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Research Article

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Deficiency of 3-Methylglutaconyl-Coenzyme A Hydratase in Two Siblings with 3-Methylglutaconic Aciduria

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Abstract

We studied two patients with 3-methylglutaconic aciduria in order to determine the molecular defect. A new assay for 3-methylglutaconyl-coenzyme A (CoA) hydratase has been developed in which the substrate, [5-^{14}C]3-methylglutaconyl-CoA, was synthesized using 3-methylcrotonyl-CoA carboxylase purified from bovine kidney. In this assay the products of the reaction are isolated by reverse-phase high performance liquid chromatography and the rates of conversion from substrate are measured. The Michaelis constant for 3-methylglutaconyl-CoA in normal fibroblasts was 6.9 μmol/liter. The mean activity of 3-methylglutaconyl-CoA hydratase in control fibroblasts was 495 pmol/min per mg protein. In the two patients the values were 11 and 17 pmol/min per mg protein, or 2–3% of normal.

Introduction

3-Methylglutaconic aciduria is a recently described disorder of leucine metabolism, characterized by an abnormal organic acid profile in which there is excessive excretion of 3-methylglutaconic acid and 3-methylglutaric acid (1–4). Nine patients have been reported to have this disorder (1–6). Most of these patients (2, 3, 5) appeared normal for the first 4–9 mo of life, then developed severe neurological degeneration. In two siblings (1) the phenotype was very different. Prominent clinical manifestations were limited to retardation of speech, and large quantities of 3-hydroxyisovaleric acid, as well as 3-methylglutaconic acid and 3-methylglutaric acid, were excreted in the urine. These compounds are all on the degradative pathway for leucine (Fig. 1). In these patients the excretion of 3-hydroxy-3-methylglutaric acid (HMG)[1] was not elevated. Therefore, it has been postulated that the site of the enzyme defect is in the enzyme, 3-methylglutaconyl-coenzyme A (CoA) hydratase (E.C. 4.2.1.18) (7). However, this has not previously been established. We have developed a new method for the direct assay of 3-methylglutaconyl-CoA hydratase (3-MG-CoA hydratase). We have studied fibroblasts derived from the two siblings described by Duran et al. (1), and have documented a deficiency of 3-MG-CoA hydratase.

Methods

Materials. Adenosine-5'-triphosphate (ATP), NADH, 3-methylcrotonyl-CoA, 3-hydroxybutyrate dehydrogenase (Type IV), polyethylene glycol 6000, and DEAE cellulose were purchased from Sigma Chemical Co. (St. Louis, MO). Sephadex G-10, Sepharose 6B, and DEAE-Sephadex A-25 resins were obtained from Pharmacia Fine Chemicals (Piscataway, NJ). Hydroxylapatite (Bio-Gel HTP) was purchased from Bio-Rad Laboratories (Richmond, CA) and PM 10 ultrafiltration membranes were obtained from Amicon Corp. (Danvers, MA). NaH^{14}CO$_3$ (9.2 mCi/mmol) was purchased from New England Nuclear (Boston, MA). All other reagents were of the highest purity available. The reverse-phase high performance liquid chromatography (HPLC) column employed has been described by DeBuysere and Olson (8).

Purification of 3-methylcrotonyl-CoA carboxylase (MCC). For the enzymatic synthesis of [5-^{14}C]3-MG-CoA, MCC was purified from frozen bovine kidney by a modification of the method of Lau et al. (9). The MCC in the crude extract was precipitated with polyethylene glycol 6000 and chromatographed on DEAE cellulose. Blue dextran-Sepharose chromatography was omitted. Instead, after hydroxylapatite chromatography, gel filtration was performed on a column of Sepharose 6B (1 cm inside diameter [i.d.] × 47.5 cm) equilibrated and eluted with 20 mmol/liter potassium phosphate buffer, pH 7.0, containing 0.1 mmol/liter EDTA, 0.1 mmol/liter dithiothreitol, and 10% glycerol. The specific activity of the 600-fold purified MCC was 1.6 μmol/min per mg protein and the yield was 6%. MCC activity was assayed in 100-μl assays modified from the method described by Weyler et al. (10), by fixation of NaH^{14}CO$_3$ into acid nonvolatile products using 3-methylcrotonyl-CoA and ATP as substrates.

Preparation of [5-^{14}C]3-MG-CoA. The product of MCC is the *trans* isomer (*E*-form) of 3-MG-CoA (11). That this is the isomer which is the substrate for 3-MG-CoA hydratase is indicated by the fact that *E*-3-MG-CoA is the product of the reverse reaction, the dehydration of 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) catalyzed by 3-MG-CoA hydratase (12). The purified MCC was used for the enzymatic synthesis of [5-^{14}C]*E*-3-MG-CoA from NaH^{14}CO$_3$, 3-methylcrotonyl-CoA, and ATP. Purified MCC (~0.025 U) was added to 1 ml of solution consisting of 100 mmol/liter Tris-HCl, pH 8.0, 50 mmol/liter KCl, 8 mmol/liter $MgCl_2$, 0.25 mmol/liter EDTA, 3 mmol/liter ATP, 1.8 mmol/liter 3-methylcrotonyl-CoA, and 10 mmol/liter NaH^{14}CO$_3$ (9.2 mCi/mmol). The mixture was incubated for 2 h at 30°C. The pH was adjusted to 2–3 with 5 mol/liter HCl, and unreacted H^{14}CO$_3^-$ was removed as $^{14}CO_2$ by the addition of small pieces of dry ice. The [5-^{14}C]3-MG-CoA product was purified by chromatography on a DEAE-Sephadex A-25 column (0.6 cm i.d. × 30 cm) using a linear gradient of LiCl (140 ml 0.065 mol/liter to 140 ml 0.2 mol/liter) in 5 mmol/liter HCl, modified from the

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1. *Abbreviations used in this paper:* CoA, coenzyme A; 3-HB, 3-hydroxybutyric acid; HMG, 3-hydroxy-3-methylglutaric acid; HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA; HPLC, high performance liquid chromatography; i.d., inside diameter; MCC, 3-methylcrotonyl-CoA-carboxylase; 3-MG-CoA, 3-methylglutaconyl-CoA.

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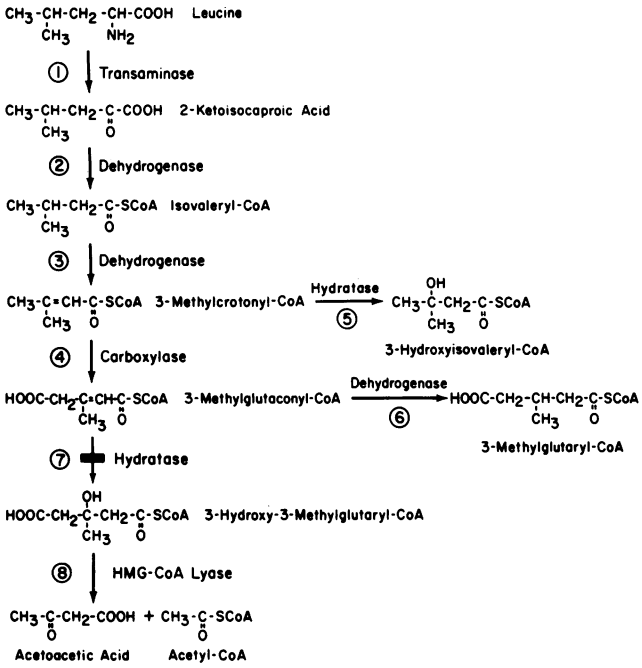

Figure 1. Catabolic pathway for leucine. The 3-MG-CoA hydratase is numbered 7.

method of Bartlett et al. (13). Chromatography was performed at room temperature and a flow rate of 0.7 ml/min. The fractions containing [5-^{14}C]3-MG-CoA were pooled, lyophilized, and LiCl was removed by chromatography on a Sephadex G-10 column (0.6 cm i.d. × 50 cm), equilibrated and eluted with distilled water.

The specific activity of the purified [5-^{14}C]3-MG-CoA was determined by enzymatic hydration to [5-^{14}C]HMG-CoA with excess crotonase (Sigma Chemical Co.) and comparison of this with authentic [3-^{14}C]HMG-CoA of known specific activity (5.5 mCi/mmol) (New England Nuclear). Both compounds were analyzed by reverse-phase HPLC (8) with the quantities determined from chromatographic peak areas with a 254-nm detector, and the radioactivity in collected fractions was determined with a liquid scintillation counter. The results were corrected for recoveries of authentic [3-^{14}C]HMG-CoA. The mean value for duplicate determinations of the specific activity of [5-^{14}C]HMG-CoA derived from [5-^{14}C]3-MG-CoA was 5.8 mCi/mmol. This was 63% of the specific activity of the precursor $NaH^{14}CO_3$, indicating considerable dilution by atmospheric CO_2 in the pH 8 buffer during the enzymatic synthesis of [5-^{14}C]3-MG-CoA. The mean radiochemical purity on reverse-phase HPLC of the [5-^{14}C]HMG-CoA derived from [5-^{14}C]3-MG-CoA was 69%, with essentially all of the rest of the radioactivity in [^{14}C]3-methylglutaconic acid, which presumably came from nonspecific hydrolysis of [5-^{14}C]3-MG-CoA. In the assays of 3-MG-CoA hydratase, after incubation the CoA esters were hydrolyzed and on chromatography the [^{14}C]3-methylglutaconic acid represents both [5-^{14}C]3-MG-CoA substrate and [^{14}C]3-methylglutaconic acid impurity (Fig. 2, *bottom*). The concentration of substrate [5-^{14}C]3-MG-CoA was determined by measuring its radioactivity and correcting for a specific activity of 5.8 mCi/mmol and radiochemical purity of 69%. The amounts of radioactive products of 3-MG-CoA hydratase were calculated from the specific activity of 5.8 mCi/mmol. The solution containing [5-^{14}C]3-MG-CoA was concentrated to 0.27 mmol/liter and stored at −20°C.

3-MG-CoA hydratase assay. Fibroblasts derived from two siblings with 3-methylglutaconic aciduria (1) and control subjects were cultured in Eagle's minimal essential medium with 10% fetal calf serum. The cells were harvested by trypsinization, pelleted by centrifugation, and washed with Tris-buffered saline. Lymphocytes were isolated from whole venous blood by density gradient centrifugation (14). Fibroblast or lymphocyte pellets were suspended in 50 mmol/liter Tris-HCl buffer, pH 7.5, containing 0.75 mmol/liter Na_2EDTA (~5 × 10^6 cells/ml). The cell suspensions were lysed while chilled in an ice bath by sonic disruption for 2 × 10 s (28 W) with a 60-s pause between bursts using a Sonifer Cell Disruptor Model W-185 (Heat Systems Ultrasonics, Inc., Farmingdale, NY).

The standard assay contained (final volume, 100 μl): cell sonicate (~20–30 μg protein), 100 mmol/liter potassium-phosphate buffer, pH 7.0, 0.055 mmol/liter [5-^{14}C]3-MG-CoA (~0.032 μCi), 3-hydroxybutyrate dehydrogenase (2.5 U/ml), and 2 mmol/liter NADH. The mixture without cell sonicate constituted the blank control. Incubations were carried out at 30°C for 30 min. The reaction was stopped by the addition of 10 μl of ice cold 4.2 mol/liter perchloric acid. The solution was centrifuged for 1 min at top speed in an Eppendorf microcentrifuge. The resultant supernatant fluid was neutralized by addition of 13 μl of 1.5 mol/liter potassium carbonate solution containing 0.25 mol/liter triethanolamine. Insoluble potassium perchlorate was removed by centrifugation. The resulting supernatant was adjusted to pH 11–12 by addition of ~23 μl of 1 mol/liter KOH. The sample was cooled on ice and centrifuged for 1 min at top speed. The resulting supernatant fluid was transferred to another tube and coenzyme-A esters were hydrolyzed by heating at 60°C for 30 min. The hydrolyzed sample was adjusted to pH 2–3 by addition of 1 mol/liter phosphoric acid (~38 μl) and 100 μl were analyzed by HPLC.

Reverse-phase chromatography of radioactive products. A guard column (0.41 cm i.d. × 2 cm) filled with SC30-40 micron reverse-phase C18 ODS pellicular particles was connected to a 0.46 cm i.d. × 25 cm Spherisorb ODS II, 5-micron column (Custom LC, Inc., Houston, TX). The mobile-phase solvents were 0.05 mol/liter potassium-phosphate buffer, pH 2.1 (Buffer A), from 0 to 35 min of elution, 55% Buffer A and 45% methanol from 35 to 60 min of elution, followed by 30 min of Buffer A to return to the starting conditions. The chromatographic

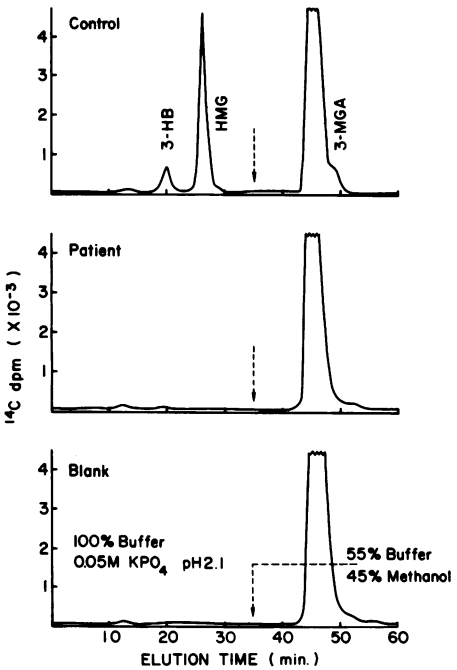

Figure 2. Reverse-phase HPLC separation of the hydrolyzed reaction mixtures after the assay of 3-MG-CoA hydratase in fibroblast lysates. 3-MGA, 3-methylglutaconic acid. The arrow designates the time at which the eluting buffer was changed.

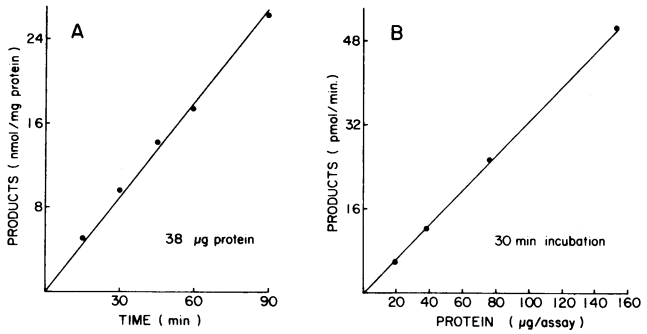

Figure 3. Linearity of the assay for the activity of 3-MG-CoA in normal fibroblast lysates with time (*A*) and with protein content (*B*). The points plotted represent the sums of the products, [^{14}C]3-hydroxybutyric acid and [^{14}C]3-hydroxy-3-methylglutaric acid.

separations were performed at room temperature and a flow rate of 0.44 ml/min, and 1-min fractions were collected. Radioactivity was determined by liquid scintillation counting with 5 ml of fluor. Studies on the separation of the substrates and products as acyl-CoA esters by reverse-phase HPLC (8) showed that there was a small amount of isomerization of the substrate *E*-3-MG-CoA to *Z*-3-MG-CoA. The *Z*-isomer coeluted with HMG-CoA, the product of 3-MG-CoA hydratase. Therefore, the acyl-CoA substrates and products were hydrolyzed with base to the acids, and the reverse-phase HPLC conditions were modified to separate the free acids. The base hydrolysis caused considerable isomerization of the free 3-methylglutaconic acid.

Results

The assay of 3-MG-CoA hydratase utilized as substrate [5-^{14}C]3-MG-CoA prepared enzymatically and the products were analyzed by reverse-phase HPLC after hydrolysis of the CoA esters to free acids. 3-Hydroxybutyrate dehydrogenase and NADH were included in the assays because the product [^{14}C]3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) is partially cleaved by endogenous HMG-CoA lyase to yield [^{14}C]acetoacetate. The [^{14}C]acetoacetate was therefore converted to the more stable [^{14}C]3-hydroxybutyric acid in the reaction catalyzed by 3-hydroxybutyrate dehydrogenase.

Fig. 2 shows reverse-phase HPLC chromatograms of reaction mixtures for the assay of 3-MG-CoA hydratase. In control fibroblast lysates there were significant radioactive peaks of [^{14}C]3-hydroxybutyric acid (3-HB) and [^{14}C]3-hydroxy-3-methylglutaric acid (HMG), whereas in these areas essentially no activity was detectable in the assay blank, which lacked cell lysate. In the assay of fibroblasts from patient 1 with 3-methylglutaconic aciduria, no peaks of radioactivity were found in the areas of 3-hydroxybutyric acid and HMG.

The activity of 3-MG-CoA hydratase was calculated as the sum of the isotope in the products 3-hydroxybutyric acid and HMG and expressed as pmol/min per mg protein. The activity of the enzyme in fibroblast sonicates was proportional to the time of incubation up to at least 90 min (Fig. 3 *A*) and to protein concentration up to at least 150 μg protein per assay (Fig. 3 *B*). In lymphocyte sonicates enzyme activity was proportional to the time of incubation up to at least 60 min and to protein concentration up to at least 90 μg protein per assay.

The kinetic properties of the enzyme in fibroblast and lymphocyte lysates were determined. 3-MG-CoA hydratase activity in fibroblasts from three different controls followed very similar curves (Fig. 4 *A*) as a function of 3-MG-CoA concentration. Fig. 4 *B* shows Lineweaver-Burk plots of the same data. The mean Michaelis constant (K_m) value for 3-methylglutaconyl-CoA in control fibroblasts was 6.9 μmol/liter (range, 6.5 to 7.5) and the values of maximum velocity (V_{max}) ranged from 568 to 614 pmol/min per mg protein. The K_m values for normal lymphocytes were 9.3 and 9.5 μmol/liter and the V_{max} values for normal lymphocytes were 1,089 and 1,359 pmol/min per mg protein. The total activity of 3-MG-CoA hydratase in control fibroblast sonicate did not vary greatly with changing pH from pH 6.0 to 8.0 (Fig. 5), although there was an optimum at pH 7.0 to 7.5. The proportion of isotopes in HMG was much greater at pH below 7.0, whereas above 7.0 the proportion of total isotope found in 3-hydroxybutyric acid increased appreciably.

On the basis of the data obtained the standard assay was developed and incubations were carried out for 30 min at 30°C in the presence of 0.055 mmol/liter of [5-^{14}C]3-MG-CoA. The mean activity in lymphocytes from four normal individuals, each assayed one time, was 822 pmol/min per mg protein with a range of 442 to 1,103. The data on the activities of 3-MG-CoA

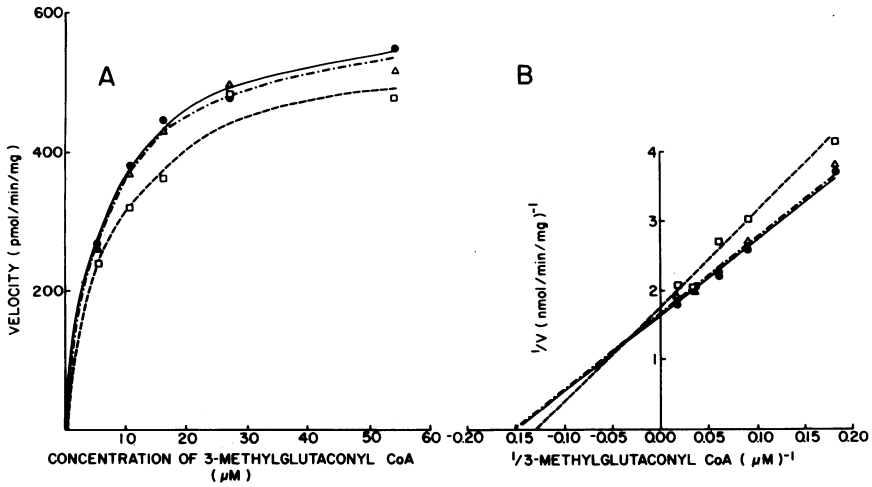

Figure 4. Kinetics of the activity of 3-MG-CoA hydratase in lysates of fibroblasts derived from three different control individuals. (*A*) Michaelis-Menten plot. (*B*) Lineweaver-Burk plot.

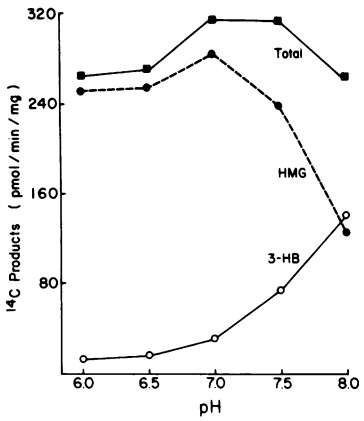

Figure 5. pH activity profile. ^{14}C-products of [5-^{14}C]3-methylglutaconyl-CoA in a control fibroblast sonicate as a function of pH.

hydratase in lysates prepared from fibroblasts of the two patients with 3-methylglutaconic aciduria and five controls are shown in Table I. The mean value for controls was 495 pmol/min per mg protein. Enzyme activities for patients 1 and 2 were 11 and 17 pmol/min per mg protein, or ~2–3% of normal.

Discussion

We have developed a new method for the assay of 3-MG-CoA hydratase. In this assay the substrate was [5-^{14}C]3-MG-CoA synthesized in the reaction catalyzed by 3-methylcrotonyl-CoA carboxylase purified from bovine kidney. The products of the hydratase reaction were isolated, their isotope content determined, and the sum of the products formed from the precursor were the measure of the activity of the enzyme. The reaction was linear with time and enzyme content. K_m values for 3-methylglutaconyl-CoA in normal human fibroblasts and lymphocytes were ~6.9 and 9.4 μmol/liter, respectively.

Using this method, the activity of 3-MG-CoA hydratase was determined in fibroblasts from two patients with 3-methylglutaconic aciduria. A marked deficiency of enzyme activity was found in the two siblings, which provided direct evidence for a deficiency of the hydratase in these patients. Defects in all eight enzymes involved in leucine degradation have now been reported.

Table I. 3-Methylglutaconyl-CoA Hydratase Activity in Cultured Fibroblasts of Patients with 3-Methylglutaconic Aciduria and Controls

Subject	Hydratase activity
	pmol/min per mg protein
Control	
1 (n = 3)	593±30
2 (n = 3)	498±67
3 (n = 3)	485±29
4 (n = 3)	542±29
5 (n = 4)	355±24
Mean of controls (±1 SD)	495±89
Patient	
1 (n = 3)	11±4
2 (n = 3)	17±5

n = number of determinations.

The patients studied had a phenotype different from other patients in whom 3-methylglutaconic aciduria has been described (2–6). The similarities of the siblings we have studied indicate that this is the clinical picture of 3-MG-CoA hydratase deficiency. Both had retardation of speech development (1). In one this was the only abnormality. His older brother was also slow in motor development, having walked first at 2 yr of age, and had a short attention span. He had had an unexplained episode of unconsciousness which lasted nearly a day. He responded to an 18-h fast with symptomatic hypoglycemia and a metabolic acidosis. Fasting did not produce hypoglycemia in his sibling. 3-MG-CoA hydratase deficiency is likely to be an autosomal recessively transmitted disorder. There was no known consanguinity of the parents of our patients. The assay developed should be suitable for the detection of heterozygosity, but fibroblasts on the parents have not been available for study. Two siblings reported from Sweden had a slowly progressive encephalopathy, but the amounts of 3-methylglutaconic acid excreted in the urine were considerably smaller than in the patients we studied with 3-MG-CoA hydratase deficiency. Two other infants reported from Sweden (3) had a similar progressive neurologic degenerative disease. We have studied the activity of 3-MG-CoA hydratase in fibroblasts from three patients with a similar clinical picture (4, 5) and found it to be normal (6). Thus, the neurological degenerative form of 3-methylglutaconic aciduria does not appear to be due to a primary deficiency of 3-MG-CoA hydratase.

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