized in our laboratory by a method previously described (14).

For the MS and CS assays summarized in Tables I, II, and IV and Figs. 1–4, skin fibroblasts were used from two unrelated MCD patients we studied earlier (S.G. and G.W.; 2, 5, 15, 16), six normal male infants, and two normal adults. For the CS assays summarized in Table V, we assayed ADH activities in unrelated patients A.M. and R.B., identified clinically by Truscott et al. (6), Gregersen et al. (1), and Kolvraa et al. (3), respectively; patients X.D., S.F., B.M., and B.V. presented with straight-chain C_6-C_{10}-dicarboxylic aciduria (all), hyperammonemia and acidosis (S.F., M.B.), and hypoketotic hypoglycemia (X.D., B.M., B.V.). Fibroblasts from X.D., S.F., and B.M. were provided by Drs. Priscille Divry, Hôpital DeBrousse, Lyon, France, Ira Brandt, Indiana University, Indianapolis, and Hélène Ogier, Hôpital Necker-Enfants Malades, Paris, respectively. A liver sample was obtained from B.V. postmortem by Dr. M. Duran, University Children's Hospital, Utrecht, and frozen at −70°C until assay; a fibroblast line was not established on this patient. Control liver samples from six infant and adult patients without ADH deficiencies

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1. *Abbreviations used in this paper:* ADH, acyl-CoA dehydrogenase; CoA, coenzyme A; CS, cell sonic supernatants; ETF, electron-transfer(ring) flavoprotein; FAD, flavin adenine dinucleotide; IVDH, isovaleryl-CoA dehydrogenase; MCADH, medium-chain acyl-CoA dehydrogenase; MCD, medium-chain acyl-CoA dehydrogenase deficiency; MS, fibroblast mitochondrial sonic supernatants; PMS, phenazine methosulfate; SCADH, short-chain acyl-CoA dehydrogenase.

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were obtained 1–5 h postmortem and frozen at −70°C before assay. Full clinical descriptions of all MCD patients will be published separately.

Cells 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 medium was changed weekly. For MS assays, cells from three 750-cm^2 glass roller bottles of each cell line (passage numbers 9–18) were harvested by trypsinization, and mitochondria were isolated by a method described earlier (17), except that digitonin treatment of the mitochondria was omitted. Mitochondria were sonicated in 0.4–0.6 ml of 20 mM KPi, pH 7.6, using a sonic disruptor (model 300 with a microtip; Fisher Scientific Co., Allied Corp., Pittsburgh, PA) at 0°C for 30 s; the sonicates were then centrifuged at 100,000 *g* for 1 h. The MS were then assayed as described below. CS were obtained from two 150-cm^2 culture flasks for each cell line (passage numbers 7–13); the cells were sonicated in 0.4–0.6 ml of 10 mM KPi, pH 7.6, containing 20 μM FAD, using the sonic disruptor (Fisher Scientific Co.) at 0°C for 1 min, and centrifuged 100,000 *g* for 1 h. The CS obtained were assayed immediately.

The ETF-linked dye-reduction MCADH assay was done in fibroblast MS as described earlier with 0.2 mM *N*-ethylmaleimide, 50 μM octanoyl-CoA, and 440 pmol of pure pig liver ETF provided by Dr. Carole L. Hall, Georgia Institute of Technology (2). The tritium-release assay was performed in duplicate as described earlier (14, 17), with these modifications: the reaction mixture contained 0.1 ml of sonic supernatant (MS: 0.1–0.3 mg protein; CS: 1.0–2.5 mg protein), 10 mM PMS, and 100 μM [2,3-^{3}H]acyl-CoAs in MS assays, and 15 mM PMS, 20 μM FAD, and 200 μM [2,3-^{3}H]acyl-CoAs in CS assays. The final volume was 0.13 ml; 20 μM FAD was added in the MS experiments displayed in Figs. 1 and 2. The assay mixture was incubated in 1-ml capped tubes at 37°C for 15 min in all MS experiments and for 10 min in all CS experiments; the reaction was stopped by cooling to 0°C. The ^3H_2O formed was separated from unreacted substrate by anion exchange chromatography on an AG-1 column (Bio-Rad Laboratories, Richmond, CA) and counted as described earlier (14). For patient B.V. and autopsied controls, human liver mitochondria were isolated using our published method for rat liver mitochondria (14). Liver MS was assayed with the tritium-release assay used for fibroblast MS, once immediately after isolation and again after storage for 2 wk at −70°C; ADH activities in B.V. and control MS were identical in both experiments (data not shown; $P > 0.2$).

In CS experiments, each tritium-release assay was performed in quadruplicate: one set of duplicates was counted directly, while the second set was lyophilized as we described previously (14, 18). Although most of the radioactivity passing through the anion exchange column is contained in ^3H_2O and is lyophilizable (>95% in control MS; 67–86% in control CS), some nonlyophilizable ^{3}H compounds, such as ^{3}H-acyl carnitines, are produced during the assay and remain after lyophilization of the eluate (14, 18, 19). The nonlyophilizable radioactivity, termed artifactual activity, is subtracted from total radioactive counts, yielding accurate quantitation of ^3H_2O released by enzymatic detritiation of the substrate. In the experiments summarized in Tables I, II, IV, and V, control and MCD MS and CS from each cell line were assayed individually. In Figs. 1–4, control and MCD MS and CS from several different cell lines of the same type were pooled before assay; the data display rates of ^3H_2O formation corrected for nonlyophilizable artifactual activity. The estimate of variance used is the standard error of the mean. Means were compared with the *t* test; all *P* values given are two-tailed and compare MCD ADH activities to the respective control values unless otherwise stated.

Results

In studies of ADH activities in isovaleric acidemia, Rhead and Tanaka (17) developed assays for short-chain acyl-CoA dehydrogenase (SCADH) and isovaleryl-CoA dehydrogenase (IVDH) that used [2,3-^{3}H]butyryl- and -isovaleryl-CoAs as substrates (14). Enzymatic removal of ^{3}H from carbons 2 and 3 of the substrate forms ^3H_2O, which is separated quantitatively from unreacted substrate by anion exchange chromatography. Here we describe a tritium-release assay for MCADH activity in fibroblasts that uses [2,3-^{3}H]octanoyl-CoA as substrate. In both MS and CS, tritium release from this substrate displayed characteristics similar to those observed with [2,3-^{3}H]isovaleryl- and -butyryl-CoAs. In fibroblast and liver MS, tritium release from all three substrates is linear with respect to added protein and time in the presence of PMS and FAD (Figs. 1 and 2; 14, 17). In CS, tritium release from [2,3-^{3}H]butyryl- and -octanoyl-CoAs was linear with respect to protein (Fig. 3). The deviation from linearity observed with [2,3-^{3}H]isovaleryl-CoA obliged us to use a fairly constant amount of CS protein (1.5–2.5 mg/assay) when employing this substrate. In CS, the rate of tritium release from [2,3-^{3}H]butyryl- and -octanoyl-CoAs declined after 10 min (Fig. 4). Thus, for convenience, CS assays with all three substrates were terminated at 10 min. In control fibroblast and liver MS incubated with [2,3-^{3}H]octanoyl-CoA, 96±5 and 98±2% of the total radioactivity passing through the column were lyophilizable, respectively, and represented ^3H_2O formation. In control CS incubated with this substrate, 86% of the total radioactivity was contained in ^3H_2O (Tables I, III, and IV).

Without PMS or FAD, [2,3-^{3}H]octanoyl-CoA detritiation in MCD MS was 54% of control ($P < 0.05$; Table I); in both control and MCD MS, similar proportions of the eluate radioactivity were contained in ^3H_2O. Since PMS, an artificial electron acceptor, greatly stimulated tritium release by fibroblast and rat liver MS and by the purified pig and rat liver enzymes under similar assay conditions (14, 17), we used this compound in our assay. Adding 10 mM PMS raised control MS ADH activities 16-fold, while MCD MCADH activity fell to 5% of control ($P < 0.01$), and SCADH activity was 73% of control ($P > 0.1$; Table I). MCADH activity in MCD MS remained 5.5% ($P < 0.01$) of control after addition of 20 μM FAD in the presence

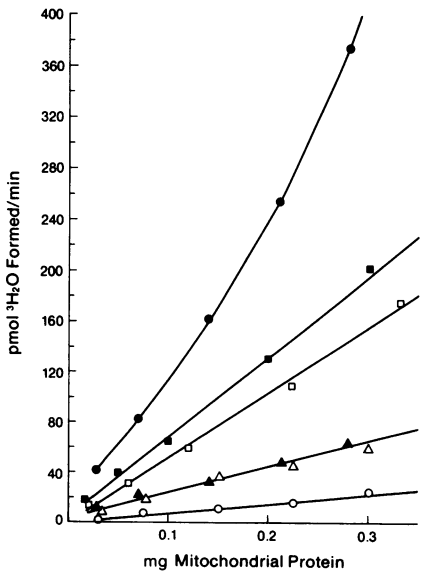

Figure 1. The dependence of [2,3-^{3}H]acyl-CoA dehydrogenation on added mitochondrial protein. Varying amounts of MS were used as the enzyme source. Tritium-release assays are described in Methods. Closed and open symbols correspond to control and MCD mitochondria, respectively. Substrates are [2,3-^{3}H]octanoyl-CoA (●, ○), -butyryl-CoA (■, □), and -isovaleryl-CoA (▲, △).

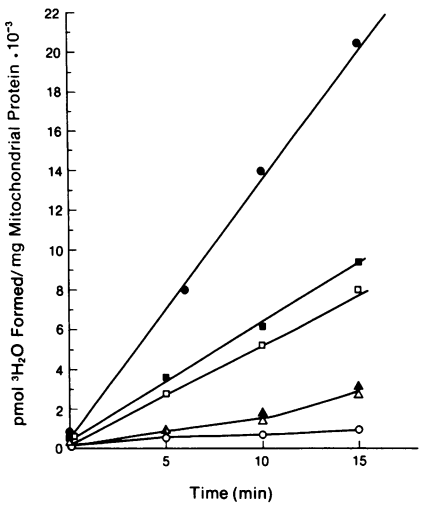

Figure 2. Time course of [2,3-^{3}H]acyl-CoA dehydrogenation in fibroblast mitochondria. MS were used as the enzyme source. The tritium-release assays are described in Methods; the reaction was stopped at the indicated intervals. The symbols for cell lines and substrates are defined in the legend to Fig. 1.

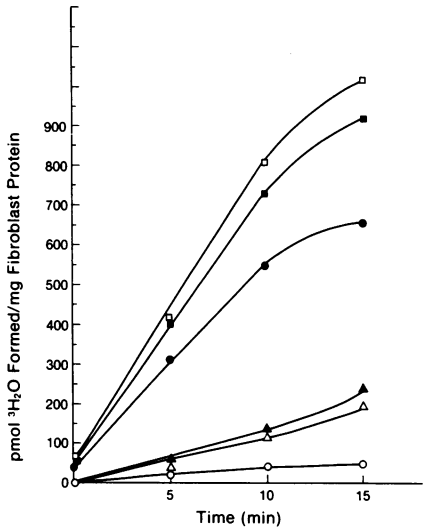

Figure 4. Time course of [2,3-^{3}H]acyl-CoA dehydrogenation in fibroblasts. Fibroblast sonic supernatants were used as the enzyme source. Tritium-release assays are described in Methods; the reaction was stopped at the indicated intervals. Symbols are defined in the legend to Fig. 1.

of PMS. Similarly, FAD addition did not raise control or MCD MCADH activities in the ETF-linked dye-reduction assay ($P > 0.8$; Table II). Lyophilization studies revealed that rates of ^3H_2O formation in MCD MS were only 3% of control (see footnote to Table I). SCADH and IVDH activities in MCD MS were 86 and 96% of control, respectively ($P > 0.1$), with added FAD and PMS (Table I). In separate experiments, equal amounts of MCD and control fibroblast MS were mixed, revealing 51% of control specific activity and confirming that no MCADH inhibitor is present in MCD MS (4; $n = 2$, data not shown).

In liver MS from patient B.V., SCADH and MCADH activities were 99 and 15% of control, respectively (Table III). Control MCADH to SCADH activity ratios were 6.7-fold higher than the patient's, consistent with an isolated MCADH deficiency. These observations do not result from postmortem ADH activity losses since MS isolated from rat liver incubated in situ at 25°C for 5 h before freezing retained ≥80% of control SCADH and MCADH activities (data not shown).

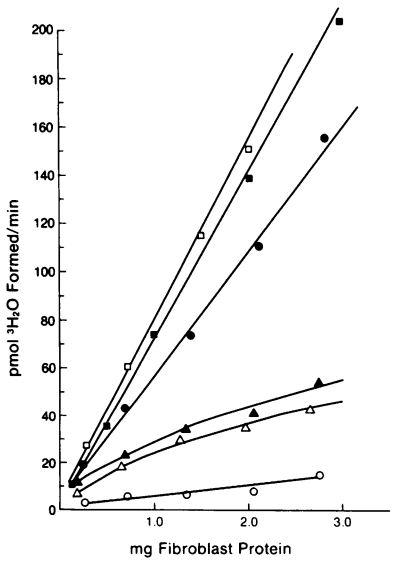

Figure 3. The dependence of [2,3-^{3}H]acyl-CoA dehydrogenation on added fibroblast protein. Varying amounts of fibroblast sonic supernatants were used as the enzyme source. Tritium-release assays are described in Methods; symbols are defined in the legend to Fig. 1.

We then employed this assay method to measure ADH activities in MCD CS. With 15 mM PMS and 20 μM FAD, apparent MCADH activity in MCD CS was 23% ($P < 0.05$) of control, and SCADH and IVDH activities were 118 and 121% of control, respectively ($P > 0.1$; Table IV). While these data demonstrate defective MCADH activity in MCD CS, the proportion of residual apparent MCADH activity in MCD CS is higher than predicted from the MS assays. Using a lyophilization procedure to quantitate precisely the ^3H_2O formed (14, 17), MCADH activity in MCD CS was determined to be only 8% of control ($P < 0.01$), while SCADH and IVDH activities were 112 and 86% of control, respectively ($P > 0.1$; Table IV), demonstrating MCADH deficiency in crude MCD fibroblast preparations. We then assayed ADH activities in CS from five other patients with the clinical presentation and organic aciduria of MCD, in four of whom ADH activities had not been assayed previously (Table V). SCADH, MCADH, and IVDH activities were 73–85% ($P > 0.1$), 4–9% ($P < 0.01$), and 105–135% ($P > 0.1$) of control, respectively, in these cell lines, similar to the first two MCD patients we studied here (Table IV; $P > 0.1$).

Discussion

Since the tentative identification of the first case in 1976, MCD has become recognized as a clinically and biochemically distinct inborn error of fatty acid metabolism (1, 4–6). While some patients are asymptomatic, the clinical phenotype is commonly characterized by episodic hypoglycemia, often provoked by fasting, unaccompanied by ketosis, and frequently associated with urinary excretion of straight-chain C_6-C_{10}-ω-dicarboxylic acids. These compounds are also excreted in other disorders, such as systemic carnitine deficiency and the multiple acyl-CoA dehy-

Table I. Enzymatic Detritiation of [2,3-^{3}H]Acyl-CoAs in MS

	[2,3-^{3}H]Butyryl-CoA		[2,3-^{3}H]Octanoyl-CoA			[2,3-^{3}H]Isovaleryl-CoA
Origin of mitochondria	Additions: PMS	PMS/FAD	None	PMS	PMS/FAD	PMS/FAD
			pmol product/mg protein per min±SEM			
Normal controls (*n*)	493±85 (5)	646±88 (3)	83±6.0* (3)	1,376±215 (4)	1,490±257§ (4)	175±44 (2)
MCD patients (*n*)	362±70 (5)	556±38 (3)	45±8.0‡ (3)	66±10 (4)	82±10‖ (4)	168±37 (2)

100 μM [2,3-^{3}H]butyryl- (10 mCi/mmol), -octanoyl- (5 mCi/mmol), and -isovaleryl-CoAs (10 mCi/mmol) were incubated with 0.5–2.0 mg MS protein and 20 μM FAD and/or 10 mM PMS for 15 min at 37°C. The ^3H_2O formed was separated from unreacted substrate by passage of the reaction mixture over an AG-1 anion exchange column; duplicate samples of the column eluates were then lyophilized at pH 6. The proportion of lyophilizable radioactivity represents ^3H_2O formed by detritiation of the substrates. Proportion of lyophilizable radioactivity: * 70±7% (*n* = 3); ‡ 64±7% (*n* = 3); § 96±5% (*n* = 2); ‖ 48±3% (*n* = 2).

drogenation disorders (12, 13), producing a generalized impairment of fatty acid oxidation. However, in three MCD patients, we demonstrated that accumulation and excretion of dicarboxylic acids resulted from an isolated deficiency in medium-chain fatty acid catabolism, since MCD fibroblasts and fibroblast mitochondria demonstrated specific deficiencies in [1-^{14}C]octanoate oxidation (2). Since MCADH is rate-limiting for β-oxidation of medium-chain-length fatty acids, we assayed MCADH with an ETF-linked dye-reduction assay. Using octanoyl-CoA as substrate, MCADH activity was only 5% of control in MCD MS. Other investigators have confirmed independently MCADH deficiency in MCD (3–5).

In demonstrating a specific IVDH deficiency in isovaleric acidemia, Rhead and Tanaka (14, 17) developed a sensitive, specific, and reproducible assay for the ADH in which ^{3}H release from [2,3-^{3}H]acyl-CoAs into solvent water reflects enzymatic dehydrogenation of the substrate. This method quantitates enzyme-catalyzed exchange of tritium with solvent occurring without net substrate oxidation, as well as the enhancement of substrate detritiation and oxidation by appropriate electron acceptors such as PMS or ETF, or their combination with dichlorophenol indophenol (14). Unlike the dye-reduction assay, the tritium-release assay is free from interferences by both nonspecific endogenous reductants and CoASH derived from thioesterase-catalyzed acyl-CoA hydrolysis (2, 20). This method can accurately measure ADH activities in crude tissue homogenates, intact and sonicated mitochondria, and purified enzymes, and has been extensively characterized in the rat liver and human fibroblast systems using [2,3-^{3}H]isovaleryl- and -butyryl-CoAs as substrates (14, 17, 18); here we use an analogous assay with [2,3-^{3}H]octanoyl CoA to assay MCADH activity in MCD fibroblasts and fibroblast and liver mitochondria.

Assayed without added electron acceptors such as ETF or PMS, apparent MCADH activity in MCD MS appeared relatively elevated (54% of control), which suggests that some residual MCADH-mediated substrate binding and catalysis of tritium exchange occurred in MCD. However, addition of PMS, a low molecular weight electron acceptor for all known straight- and branched-chain ADHs (21–24), increased MCADH activity 16-fold in control MS but only 47% in MCD MS, and revealed MCD MCADH activity to be 5% of control. In vivo, MCADH, reduced by substrate, can only be reoxidized by ETF, a heterodimeric protein of 60,000 mol wt containing one FAD per mole (24–26). While the available data suggest that ETF activity is limiting for ADH turnover in vivo and in vitro (21–26; other data not shown), the profound MCADH deficiency of MCD severely limits medium-chain acyl-CoA oxidation in affected patients, intact fibroblasts, and fibroblast mitochondria (1–5). The failure of MCADH in MCD to catalyze net octanoyl-CoA oxidation could result from defective reoxidation of a mutant MCADH holoenzyme by ETF, following normal substrate binding to and dehydrogenation by the enzyme. However, since PMS does not raise MCADH activity in MCD MS, defective

Table II. Effect of FAD on MCADH Activity in MS

	Addition	
Origin of mitochondria	None	FAD
	pmol DCIP reduced/mg protein per min±SEM	
Normal controls (*n*)	1,850±434 (6)	1,790±379 (6)
MCD patients (*n*)	134±34 (7)	106±20 (7)

0.2–0.4 mg of fibroblast MS protein was preincubated at 37°C for 1 min with and without 20 μM FAD. MCADH activity was then assayed as described (2) with 50 μM octanoyl-CoA, 440 pmol pig liver ETF, 0.2 mM *N*-ethylmaleimide, and 70 μM dichlorophenol indophenol (DCIP). The rate of DCIP reduction was calculated from the initial absorbance change at 600 nm.

Table III. Enzymatic Detritiation of [2,3-^{3}H]Acyl-CoAs in Liver MS

Origin of mitochondria	[2,3-^{3}H]Butyryl-CoA	[2,3-^{3}H]Octanoyl-CoA
	pmol product/mg protein per min±SEM	
Normal control liver (*n*)	1,020±103* (12)	3,190±398§ (12)
MCD patient (B.V.) (*n*)	1,010±216‡ (2)	471±129‖ (2)

100 μM [2,3-^{3}H]butyryl- (10 mCi/mmol) and -octanoyl-CoAs (5 mCi/mmol) were incubated with 0.2–0.5 mg liver MS protein, 20 μM FAD and 10 mM PMS for 15 min at 37°C. The ^3H_2O formed was separated from unreacted substrate by passage of the reaction mixture over an AG-1 anion exchange column; duplicate samples of the column eluates were then lyophilized at pH 6. The proportion of lyophilizable radioactivity represents ^3H_2O formed by detritiation of the substrates. Proportion of lyophilizable radioactivity: * 96±4% (*n* = 6); ‡ 100% (*n* = 1); § 98±2% (*n* = 6); ‖ 96% (*n* = 1).

Table IV. Enzymatic Detritiation of [2,3-^{3}H]Acyl-CoAs in CS

Origin of cell lines	[2,3-^{3}H]Butyryl-CoA			[2,3-^{3}H]Octanoyl-CoA			[2,3-^{3}H]Isovaleryl-CoA		
	Apparent activity	Nonlyophilizable artifact	^3H_2O formation	Apparent activity	Nonlyophilizable artifact	^3H_2O formation	Apparent activity	Nonlyophilizable artifact	^3H_2O formation
	pmol product/mg protein per min±SEM						*pmol product/mg protein per min±SEM*		
Normal controls (*n*)	92±5.0 (16)	18±1.0 (8)	74±5.0 (8)	66±4.0 (16)	9.0±0.6 (10)	57±4.0 (10)	21±4.0 (16)	7.3±1.0 (8)	14±4.0 (8)
MCD patients (*n*)	108±9.0 (8)	25±1.5 (4)	83±9.0 (4)	15±1.0 (8)	10.5±0.6 (6)	4.5±0.6 (6)	25±8.0 (8)	13±3.0 (4)	12±4.0 (4)

200 μM [2,3-^{3}H]butyryl- (10 mCi/mmol), -octanoyl- (5 mCi/mmol), and -isovaleryl-CoAs (10 mCi/mmol) were incubated with 1.5–2.5 mg CS protein, 20 μM FAD, and 15 mM PMS at 37°C for 10 min. After passage of the reaction mixture over AG-1 anion exchange resin, duplicate samples were lyophilized at pH 6 and the nonlyophilizable radioactivity remaining was taken as artifactual activity. Lyophilizable radioactivity represents ^3H_2O formed by detritiation of the substrates.

electron transfer from MCADH to electron acceptors probably does not contribute to deficient MCADH activity in MCD. These observations effectively localize the catalytic defect in MCD to deficient substrate binding and/or dehydrogenation by MCADH. This conclusion is strengthened by our assays being performed in a mitochondrial matrix subfraction, rather than cell or tissue homogenates. In addition, the tritium-release method possesses the catalytic specificity to support this conclusion, while assays using ETF as an electron acceptor do not (2, 4).

Other investigators have not systematically studied the effects of FAD addition on MCD MCADH activity in vitro, although Gregersen et al. (3) found no decrease in MCD MCADH activity when they lowered FAD concentration from 250 to 1 μM. Addition of 20 μM FAD, the ionically bound cofactor for all known ADH (21, 23, 24), increased MCADH activity little in control or MCD MS; MCD MCADH activity remained low at 5–6% of control using both the tritium-release and dye-reduction assays. These observations suggest that defective FAD binding to an altered MCADH apoenzyme does not explain MCADH deficiency in MCD. Since the Michaelis constants (K_m's) of the ADH for FAD are quite low, ranging from 0.43 to 2.4 μM (21–23), these data do not exclude the existence of a mutant MCADH apoenzyme in MCD with a K_m for FAD 100-fold greater than normal and/or the accelerated degradation of such a protein in vivo (27).

Combining enzyme preparations from two MCD cell lines did not change MCD MCADH activities in either MS or CS (Figs. 1–4), which suggests that no intragenic complementation is occurring between these cell lines under our assay conditions. Mixing of control and MCD MS in equal proportions yielded MCADH activities within 1% of the predicted values, effectively excluding the existence of an MCADH inhibitor in MCD (n = 2, data not shown). These mixing studies are informative, since deficiencies of other β-oxidation enzymes, such as 2-enoyl-CoA hydratase (crotonase; EC 4.2.1.17) or 3-ketoacyl-CoA thiolase (EC 2.3.1.16) could lead to intramitochondrial accumulation of 2-enoyl- or 3-ketoacyl-CoAs, potent inhibitors of the ADH (28). In sum, these data suggest that the altered MCADH holoenzyme in MCD MS catalyzes essentially no tritium release from [2,3-^{3}H]octanoyl-CoA. This catalytic defect is not corrected by addition of either physiologic (i.e., ETF; 2, 4) or nonphysiologic electron acceptors (i.e., PMS) or of FAD, and is not caused by a MCADH inhibitor present in MCD cell lines.

Table V. ^3H_2O Formation from [2,3-^{3}H]Acyl-CoAs in CS

Origin of cell lines	[2,3-^{3}H]Butyryl-CoA	[2,3-^{3}H]Octanoyl-CoA	[2,3-^{3}H]Isovaleryl-CoA
	pmol product/mg protein per min±SEM		
Normal controls (n)	66±5.3 (14)	55±8.8 (19)	20±3.7 (14)
MCD patients (n)			
S.F.	48±2.8 (2)	4.0±2.6 (2)	27±7.7 (2)
A.M.	53±4.1 (2)	2.1±1.4 (2)	23±7.9 (2)
X.D.	56±3.7 (2)	4.5±1.7 (3)	21±8.2 (2)
R.B.	54±1.0 (3)	4.7±1.3 (2)	25±6.3 (2)
B.M.	55±8.7 (4)	5.0±2.3 (4)	22±1.8 (3)

The assay conditions are identical to those described in Table IV; apparent and nonlyophilizable artifactual activities are omitted for clarity.

In MCD MS assayed with [2,3-^{3}H]butyryl-CoA and PMS, SCADH activity was 73% of control ($P > 0.1$). Addition of 20 μM FAD raised control SCADH activities 31% and revealed MCD SCADH activity to be 86% of control ($P > 0.1$). Using the dye-reduction assay, we found SCADH activity in MCD MS to be somewhat lower at 55% ($P < 0.05$) of control (2). These moderate reductions in MCD SCADH activity may reflect the consequences of absent MCADH activity on the dehydrogenation of butyryl-CoA. Mammalian MCADHs, isolated from pig and rat liver, possess varying activities toward butyryl-CoA (21, 24, 29), although the chain-length specificity of human fibroblast MCADH is not known. IVDH activities were similar in both control and MCD MS. We anticipated this result, since isovaleryl-CoA is dehydrogenated by a specific and distinct IVDH. Pure rat liver IVDH has no activity toward hexanoyl- or octanoyl-CoA; conversely, rat liver MCADH cannot dehydrogenate any branched-chain acyl-CoA (21, 22).

We then modified the method for ADH assay in whole fibroblast preparations (CS), since an effective assay for ADH activity in CS would speed diagnosis of MCD and related disorders. In our first three experiments, apparent MCADH activity in MCD CS proved to be 23% of control. While these observations were generally consistent with the results of both the dye-reduction and tritium-release MS assays, apparent residual MCADH activity in MCD CS was more elevated than we predicted. However, some of our earlier observations can explain this discrepancy. In sonicated rat tissue homogenates incubated with PMS and [2,3-^{3}H]acyl-CoAs, the proportion of nonlyophilizable radioactivity in column eluates ranged from 3 to 81% (18; other data not shown). This nonlyophilizable artifactual radioactivity results from passage through the anion exchange column of neutral or cationic complexes of [2,3-^{3}H]acyl CoAs with basic proteins and/or amino acids and of amphoteric [2,3-

^{3}H]acyl-carnitines. In fibroblast CS, lyophilization of column eluates yielded artifactual activities of up to 25 pmol/mg protein per min. Nonlyophilizable artifactual activities detected with [2,3-^{3}H]butyryl- and -isovaleryl-CoAs were higher in MCD CS than in control ($P < 0.02$), but identical in control and MCD CS incubated with [2,3-^{3}H]octanoyl-CoA ($P > 0.1$). While we cannot easily explain these differences, they may be related to perturbations in intracellular acyl-CoA/CoASH and/or acylcarnitine/carnitine ratios produced by accumulation of medium-chain acyl-CoAs in MCD cells (1, 3–6, 30). Subtracting these artifactual activities from the apparent CS activities yields ADH activities due only to ^3H_2O formation and clearly demonstrates MCADH deficiency in MCD CS (8% of control). Subsequently, in fibroblasts or liver from six other patients with the characteristic clinical phenotype and organic aciduria, we successfully confirmed MCADH deficiency and the diagnosis of MCD. Including our present data, we have studied 9 of the 14 published MCD patients in whom MCADH deficiency has been confirmed in vitro (2–5, 31, 32). We have found no evidence for significant biochemical heterogeneity in MCD, since SCADH, MCADH, IVDH, and long-chain ADH activities are similar in all cell lines we studied with the tritium-release and/or dye-reduction methods (2; other data not shown). Thus, clinical heterogeneity in MCD probably reflects individual differences in diet, gluconeogenesis, glucose homeostasis, and/or efficiency of acyl-CoA detoxification (1, 4–8).

Other investigators have also developed assays for ADH activities in MCD cell homogenates. Stanley et al. (4) used a complex assay that quantitated ADH activity by following ETF reduction fluorometrically, but which did not permit calculation of specific activities. With octanoyl-CoA as substrate, liver homogenates from their three patients had <2.5% of control MCADH activity; liver SCADH and IVDH activities appeared identical to control, using butyryl-CoA and isovaleryl-CoA as substrates, respectively. Kolvraa et al. (3) and Divry et al. (5) quantitated decanoyl-CoA conversion to 2-decenoyl-CoA and found 28 and 20% of control MCADH activity in fibroblast homogenates from MCD patients R.B. and S.G., respectively (3, 5), results substantially higher than the 8–9% residual MCADH activities we found in the same cell lines (Tables IV and V). These high residual activities suggest that peroxisomal acyl-CoA oxidase is active in their preparations (33, 34). This flavoenzyme catalyzes the formation of 2-enoyl-CoA and H_2O_2 from acyl-CoAs and is most active with C_{12}-C_{18}-acyl-CoAs, although C_6-C_8-acyl-CoAs are also oxidized (33). Both the tritium-release and 2-enoyl-CoA methods (3) will detect acyl-CoA oxidation catalyzed by the peroxisomal enzyme, while assays using ETF as an electron acceptor will not (2, 4). Peroxisomal acyl-CoA oxidase activity might also explain the high apparent K_m of fibroblast MCADH toward octanoyl-CoA that Gregersen and Kolvraa (35) found in one MCD cell line; they concluded that decreased amounts of MCADH apoenzyme are made in MCD. However Ikeda and co-workers (36) found no cross-reacting-material-negative or molecular weight MCADH variants in 10 other MCD cell lines. Demonstration of genetic heterogeneity in MCD must await enzymatic, immunochemical, complementation, and restriction enzyme studies in other MCD cell lines.

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