

# Ethylmalonyl-CoA Decarboxylase, a New Enzyme Involved in Metabolite Proofreading\*

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Carole L. Linster<sup>‡</sup>, Gaëtane Noël<sup>‡</sup>, Vincent Stroobant<sup>§</sup>, Didier Vertommen<sup>\*1</sup>, Marie-Françoise Vincent<sup>||</sup>, Guido T. Bommer<sup>‡</sup>, Maria Veiga-da-Cunha<sup>‡</sup>, and Emile Van Schaftingen<sup>‡2</sup>

From <sup>‡</sup>Welbio and the Laboratory of Physiological Chemistry, the <sup>§</sup>Ludwig Institute for Cancer Research, and the <sup>\*1</sup>Hormone and Metabolic Research Unit, de Duve Institute, Université Catholique de Louvain, 1200 Brussels, Belgium and the <sup>||</sup>Laboratory of Metabolic Diseases, Cliniques Universitaires Saint-Luc, B-1200 Brussels, Belgium

**Background:** Acetyl-CoA and propionyl-CoA carboxylases slowly form ethylmalonyl-CoA as a side activity.

**Results:** A new enzyme, ethylmalonyl-CoA decarboxylase, was identified in mammalian tissues and shown to be encoded by the *ECHDC1* gene. Knocking down this gene increased the formation of ethylmalonate from butyrate in cultured cells.

**Conclusion:** Ethylmalonyl-CoA decarboxylase may be a new “metabolite proofreading” enzyme.

**Significance:** Its deficiency may cause ethylmalonic aciduria.

A limited number of enzymes are known that play a role analogous to DNA proofreading by eliminating non-classical metabolites formed by side activities of enzymes of intermediary metabolism. Because few such “metabolite proofreading enzymes” are known, our purpose was to search for an enzyme able to degrade ethylmalonyl-CoA, a potentially toxic metabolite formed at a low rate from butyryl-CoA by acetyl-CoA carboxylase and propionyl-CoA carboxylase, two major enzymes of lipid metabolism. We show that mammalian tissues contain a previously unknown enzyme that decarboxylates ethylmalonyl-CoA and, at lower rates, methylmalonyl-CoA but that does not act on malonyl-CoA. Ethylmalonyl-CoA decarboxylase is particularly abundant in brown adipose tissue, liver, and kidney in mice, and is essentially cytosolic. Because *Escherichia coli* methylmalonyl-CoA decarboxylase belongs to the family of enoyl-CoA hydratase (ECH), we searched mammalian databases for proteins of uncharacterized function belonging to the ECH family. Combining this database search approach with sequencing data obtained on a partially purified enzyme preparation, we identified ethylmalonyl-CoA decarboxylase as *ECHDC1*. We confirmed this identification by showing that recombinant mouse *ECHDC1* has a substantial ethylmalonyl-CoA decarboxylase activity and a lower methylmalonyl-CoA decarboxylase activity but no malonyl-CoA decarboxylase or enoyl-CoA hydratase activity. Furthermore, *ECHDC1*-specific siRNAs decreased the ethylmalonyl-CoA decarboxylase activity in human cells and increased the formation of ethylmalonate, most particularly in cells incubated with butyrate. These findings indicate that ethylmalonyl-CoA decarboxylase may correct a side activity of acetyl-CoA carboxylase and suggest that its mutation may be involved in the development of certain forms of ethylmalonic aciduria.

Specificity is a remarkable feature of enzymes, compared with other catalysts, and it is usually assumed that this specificity, although generally not absolute, is high enough in the case of enzymes of intermediary metabolism to avoid the formation of significant amounts of side products. Such side products are unlikely to be metabolized at significant rates; they may therefore accumulate and possibly perturb cell physiology. Recent findings indicate, however, that enzyme specificity is not always sufficient and that a few enzymes exist that serve to eliminate non-classical metabolites resulting from the side activity of enzymes of intermediary metabolism. These enzymes, which are a variety of metabolite repair enzymes (the other ones act on chemically damaged metabolites (1)), play a role analogous to that of the proofreading activities that serve to increase the fidelity of DNA replication and the formation of aminoacyl-tRNAs. We propose to name them “metabolite proofreading enzymes.”

A good illustration of the importance of metabolite repair is the metabolic disorder L-2-hydroxyglutaric aciduria (2). L-2-Hydroxyglutaric aciduria, a severe neurological disorder (3, 4), is due to a deficiency in L-2-hydroxyglutarate dehydrogenase, a mitochondrial enzyme serving to destroy L-2-hydroxyglutarate (5–7). The latter has no known metabolic role but appears to be formed by mitochondrial L-malate dehydrogenase due to a minor side activity of this enzyme on  $\alpha$ -ketoglutarate (8, 9). This activity amounts to about  $10^7$  times less than the main activity of this enzyme (on oxaloacetate), yet, taking into account the intracellular concentration of  $\alpha$ -ketoglutarate, it is sufficient to account for the formation of several g of L-2-hydroxyglutarate per day. The absence of a known role for L-2-hydroxyglutarate in mammals and its apparent toxicity indicate that L-2-hydroxyglutarate dehydrogenase plays the role of a metabolite proofreading enzyme.

A few other examples of metabolite proofreading enzymes are known. A hydrated form of NADH (NADHX) formed by glyceraldehyde 3-phosphate dehydrogenase (10–12), is reconverted to NADH by an ATP-dependent enzyme (13, 14) that has recently been molecularly identified (15). The activity of this widely distributed stereospecific enzyme, which also acts

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<sup>1</sup> Collaborateur Logistique of the FRS-FNRS.

<sup>2</sup> To whom correspondence should be addressed: de Duve Institute, Université Catholique de Louvain, BCHM 7539, Ave. Hippocrate 75, B-1200 Brussels, Belgium. Tel.: 32-2-7647564; Fax: 32-2-7647598; E-mail: emile.vanschaftingen@uclouvain.be.

on NADPHX, is complemented by an NAD(P)HX epimerase (15). Furthermore, a GDP-glucose phosphorylase that corrects a side activity of the GDP-mannose-forming enzyme in mammalian cells and *Caenorhabditis elegans* has been described (16).

Because few examples of metabolite proofreading enzymes are known and because of the potential importance of this process, we decided to actively search for such enzymes. Two examples of metabolic enzymes displaying an obvious lack of specificity are propionyl-CoA carboxylase and acetyl-CoA carboxylase. Propionyl-CoA carboxylase significantly acts on butyryl-CoA, which is converted to ethylmalonyl-CoA at a rate amounting to about 2% of the rate at which propionyl-CoA is carboxylated (17). Acetyl-CoA carboxylase displays a similar lack of substrate specificity, being able to synthesize methylmalonyl-CoA and ethylmalonyl-CoA (18–20).

The purpose of the present work was to search for an enzyme that would “correct” a side activity of acetyl-CoA and propionyl-CoA carboxylases by breaking down ethylmalonyl-CoA. We report here that mammalian tissues indeed contain an ethylmalonyl-CoA decarboxylase, an enzyme that has not been described before. We identify this enzyme as the expression product of the *ECHDC1* (enoyl-CoA hydratase domain containing 1) gene, a member of the enoyl-CoA hydratase family, and provide evidence for its role in metabolite repair.

### EXPERIMENTAL PROCEDURES

**Materials**—Reagents, of analytical grade whenever possible, were from Sigma, Acros (Geel, Belgium), Roche Applied Science, or Merck. [ $^{14}\text{C}$ ]NaHCO<sub>3</sub>, DEAE-Sepharose, phenyl-Sepharose, and Q-Sepharose resins as well as NAP-5 columns were obtained from GE Healthcare. The nickel-NTA Superflow column was from Qiagen, and the Blue-Trisacryl resin was from Sigma. Enzymes used for PCR and cloning purposes were obtained from Fermentas (Sankt Leon-Rot, Germany).

**Purification of Ethylmalonyl-CoA Decarboxylase**—Frozen rat liver (~10 g) was homogenized with 3 volumes of buffer A (25 mM Hepes, 5 mM potassium phosphate, both at pH 7.1, 2  $\mu\text{g}/\text{ml}$  leupeptin, 2  $\mu\text{g}/\text{ml}$  antipain, and 1 mM dithiothreitol) containing 100 mM KCl. The homogenate was centrifuged for 10 min at 10,000  $\times g$  and 4 °C. The resulting supernatant was enriched with 2% (w/v) PEG and recentrifuged as above. This supernatant was diluted 2-fold with homogenizing buffer without KCl and loaded onto a DEAE-Sepharose column, and a NaCl gradient (0–500 mM in 2  $\times$  75 ml of buffer A) was applied to elute the enzymatic activity.

A sample (15 ml) of the purified protein was applied onto a Blue-Trisacryl column (1.5-ml gel bed). The column was washed with 5 mM Hepes, 5 mM potassium phosphate, both at pH 7.1, 2  $\mu\text{g}/\text{ml}$  leupeptin, 2  $\mu\text{g}/\text{ml}$  antipain, and 1 mM dithiothreitol. The protein was eluted with a stepwise salt gradient (0, 100, 200, 300, 400, 500, 600, 800, and 1000 mM NaCl in the same buffer), and 2-ml fractions were collected. The active fractions (~5 ml) were enriched with 0.5 M NaCl and applied onto a phenyl-Sepharose column (1.5-ml bed volume) equilibrated with buffer A containing 0.5 M NaCl. The retained proteins were eluted with a decreasing gradient of NaCl followed by an increasing gradient of ethylene glycol (0–70%). The most puri-

fied fraction of rat liver ethylmalonyl-CoA decarboxylase obtained after the three-step purification procedure described above was digested with trypsin and analyzed by LC-tandem mass spectrometry essentially as described previously (21).

**Tissue Fractionation**—Tissue fractionation was performed by differential centrifugation as described by de Duve *et al.* (22) starting from the livers of overnight starved rats, to obtain fractions enriched in mitochondria, lysosomes, or microsomes (designated fractions M, L, or P, respectively) and a final supernatant, containing cytosolic proteins. Marker enzymes were determined to ensure that the fractionation was successful. Triton X-100 (0.1%) was included in all assays, to ensure membrane permeability. This concentration of detergent does not inhibit ethylmalonyl-CoA decarboxylase. Protein concentration was determined using the Lowry assay.

**Partial Purification of Propionyl-CoA Carboxylase**—The flow-through of the first DEAE-Sepharose column was diluted 2-fold with buffer A and reapplied onto a DEAE-Sepharose column, which was developed with the same gradient as above. The enzyme was assayed in the eluted fractions. The most active fractions were treated with 10% (w/v) PEG. The pellet was resuspended in one-tenth of the initial volume of buffer A and applied onto a column of Blue-Trisacryl (2-ml gel bed) equilibrated with buffer A. The column was washed with buffer A, and proteins were eluted with a salt gradient in the same buffer. The active fractions were eluted with ~750 mM NaCl and were used to prepare radiolabeled ethylmalonyl-CoA, methylmalonyl-CoA, and malonyl-CoA.

**Preparation of [ $^{14}\text{C}$ ]Ethylmalonyl-CoA**—The reaction mixture (1 ml) contained 100 mM Tris, pH 8, 10 mM potassium phosphate, 1 mM dithiothreitol, 2 mM ATP-Mg, 5 mM MgCl<sub>2</sub>, 1 mM butyryl-CoA, 40  $\mu\text{Ci}$  of [ $^{14}\text{C}$ ]NaHCO<sub>3</sub> (55 mCi/mmol), and 200  $\mu\text{g}$  of propionyl-CoA carboxylase. 5 mM glucose and 2  $\mu\text{l}$  of hexokinase were added to arrest the reaction after 15 min of incubation at 30 °C. The sample was diluted 2-fold in 5 mM Hepes, pH 7.1, and applied onto a Q-Sepharose column (3 ml) equilibrated with the same buffer. The radioactivity was eluted with 5 ml of 150 mM NaCl, followed by 5 ml of 300 mM NaCl. We noted that ethylmalonyl-CoA treated with acid to eliminate unreacted HCO<sub>3</sub><sup>-</sup> was partially resistant to decarboxylation by ethylmalonyl-CoA decarboxylase, suggesting that it was racemized; we therefore avoided the use of an acidification step in the preparation of [ $^{14}\text{C}$ ]ethylmalonyl-CoA. The yield of radioactive ethylmalonyl-CoA amounted to ~25–40% of the initial radioactivity. Radiolabeled methylmalonyl-CoA and malonyl-CoA were prepared similarly but with 0.2 mM propionyl-CoA and 2 mM acetyl-CoA, respectively.

**Chemical Synthesis of Non-labeled Ethylmalonyl-CoA**—Ethylmalonyl-CoA was synthesized according to a modified procedure of Coleman and Huang (23) using acyl adenylate as the thioacylating reagent. Briefly, ethylmalonyl-AMP was prepared from ethylmalonic acid and adenosine monophosphate as follows. To a solution of ethylmalonic acid (50  $\mu\text{mol}$ ) in dimethylformamide and AMP (45  $\mu\text{mol}$ ) was added a solution of dicyclohexylcarbodiimide (50  $\mu\text{mol}$ ) in dimethylformamide with stirring. The reaction was allowed to proceed for 1 h, and ethylmalonyl-AMP was directly purified by HPLC. Ethylmalonyl-CoA was prepared by incubating equal amounts of ethylmalonyl-

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nyl-AMP (2  $\mu\text{mol}$ ) and CoA (2  $\mu\text{mol}$ ) in a solution containing 100 mM imidazole (pH 7) for 2 h at room temperature. Finally, ethylmalonyl-CoA was isolated by HPLC and characterized by UV and mass spectrometry analyses, whose results were consistent with its structure.

**Propionyl-CoA Carboxylase Assay**—Samples to be analyzed (25  $\mu\text{l}$ ) were incubated with 100 mM Tris, pH 8, 10 mM potassium phosphate, 1 mM dithiothreitol, 2 mM ATP-Mg, 5 mM  $\text{MgCl}_2$ , 0.2 mM propionyl-CoA, and 100,000 cpm [ $^{14}\text{C}$ ]NaHCO<sub>3</sub> for 20 min at 30 °C in a total volume of 100  $\mu\text{l}$ . The reaction was stopped by the addition of 50  $\mu\text{l}$  of 10% (w/v) trichloroacetic acid and 500  $\mu\text{l}$  of water. The mixture was centrifuged, and the resulting supernatant was incubated for 1 h at 37 °C and flushed with air three times at 20-min intervals to eliminate radioactive CO<sub>2</sub>. The samples were then counted for radioactivity.

**Ethylmalonyl-CoA Decarboxylase Assay**—The enzyme was assayed through the decrease in acid-stable [ $^{14}\text{C}$ ]ethylmalonyl-CoA in a reaction mixture at 30 °C containing 20 mM Hepes, pH 7.1, 1 mM  $\text{MgCl}_2$ , 5 mM ATP-Mg, and 8000 cpm ethylmalonyl-CoA in a final volume of 100  $\mu\text{l}$ . The reaction was stopped with 50  $\mu\text{l}$  of 10% trichloroacetic acid, 500  $\mu\text{l}$  water was added, and the samples were incubated at 37 °C for 30 min with flushing. Control incubations were run without enzyme to take into account a modest spontaneous decarboxylation (~5% in 20 min). One unit of enzyme is the amount that causes 50% degradation of the substrate in 1 min in a final volume of 1 ml. Note that the assay is more easily performed by measuring the disappearance of ethylmalonyl-CoA than by measuring the appearance of radiolabeled CO<sub>2</sub>, which requires efficient trapping of CO<sub>2</sub>. To measure the enzyme activity in tissues, we chose dilutions allowing us to obtain between 20 and 75% of substrate conversion. Semilogarithmic plots were used to calculate the activity.

**Preparation of Recombinant Mouse ECHDC1**—A 5'-primer containing the ATG codon (CGACATATGGCAAATGCTTTTGGACTTC) in an NdeI site (in boldface type) and a 3'-primer containing the putative stop codon (TTTGCGGCCGCCTATTTAGTATGCTTTCCTTCTTA) flanked by a NotI site were used to PCR-amplify the coding sequence of ECHDC1 from mouse brain cDNA with Pfu polymerase. An ~900-bp product was obtained. This fragment was restricted with NdeI and NotI and ligated into the pET28a plasmid in order to express ECHDC1 as an N-terminally His-tagged protein. The resulting plasmid was checked by sequencing and used to transform *Escherichia coli* BL21 cells.

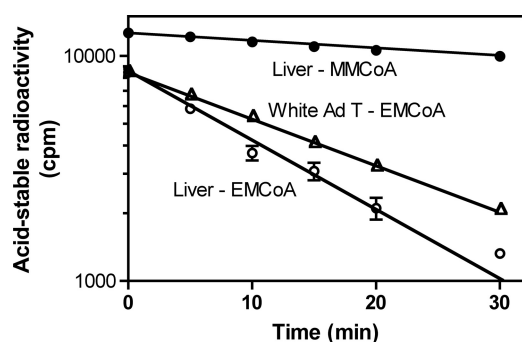
The resulting bacteria were grown in 400 ml of Luria-Bertani (LB) medium containing kanamycin (50 mg/liter). The culture was grown at 37 °C until A<sub>600</sub> reached 0.5–0.6. After cooling on ice for 20 min, the inducer isopropyl-1-thio- $\beta$ -D-galactopyranoside was added to a final concentration of 0.4 mM, and the culture was pursued at 18 °C for 20 h. The cells were collected by centrifugation, resuspended in 20 ml of lysing buffer (20 mM Hepes, pH 7.1, 0.5 mM phenylmethylsulfonyl fluoride, 5  $\mu\text{g}/\text{ml}$  leupeptin, 5  $\mu\text{g}/\text{ml}$  antipain, and 1 mg/ml lysozyme), and submitted to three cycles of freezing and thawing. The bacterial extract was incubated on ice for 1 h with 0.1 mg/ml DNase I in the presence of 10 mM MgSO<sub>4</sub> and centrifuged for 30 min at

10,000  $\times g$ . The resulting supernatant (~20 ml) was diluted 3-fold with buffer B (25 mM Hepes, pH 7.4, 5  $\mu\text{g}/\text{ml}$  leupeptin, 5  $\mu\text{g}/\text{ml}$  antipain, 300 mM NaCl, and 20 mM imidazole) and loaded onto a nickel-NTA Superflow column (1 ml of resin). The column was washed with buffer B containing 5 mM imidazole until A<sub>280</sub> had reached less than 0.01, and the His-tagged protein was eluted with buffer B containing 250 mM imidazole. The purified protein was analyzed by SDS-PAGE, desalted on a NAP-5 column equilibrated with buffer C (25 mM Hepes, pH 7.1, 5  $\mu\text{g}/\text{ml}$  leupeptin, 5  $\mu\text{g}/\text{ml}$  antipain, and 100 mM KCl), supplemented with 10% (v/v) glycerol, and stored at –70 °C.

**siRNA Transfection of HEK293T Cells and Quantitative Real-time PCR**—HEK293T cells were maintained and transfected with siRNAs as described (16). Control siRNA (Dharmacon ON-TARGETplus non-targeting pool from Thermo Scientific (Epsom, UK)) or ECHDC1 siRNA (Dharmacon ON-TARGETplus SMARTpool from Thermo Scientific) was added at a final concentration of 50 nM using Lipofectamine 2000 (Invitrogen) as the transfection reagent. After 48–72 h, protein or total RNA was extracted from cells as described previously (16). Protein concentration was determined by the Lowry assay after protein precipitation with 10% trichloroacetic acid, and RNA concentrations were determined by measuring the absorbance at 260 nm. Ethylmalonyl-CoA decarboxylase activity was assayed in the cleared lysates by the method described above. To measure ECHDC1 mRNA transcript levels, cDNA synthesis and quantitative real-time PCR were performed as described previously (16) using RevertAid<sup>TM</sup> H Minus reverse transcriptase (Fermentas) and SYBR Green fluorescent dye (Bio-Rad), respectively. Primers used for amplification can be obtained upon request.

**Assay of Short Chain Acyl-CoAs**—Short chain acyl-CoAs were separated by reverse-phase HPLC using the method described by Deutsch *et al.* (24), except that a 5- $\mu\text{m}$  Pursuit XRs C<sub>18</sub> column, 250  $\times$  4.6 mm (Varian), was used. Retention times of methylmalonyl-CoA, ethylmalonyl-CoA, propionyl-CoA, and butyryl-CoA were 15.5, 18.5, 27.4, and 31.0 min, respectively. The concentration of those compounds in analyzed samples was calculated by comparison of the corresponding peak areas with the peak area of a known amount of standard butyryl-CoA obtained by monitoring UV absorbance at 260 nm.

**Assay of Extracellular Ethylmalonate**—Cell media (1 ml) were treated with hydroxylamine hydrochloride in the presence of mandelic acid as an internal standard. Following acidification with HCl and saturation with NaCl, extraction was performed with ethyl acetate and diethyl ether. After evaporation of the combined extracts under a stream of nitrogen, derivatization was achieved with *N,O*-bis(trimethylsilyl)trifluoroacetamide. Analysis was performed by gas chromatography (Hewlett-Packard 6890 series GC system equipped with a 30 m  $\times$  0.25-mm fused silica capillary column CP-SIL 8CB from Varian) with helium as carrier gas, coupled to a Hewlett-Packard 5973 mass-selective detector under electron impact fragmentation and with the scan and selective ion monitoring modes for data acquisition. Ethylmalonic acid was identified and quantified by comparing its retention time, mass spectrum, and detector response with those of the commercial compound. For quantification, the extracted ion chromatogram at



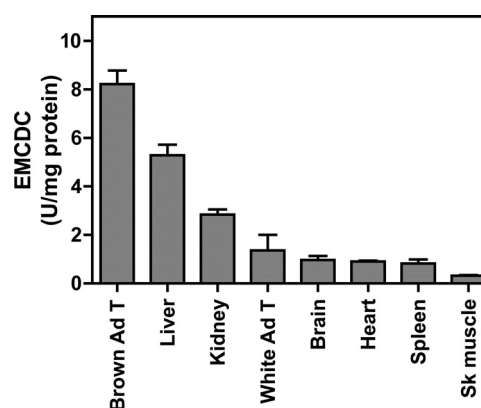
**FIGURE 1. Time course of ethylmalonyl-CoA decarboxylation in different tissue extracts.** Radiolabeled ethylmalonyl-CoA or methylmalonyl-CoA ( $\sim 8000$  cpm;  $0.5 \mu\text{M}$ ) was incubated with diluted mouse liver (1:10,000 final dilution in the assay) or white adipose tissue (1:1000) extracts in a final volume of  $100 \mu\text{l}$ . The incubations were arrested at the indicated times by the addition of trichloroacetic acid. The radioactivity of the samples was counted after elimination of  $^{14}\text{CO}_2$  and is represented here using a logarithmic scale. Results represent the means of four values  $\pm$  S.E. Ad T, adipose tissue; EMCoA, ethylmalonyl-CoA; MMCoA, methylmalonyl-CoA.

*m/z* 217 was used after data acquisition in the selective ion monitoring mode.

## RESULTS

**Presence of an Enzyme Catalyzing the Decarboxylation of Ethylmalonyl-CoA in Mouse Tissue Extracts**—To detect the presence of a putative ethylmalonyl-CoA decarboxylase, [ $^{14}\text{C}$ ]ethylmalonyl-CoA was prepared by carboxylation of butyryl-CoA with [ $^{14}\text{C}$ ]HCO $_3^-$  with partially purified propionyl-CoA carboxylase. The resulting ethylmalonyl-CoA was purified by chromatography on Q-Sepharose. Methylmalonyl-CoA was prepared similarly starting from propionyl-CoA. The decarboxylase activity was measured with these substrates by following the disappearance of acid-stable radioactivity. All assays in crude extracts and during the purification were performed in the presence of  $5 \text{ mM}$  ATP-Mg, which we found to slow down enzymatic hydrolysis of CoA derivatives by pyrophosphatases<sup>3</sup>; however, this did cause some inhibition of the decarboxylase activity (see below). Diluted mouse liver (1:10,000) and white adipose tissue (1:1000) extracts catalyzed the decarboxylation of ethylmalonyl-CoA ( $\sim 0.5 \mu\text{M}$ ) with first order kinetics and half-lives of  $\sim 10$  and  $20$  min, respectively (Fig. 1). About 10-fold slower decarboxylation rates were observed with methylmalonyl-CoA than with ethylmalonyl-CoA (Fig. 1). The decarboxylation rates were proportional with extract concentration, provided appropriate dilutions were used. Because of the first order kinetics of the decarboxylation rate under our experimental conditions, one unit was defined as the amount of enzyme that catalyzes the breakdown of 50% of radioactive ethylmalonyl-CoA per ml in 1 min under the conditions described (*i.e.* in the presence of  $5 \text{ mM}$  ATP-Mg). Calculations applied to the results shown in Fig. 1 indicated the presence of  $\sim 1000$  and  $50$  units of ethylmalonyl-CoA decarboxylase per g of liver and white adipose tissue, respectively.

**Tissue Distribution, Subcellular Localization, and Partial Purification of Ethylmalonyl-CoA Decarboxylase**—Similar assays were performed on extracts from different mouse tissues.



**FIGURE 2. Tissue distribution of ethylmalonyl-CoA decarboxylase.** The ethylmalonyl-CoA decarboxylase activity was determined with appropriate dilutions of the extracts prepared from the indicated tissues. The results are expressed as units/mg protein in the extract. For the definition of unit, see "Experimental Procedures." Data represent the means of 3–4 samples  $\pm$  S.E. (error bars) for each tissue. EMDCDC, ethylmalonyl-CoA decarboxylase; Ad T, adipose tissue; Sk, skeletal.

The activities are reported per mg of protein present in the extracts (Fig. 2). Brown adipose tissue, liver, and kidney contained the highest levels of enzymatic activity. Using this assay, we also tested the effect of up to 3 days of fasting on the enzymatic activity in liver, brown adipose tissue, and kidney. No significant effect was observed (not shown).

Subcellular fractionation of a rat liver extract prepared from fresh tissue (22) indicated that the enzyme was essentially ( $>90\%$ ) present in the final high speed supernatant. Specific activities of  $0.13$ ,  $0.09$ ,  $0.09$ , and  $1.52$  units/mg protein (means of two values differing by less than  $15\%$ ) were recorded in the heavy mitochondrial (M), light mitochondrial (L), microsomal (P), and final supernatant fractions. We verified that the low activity found in the mitochondrial fraction was not due to hydrolysis of the substrate by (pyro)phosphatases or deacylases by checking the behavior of this substrate on anion exchange chromatography at the end of incubations.

The enzyme was partially purified from a frozen rat liver extract by successive chromatographic steps on DEAE-Sepharose (Fig. 3), Blue-Trisacryl, and phenyl-Sepharose. These purification steps allowed us to separate ethylmalonyl-CoA decarboxylase from contaminating and potentially interfering activities such as propionyl-CoA carboxylase and methylmalonyl-CoA racemase. The overall degree of purification over the original liver extract (3-fold after the DEAE-Sepharose step; 50-fold after the Blue-Trisacryl and the Phenyl-Sepharose steps) was, however, weak, largely due to loss of activity in each of the steps. As shown in Fig. 4, the partially purified enzyme preparation acted upon ethylmalonyl-CoA about 10 times more rapidly than on methylmalonyl-CoA and not detectably on malonyl-CoA.

**Molecular Identification of Ethylmalonyl-CoA Decarboxylase**—Two complementary approaches were used to try to molecularly identify ethylmalonyl-CoA decarboxylase. The first one was a database search approach, looking for homologous proteins catalyzing a similar reaction. Two such enzymes are known: malonyl-CoA decarboxylase, an enzyme found in vertebrates, and methylmalonyl-CoA decarboxylase, an enzyme initially described in *E. coli* and that is involved in the formation

<sup>3</sup> E. Van Schaftingen and G. Noël, unpublished results.

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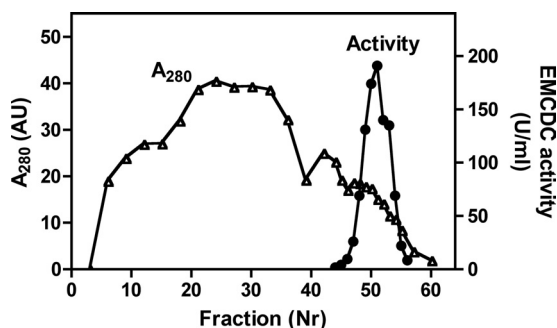


FIGURE 3. Purification of ethylmalonyl-CoA decarboxylase from rat liver by chromatography on DEAE-Sephrose. Shown is the first chromatographic step of the purification procedure. 3-ml fractions were collected. For additional details, see "Experimental Procedures." EMCDC, ethylmalonyl-CoA decarboxylase.

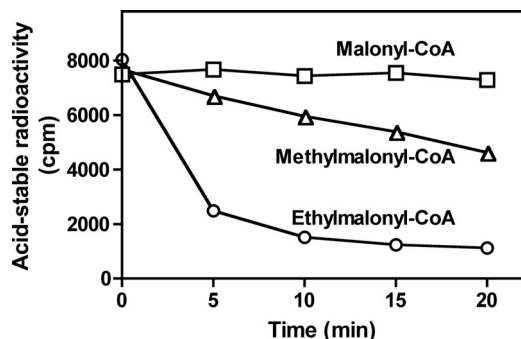


FIGURE 4. Decarboxylation of [<sup>14</sup>C]ethylmalonyl-CoA, [<sup>14</sup>C]methylmalonyl-CoA, and [<sup>14</sup>C]malonyl-CoA by rat liver ethylmalonyl-CoA decarboxylase. Radiolabeled ethylmalonyl-CoA, methylmalonyl-CoA, and malonyl-CoA were incubated with partially purified rat liver ethylmalonyl-CoA decarboxylase (~1.5 units/ml), and decarboxylation was quantified as described for the tissue extracts in legend to Fig. 1. Results are representative of two independent experiments.

of propionyl-CoA from succinyl-CoA. Except for malonyl-CoA decarboxylase itself, no homologous protein of malonyl-CoA decarboxylase was found in mammalian genomes. *E. coli* methylmalonyl-CoA decarboxylase (25) belongs to the family of enoyl-CoA hydratases. The human genome encodes 14 proteins belonging to this family. Among these proteins, only three remain functionally uncharacterized (ECHDC1, ECHDC2, and ECHDC3). Blast searches indicated that none of the three proteins seemed to be closer to *E. coli* methylmalonyl-CoA decarboxylase than other proteins of the same family, indicating that none of them qualified as an obvious ortholog of the *E. coli* protein. However, the prediction for mitochondrial localization by TargetP (26) was much stronger in the case of ECHDC2 and ECHDC3 (0.85–0.90 for the human and mouse proteins) than in the case of ECHDC1 (0.220 and 0.435 for the mouse and human proteins, respectively), suggesting that ECHDC1 is a better candidate for ethylmalonyl-CoA decarboxylase, a cytosolic protein, than the other two proteins. In addition, the tissue distribution of *ECHDC1* mRNA, as available at BioGPS (available on the World Wide Web), correlated better ( $r = 0.9430$ ;  $p \approx 0.0004$ ; 6 degrees of freedom) with the tissue distribution of the ethylmalonyl-CoA decarboxylase activity than those of *ECHDC2* ( $r = 0.6831$ , non-significant) and *ECHDC3* ( $r = 0.1676$ , non-significant).

The second approach was to determine by liquid chromatography coupled to tandem mass spectrometry the identity of the

proteins contained in the most purified fraction of ethylmalonyl-CoA decarboxylase from rat liver. Among 75 hits for which at least two distinct peptides were identified, ECHDC1 was present in the 14th position and ECHDC2 in the 49th position in terms of Sequest identification score, whereas ECHDC3 was absent. ECHDC1 was the fourth hit in terms of sequence coverage (33%), whereas ECHDC2 ranked 40th (11%) based on this criterion. Taken together, these findings indicated that ECHDC1 is an excellent candidate for ethylmalonyl-CoA decarboxylase, as was indeed confirmed by characterization of the recombinant enzyme (see below).

Blast searches indicated that ECHDC1 orthologs (*i.e.* proteins that are significantly closer to human ECHDC1 than to any other human protein) are present in all vertebrates whose genomes have been sequenced (mammalian genomes, *Gallus gallus*, *Xenopus*, fishes) and in several invertebrates like the sea urchin (*Strongylocentrotus purpuratus*), *Tribolium castaneum*, the shoulder tick (*Ixodes scapularis*), *Ciona intestinalis*, *Branchiostoma floridae*, and *C. elegans*. It is apparently absent from *Drosophila*, as indicated by the finding that the closest homolog shows less than 30% sequence identity with mammalian ECHDC1 and is much closer to human ECHDC2 than to human ECHDC1.

Fig. 5 shows an alignment of rat ECHDC1 with enzymes of the same family. The N-terminal region of the protein is not very conserved, which could have suggested the presence of a mitochondrial propeptide. However, two of the peptides identified by mass spectrometry in the protein purified from rat liver corresponded to this non-conserved region, indicating that it was not removed from the protein *in vivo*.

**Characterization of Recombinant ECHDC1**—The cDNA sequence encoding the full-length mouse protein was amplified from mouse brain cDNA and inserted into an expression vector (pET28a) with an N-terminal poly-His tag. The protein was expressed for 20 h at 18 °C and purified to homogeneity by metal affinity chromatography. SDS-PAGE analysis indicated that the protein had, as expected, a subunit molecular mass of ~34 kDa. The homogeneous recombinant enzyme had a specific activity of 8800 units/mg protein, which is ~100-fold higher than the specific activity of the most purified preparation from liver. An HPLC assay was used to verify that ECHDC1 catalyzes the decarboxylation of ethylmalonyl-CoA. As shown in Fig. 6, the enzyme converted ethylmalonyl-CoA and methylmalonyl-CoA to products that co-migrated with butyryl-CoA and propionyl-CoA, respectively.

Chemically synthesized ethylmalonyl-CoA and methylmalonyl-CoA must be mixtures of *S*- and *R*-isomers in roughly equal amounts, which, unfortunately, are not separated with the HPLC procedure that we used (see Fig. 6). It is likely that the enzyme catalyzes preferentially the decarboxylation of one of the two isomers. Evidence for this was obtained by performing decarboxylation assays with different enzyme concentrations. We obtained a clearly biphasic curve with chemically synthesized ethylmalonyl-CoA (Fig. 7A), suggesting that the two isomers are consumed with activities differing by ~2 orders of magnitude. Propionyl-CoA carboxylase synthesizes the *S*-isomer of methylmalonyl-CoA (27) and presumably also of ethylmalonyl-CoA. Similar "titrations" with different concen-

## Ethylmalonyl-CoA Decarboxylase, a Metabolite Repair Enzyme

Ratnor MAKSLLASS-LSVRTKIL-OTGVSLYNTTHGFHEEEVKKILEQFPGGSIDLQKKQNGIGIL 59  
Homsap MAKSLLKTASLSGRTKLLHQTGLSLYSTSHGFYEEVKKTLQFPGGSIDLQKEDNGIGIL 61  
Xenlae MGIFVWRSSLSMTNIRWLHHRCLSLYNSSHGFNEAKIKKKLAQFTGGSVDLKSDDGIAEI 61

Ratnor TLNNSNKMNAFSGAMMLQLLERVIELENWTEGKGLIVHGAKNTFCSGSDLNAVKALSTPE 119  
Homsap TLNNSPSRMNAFSGVMMLQLEKVELENWTEGKGLIVRGAKNTFSSGSDLNAVKSLGTPE 121  
Xenlae CINNPTRMNAFTGTMIELEERISDLENWQDGKGLIVYGAENTFCSGSDLNAVKAISNPQ 121

Ratnor NGVALSMFMQNTLTRFMRLPLISVALVQGWAMGGGAELTTACDFRLMTEESVIRFVHKEM 179  
Homsap DGMAVCMFMQNTLTRFMRLPLISVALVQGWALGGGAEFTTACDFRLMTPESKIRFVHKEM 181  
Xenlae EGMMLMQNTLTRLQRLPLVSVALIQGKALGGGAELCTTACDFRLMTEGSEIRFVHKQM 181

Ratnor GIVPSWGGASRLVEIIGSRQALKVLSGTFKLDSKEALRIGLADEVLQPSDEATALEQAQE 239  
Homsap GIIPSWGGTTRLVEIIGSRQALKVLSGALKLDSKNALNIGMVVEEVLQSSDETKSLEEAQE 241  
Xenlae GLVPGWGGAAARLIHIVGSRHALKLLSGAPRVQENALELGLADNILTGTEAG-VLSEAKN 240

Ratnor WLEQFVSGPAQVIRGLKKSVCSGRELYLEEALQNERDVLETLWGGPANLEAIAKKGKHTK 299  
Homsap WLKQFIQGPPEVIRALKKSVCSGRELYLEEALQNERDLLGTVWGGPANLEAIAKKGKFNK 301  
Xenlae WIMPIKGPSDVTRAVKKVVISGREQNLEDALRTEKEIFGTVWGGLANLQALAKGTKHK- 299

FIGURE 5. Alignment of ECHDC1 from rats, humans, and *X. laevis*. The underlined peptides have been identified by MS/MS analysis of a purified fraction of rat liver ethylmalonyl-CoA decarboxylase. The following sequences are shown: *Rattus norvegicus* NP\_001007735.1 (Ratnor), *Homo sapiens* NP\_001002030 (Homsap), and *X. laevis* NP\_001088953.1 (Xenlae). Residues that are conserved in the three sequences are indicated in boldface type.

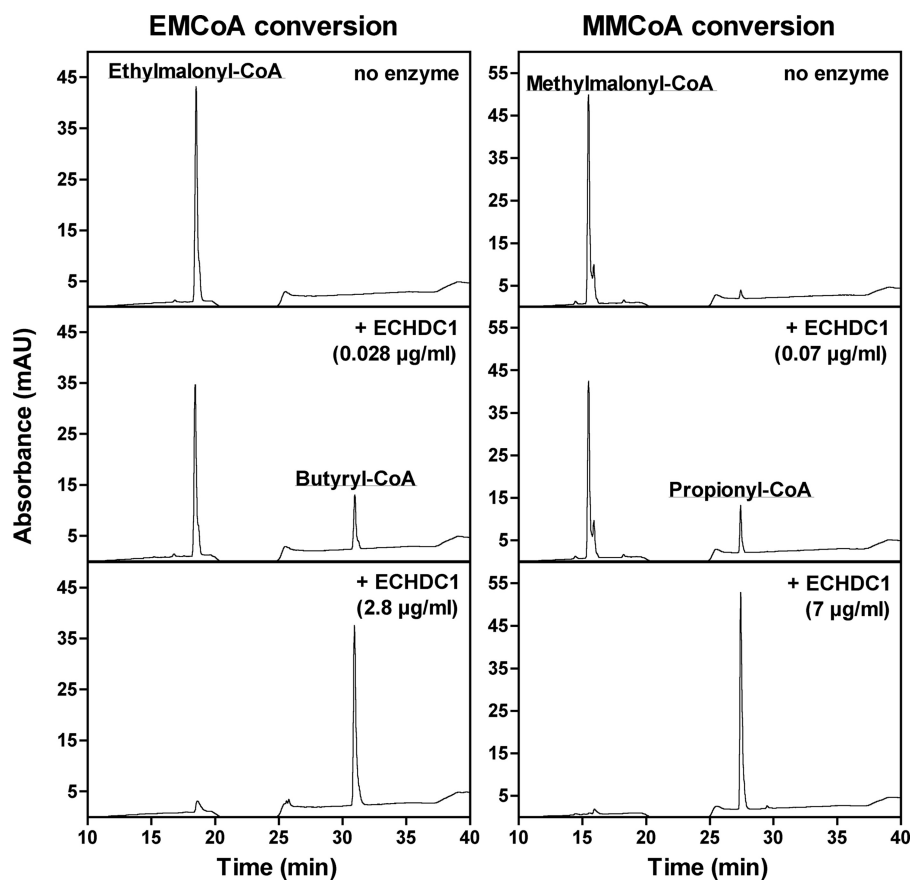
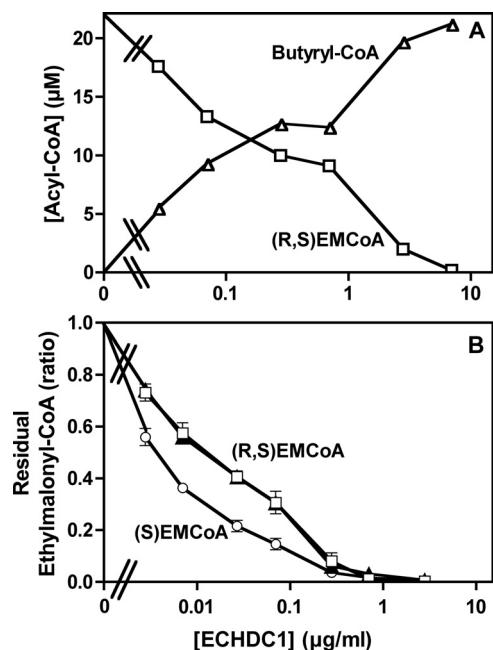


FIGURE 6. Formation of butyryl-CoA from ethylmalonyl-CoA and of propionyl-CoA from methylmalonyl-CoA catalyzed by ECHDC1. 22  $\mu$ M ethylmalonyl-CoA or methylmalonyl-CoA was incubated at 30 °C without or with recombinant ECHDC1 at the indicated concentrations, and the incubations were arrested after 20 min by the addition of perchloric acid. After adjusting the pH to about 4.5 with  $K_2CO_3$ , samples were analyzed by the reverse-phase HPLC method described under "Experimental Procedures." Shown are HPLC chromatograms obtained by monitoring UV absorbance at 260 nm. Eluting peaks were identified by comparing their retention time and UV absorption spectra with standard compounds. EMCoA, ethylmalonyl-CoA; MMCoA, methylmalonyl-CoA.

trations of recombinant ethylmalonyl-CoA decarboxylase were performed using radiolabeled ethylmalonyl-CoA produced by propionyl-CoA carboxylase or with a mixture of epimers obtained by heating this enzymatically synthesized ethylmalonyl-CoA at 100 °C for 15 or 30 min (28). As shown in Fig. 7B, a

monophasic curve was obtained with (*S*)-ethylmalonyl-CoA, whereas a biphasic curve was observed with the mixture of epimers. From these data, we conclude that ethylmalonyl-CoA decarboxylase preferentially uses (*S*)-ethylmalonyl-CoA but that it has also some activity on the *R*-form. To rule out the

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**FIGURE 7. Evidence that recombinant ethylmalonyl-CoA decarboxylase acts preferentially on (S)-ethylmalonyl-CoA.** *A*, chemically synthesized ethylmalonyl-CoA (22 μM) was incubated with the indicated concentrations of recombinant ECHDC1 as described in the legend to Fig. 6. Ethylmalonyl-CoA consumption and butyryl-CoA formation were determined by HPLC. The results shown are representative of three independent experiments. *B*, enzymatically synthesized [<sup>14</sup>C]ethylmalonyl-CoA was used as such (open circles) or after racemization by incubation for 15 min (closed triangles) or 30 min (open squares) at 100 °C. Both types of substrates were incubated for 20 min at 30 °C in the presence of the indicated concentrations of recombinant ECHDC1, and the residual acid-stable radioactivity was measured. Results represent the mean of three values ± S.E. EMCoA, ethylmalonyl-CoA.

**TABLE 1**  
Kinetic properties of recombinant ECHDC1

The values are given for the *S*-form of the substrates, assuming that the chemically synthesized compounds comprise 50% of the two forms.

Substrate	[ATP-Mg]	$K_m$	$k_{cat}$	$k_{cat}/K_m$
	<i>mM</i>	<i>μM</i>	<i>s</i> <sup>-1</sup>	<i>M</i> <sup>-1</sup> <i>s</i> <sup>-1</sup>
(S)-Ethylmalonyl-CoA	0	0.96 ± 0.08	9.5 ± 0.3	9.9 × 10 <sup>6</sup>
(S)-Ethylmalonyl-CoA	5	6.5 ± 0.5	10.0 ± 0.4	1.5 × 10 <sup>6</sup>
(S)-Methylmalonyl-CoA	0	3.1 ± 0.5	1.61 ± 0.10	0.51 × 10 <sup>6</sup>
(S)-Methylmalonyl-CoA	5	15.1 ± 0.7	1.68 ± 0.03	0.11 × 10 <sup>6</sup>

possibility that ethylmalonyl-CoA decarboxylase uses the *R*-form because of a contamination of the preparation with methylmalonyl-CoA epimerase, a metalloenzyme, we preincubated the purified enzyme preparation with EDTA for up to 16 h. This enzyme preparation still decarboxylated both forms of ethylmalonyl-CoA (results not shown).

We determined the  $K_m$  of recombinant ECHDC1 for ethylmalonyl-CoA using enzymatically synthesized radiolabeled ethylmalonyl-CoA and methylmalonyl-CoA together with variable concentrations of the chemically synthesized unlabeled compounds, which we assumed to be mixtures of the two epimers in equal amounts. As shown in Table 1, ethylmalonyl-CoA decarboxylase showed a  $K_m$  for (S)-ethylmalonyl-CoA of ~1 μM and a  $k_{cat}$  of ~10 s<sup>-1</sup> in the absence of ATP-Mg. For (S)-methylmalonyl-CoA, the  $K_m$  was about 3-fold higher, and the  $V_{max}$  ( $k_{cat}$ ) was about 6-fold lower. Thus, ethylmalonyl-CoA was an about 20-fold better substrate for ECHDC1 than methylmalonyl-CoA in terms of

catalytic efficiency, in agreement with the difference in activities observed with the enzyme purified from rat liver (see Fig. 4). The presence of ATP-Mg increased the  $K_m$  for both substrates without affecting the maximal activity, indicating that ATP-Mg acts as a competitive inhibitor. Other experiments performed with ethylmalonyl-CoA as a substrate indicated a  $K_i$  of ~1 mM for ATP-Mg. No inhibition was observed with GTP-Mg (not shown). Although we used 1 mM Mg<sup>2+</sup> in most of our enzymatic assays to mimic physiological conditions, we found that Mg<sup>2+</sup> had no effect on the activity of recombinant ECHDC1, which was also virtually unaffected by the presence of 1 mM EDTA (not shown).

We also checked whether recombinant ECHDC1 can act as an enoyl-CoA hydratase. Using a spectrophotometric assay (29) containing 30 μM butenoyl-CoA as a substrate, we did not detect any activity, leading us to conclude that the enoyl-CoA hydratase activity of ECHDC1, if any, amounts to less than 1/1000 of its ethylmalonyl-CoA decarboxylase activity.

*Confirmation of the Identity of Ethylmalonyl-CoA Decarboxylase through an siRNA Approach and Effect on the Concentration of Ethylmalonate*—Ethylmalonyl-CoA decarboxylase activity was also present in HEK293T cell extracts. To verify that this enzyme was indeed encoded by the *ECHDC1* gene, we transfected HEK293T cells with an siRNA pool specifically targeting this gene. As shown in Fig. 8A, an about 3-fold decrease in *ECHDC1* mRNA levels was measured in HEK293T cells transfected with *ECHDC1* siRNA compared with cells treated with non-targeting control siRNA, independently of the reference gene used for normalization purposes (*GAPDH* or *ACTB* (β-actin)). This decrease in *ECHDC1* mRNA was accompanied by an average decrease of about 50% in the ethylmalonyl-CoA decarboxylase activity (Fig. 8B). The knockdown efficiency in these experiments varied from 40 to 70% in terms of activity and even reached up to 85% in the loading experiments described below. We assume that this variability is mostly due to differences in cell confluence at the time of siRNA transfection, a lower cell confluence favoring knockdown efficiency in the case of the *ECHDC1* gene. These results confirm the identification of ethylmalonyl-CoA decarboxylase as the expression product of the *ECHDC1* gene.

To confirm that ethylmalonyl-CoA is a substrate of the ECHDC1 enzyme in the living cell, we tested whether knocking down the *ECHDC1* gene leads to increased ethylmalonic acid concentration in the medium of HEK293T cells incubated in the absence or presence of potential precursors (5 mM butyrate, 5 mM L-isoleucine, or 1 mM L-alloisoleucine). Extracellular ethylmalonate concentrations were very low compared with the concentration of other organic acids in the cell media, and ethylmalonate could only be detected when using the selected ion mode in our GC-MS method. As shown in Fig. 9A, a small increase in signal intensity could be detected in the extracted ion chromatogram for ion *m/z* 217 (a major ion in the mass spectrum of derivatized ethylmalonic acid) at the retention time of ethylmalonic acid (9.0 min) in the media of cells transfected with *ECHDC1* siRNA compared with cells treated with control siRNA. A 24-h incubation with butyrate led to a more than 2-fold increase in the extracellular concentration of ethyl-

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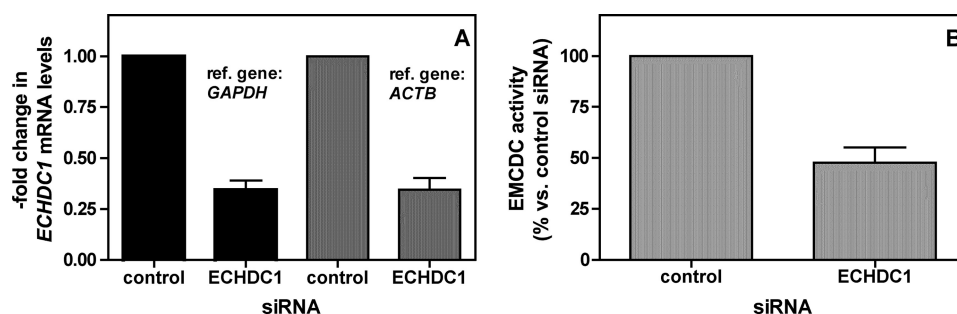


FIGURE 8. **Effect of *ECHDC1* siRNA on *ECHDC1* mRNA levels and the ethylmalonyl-CoA decarboxylase activity in human cells.** HEK293T cell cultures were stopped 48–72 h after siRNA transfection. *A*, quantitative RT-PCR was performed on cDNA derived from these cells to measure *ECHDC1* mRNA levels. *ECHDC1* expression was normalized to the abundance of *GAPDH* (black bars) and *ACTB* ( $\beta$ -actin; gray bars) mRNAs and -fold changes in expression levels in *ECHDC1* versus control siRNA-transfected cells were calculated using the  $2^{-\Delta\Delta C_t}$  method. *B*, ethylmalonyl-CoA decarboxylase activity was measured in protein extracts derived from these cells by the radioactive assay. The results shown represent the means  $\pm$  S.E. (error bars) of four biological replicates. EMDC, ethylmalonyl-CoA decarboxylase.

malonate in control cells and to a 5-fold increase in *ECHDC1* knockdown cells (Fig. 9, *B* and *C*).

L-Alloisoleucine is formed in small amounts during transamination of L-isoleucine (30), and its metabolism (through a pathway similar to that of valine metabolism) has been suggested to lead to the formation of ethylmalonyl-CoA via 2-ethylhydracrylate (31). Although we could detect ethylhydracrylate in the medium of cells incubated with L-alloisoleucine (not shown), no increases or only minimal increases in extracellular ethylmalonate were measured in this condition whether for control or *ECHDC1* knockdown cells (Fig. 9*C*). Similar results were obtained when L-isoleucine was added to the cell culture media (Fig. 9*C*).

### DISCUSSION

**Establishment of the Activity of *ECHDC1* as Ethylmalonyl-CoA Decarboxylase**—We report the existence of a new enzyme that converts ethylmalonyl-CoA to butyryl-CoA and CO<sub>2</sub>. It also catalyzes a similar reaction on methylmalonyl-CoA but with an about 20-fold lower catalytic efficiency. We also provide evidence that it acts on the ethylmalonyl-CoA form that is produced by propionyl-CoA carboxylase (*i.e.* the *S*-isomer, by analogy with the normal product of this enzyme, (*S*)-methylmalonyl-CoA) (27). The new enzyme does not act detectably on malonyl-CoA and is thus different from malonyl-CoA decarboxylase. It is therefore best described as being an ethylmalonyl-CoA decarboxylase.

We show that this enzyme is encoded by the *ECHDC1* gene. Suggestive evidence came from the observations that *ECHDC1* is present in partially purified ethylmalonyl-CoA decarboxylase and that the mouse tissue distribution of the activity of ethylmalonyl-CoA decarboxylase is similar to that of the *ECHDC1* mRNA. The proof for this identification was provided by showing that recombinant *ECHDC1* catalyzes the same reaction as ethylmalonyl-CoA decarboxylase purified from rat liver and that it is also more active on ethylmalonyl-CoA than on methylmalonyl-CoA and inactive on malonyl-CoA. Furthermore, the ethylmalonyl-CoA decarboxylase activity present in human cells could be knocked down by using *ECHDC1*-specific siRNAs.

*ECHDC1* belongs to the family of enoyl-CoA hydratases. The latter comprises a series of enzymes catalyzing diverse

reactions on acyl-CoAs, including hydration (enoyl-CoA hydratase), isomerization, thioester bond hydrolysis (3-hydroxyisopropionyl-CoA hydrolase), and decarboxylation (methylmalonyl-CoA decarboxylase) (see Ref. 32 for a review). We verified that *ECHDC1* has no crotonyl-CoA hydratase activity. Furthermore, the quantitative conversion of ethylmalonyl-CoA to butyryl-CoA that we measured with *ECHDC1* in this study indicates that it has no thioesterase activity. The common feature of all enzymes of the enoyl-CoA hydratase family is that the catalytic mechanism proceeds via a thiolate anion (32).

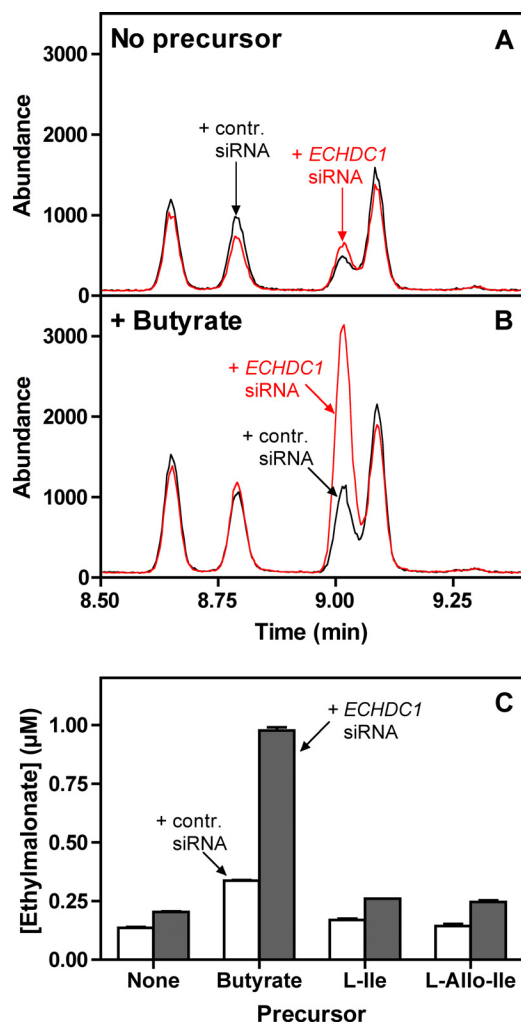
It is interesting to compare *ECHDC1* with the other decarboxylase that has been described in this family, namely *E. coli* methylmalonyl-CoA decarboxylase (25, 33). The latter was identified as an enzyme belonging to an operon permitting the formation of propionate from succinyl-CoA by the successive action of methylmalonyl-CoA mutase (*Sbm*), methylmalonyl-CoA decarboxylase (*YgfG*), and propionyl-CoA:succinate-CoA transferase (*YgfH*). Because there is apparently no methylmalonyl-CoA epimerase encoded by the *E. coli* genome (33), we presume that methylmalonyl-CoA decarboxylase is acting on the isomer that is produced by methylmalonyl-CoA mutase (*i.e.* the *R*-isomer), whereas, as mentioned above, mammalian ethylmalonyl-CoA decarboxylase acts on the *S*-isomer. This may explain that, despite belonging to the same protein family, *E. coli* *YgfG* and mammalian *ECHDC1* show low degrees of identity with each other. As a matter of fact, *YgfG* is not closer to *ECHDC1* than it is to other mammalian members of the enoyl-CoA hydratase family catalyzing very different reactions.

It is also interesting to compare the catalytic efficiency of recombinant mouse *ECHDC1* in its action on ethylmalonyl-CoA (Table 1) with that of *E. coli* methylmalonyl-CoA decarboxylase on methylmalonyl-CoA ( $1.2 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ ) (33). The 80 times higher value observed with the former enzyme supports the idea that ethylmalonyl-CoA decarboxylation is the physiological activity of *ECHDC1*.

**Is Ethylmalonyl-CoA Decarboxylase Involved in the Metabolism of Alloisoleucine?**—A potential source of ethylmalonate and ethylmalonyl-CoA is alloisoleucine. This non-standard amino acid is formed through epimerization of the  $\beta$ -carbon during transamination of isoleucine (30). Its metabolism, which



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**FIGURE 9. Consequences of knocking down ECHDC1 activity on the concentration of ethylmalonate in the medium of cells challenged with butyrate, L-isoleucine and L-alloisoleucine.** Butyrate (5 mM), L-isoleucine (5 mM), and L-alloisoleucine (1 mM) were added to the culture media of HEK293T cells that had been transfected with control or *ECHDC1* siRNAs 48 h earlier. Cultures were stopped 24 h after medium supplementation; protein extracts were prepared from cells for ethylmalonyl-CoA decarboxylase activity measurements, and organic acids were extracted from the corresponding media for subsequent GC-MS analysis. An average knockdown efficiency of about 75% was obtained for ethylmalonyl-CoA decarboxylase activity in these experiments. A and B show overlays of extracted ion chromatograms (*m/z* 217) obtained for media of control siRNA-treated cells (black line) or *ECHDC1* siRNA-treated cells (red line) incubated in the absence (A) or presence of 5 mM butyrate (B). Standard ethylmalonic acid eluted at 9.0 min in the GC-MS method used. C represents the concentrations of extracellular ethylmalonate measured for control (white bars) or *ECHDC1* knockdown (gray bars) cells incubated in the absence or presence of the indicated compounds. Peak areas of the extracted ion current were used for ethylmalonate quantification except when L-isoleucine was used for medium supplementation. In the latter condition, a 2-keto-3-methylvaleric acid (transamination product of L-isoleucine) derivative generated a peak overlapping with the ethylmalonate peak, and consequently peak height rather than peak area was used for ethylmalonate quantification. The concentrations shown represent the means  $\pm$  S.E. (error bars) of three biological replicates. L-Allo-Ile, L-alloisoleucine.

represents an undefined, presumably small fraction of L-isoleucine metabolism, seems to proceed initially in a similar manner to the metabolism of L-valine, in that the methyl branch rather than the ethyl branch (as in the normal metabolism of isoleucine) is oxidized. This leads successively to the formation of ethylacrylyl-CoA (an enoyl-CoA), ethylhydracrylyl-CoA (a 3-hydroxyacyl-CoA), ethylhydracrylate, and ethylmalonate

semialdehyde (Fig. 10). Although most of the indicated reactions have not been studied *in vitro*, indirect evidence supports this sequence of reactions as well as the identity of the enzymes suggested in Fig. 10. Thus, patients with 3-hydroxyisobutyric aciduria, which is presumably due to 3-hydroxyisobutyrate dehydrogenase deficiency, often show also accumulation of ethylhydracrylate (34), indicating that 3-hydroxyisobutyrate dehydrogenase is involved in ethylhydracrylate metabolism but implying also that ethylhydracrylyl-CoA is hydrolyzed before its alcohol function is oxidized.

The analogy with the L-valine pathway appears to stop at the level of (m)ethylmalonate semialdehyde oxidation. The valine catabolite is oxidized by methylmalonate semialdehyde dehydrogenase (ALDH6A1), a mitochondrial enzyme that oxidizes the aldehyde group by converting it to an acyl-CoA ester, while at the same time removing the initial carboxylic group and leading therefore to propionyl-CoA (35). Goodwin *et al.* (35) mention that methylmalonate semialdehyde dehydrogenase has no activity on ethylmalonate semialdehyde. By contrast, *in vivo* tracer studies obtained with 2- $^{13}\text{C}$ methylbutyrate labeled on the first carbon or the 2-methyl carbon suggest that ethylmalonate semialdehyde loses its first carbon during conversion to butyryl-CoA (36). The two findings can be reconciled by admitting the existence of a dehydrogenase catalyzing on ethylmalonate semialdehyde a similar reaction as the one catalyzed by methylmalonate semialdehyde dehydrogenase on its substrate (Fig. 10). Alternatively, a dehydrogenase could convert ethylmalonate semialdehyde to ethylmalonate, and the latter, a prochiral compound, could be stereospecifically activated to ethylmalonyl-CoA and decarboxylated by ECHDC1 (Fig. 10). However, nothing precise is known about the dehydrogenase that acts on ethylmalonate semialdehyde and about the enzyme(s) that could activate ethylmalonate to ethylmalonyl-CoA.

Many data point to the sluggishness of this “alternative” pathway of isoleucine metabolism. The finding that ethylhydracrylic acid accumulates when the normal pathway of isoleucine metabolism is blocked at the level of 2-methylbutyryl-CoA dehydrogenase suggests that the alternative R-pathway is not very efficient (37). This is also indicated by the finding that loading with (R,S)-2-methylbutyric acid, a precursor of one of the early intermediates in this pathway, leads to massive excretion of ethylhydracrylic acid, amounting to about 40% of the administered (R)-2-methylbutyric acid (36). Our own data do not support a role of ethylmalonyl-CoA decarboxylase in the metabolism of L-alloisoleucine. The subcellular localization of this enzyme does not fit with that (mitochondrial) of the known enzymes involved in L-alloisoleucine metabolism. Furthermore, the addition of this amino acid to cell cultures did not cause any detectable increase in the concentration of ethylmalonate, whether in control or *ECHDC1* knockdown cells, whereas it did cause an increase in ethylhydracrylic acid, indicating that L-alloisoleucine was metabolized. However, we cannot exclude the possibility that this absence of increase in the ethylmalonate concentration is due to the absence of appropriate enzymes for the oxidation of ethylmalonate semialdehyde in HEK293T cells.

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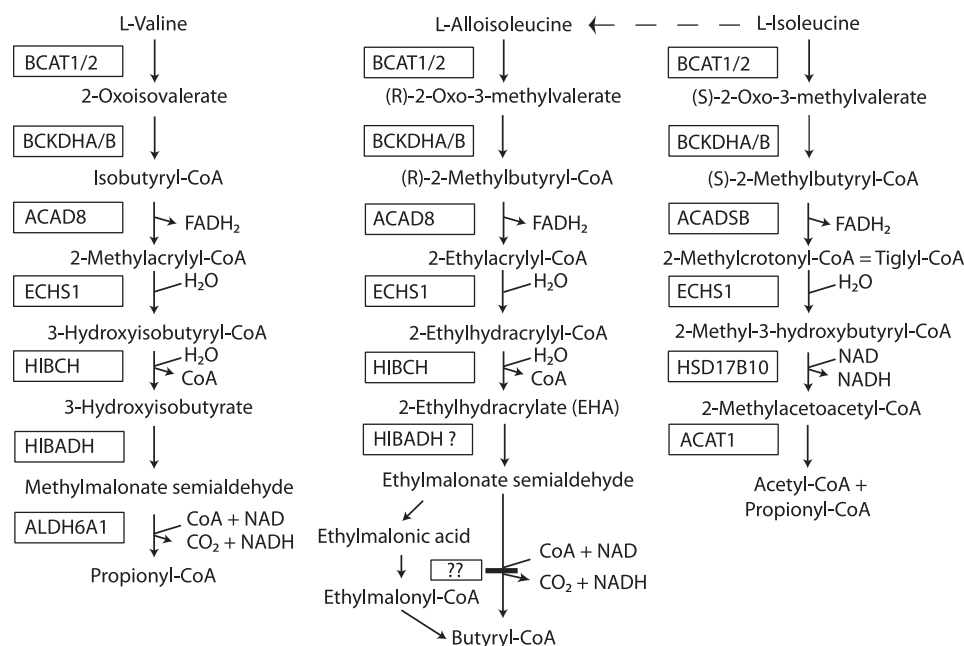


FIGURE 10. **Comparative metabolism of L-valine, L-isoleucine, and L-alloisoleucine and formation of ethylmalonate from L-alloisoleucine.** Enzymes involved in the different reactions are indicated by the human gene names. *ACAD8*, isobutyryl-CoA dehydrogenase; *ACADSB*, short/branched chain acyl-CoA dehydrogenase; *ACAT1*, acetyl-CoA acetyltransferase 1; *ALDH6A1*, methylmalonate semialdehyde dehydrogenase; *BCAT1/2*, branched chain amino acid transaminase 1 or 2; *BCKDHA/B*, branched chain keto acid dehydrogenase; *ECHS1*, short chain enoyl-CoA hydratase; *HIBADH*, 3-hydroxyisobutyrate dehydrogenase; *HIBCH*, 3-hydroxyisobutyryl-CoA hydrolase; *HSD17B10*, 2-methyl-3-hydroxybutyryl-CoA dehydrogenase.

**Metabolite Proofreading Role of Ethylmalonyl-CoA Decarboxylase**—As mentioned in the Introduction, ethylmalonyl-CoA can be formed *in vitro* both by acetyl-CoA carboxylase (20), a cytosolic enzyme, and by propionyl-CoA carboxylase (17), a mitochondrial enzyme. Our hypothesis that ethylmalonyl-CoA decarboxylase serves to eliminate ethylmalonyl-CoA made from butyryl-CoA is supported by our finding that knocking down *ECHDC1* with siRNAs increases severalfold the formation of ethylmalonate in cells incubated with butyrate. The ethylmalonyl-CoA that accumulates under such conditions is presumably hydrolyzed by thioesterases present in cells.

We were initially surprised by two properties of the ethylmalonyl-CoA decarboxylase: its main localization in the cytosol, which accounts for more than 90% of the enzymatic activity, and its relative lack of specificity, because it acts also on methylmalonyl-CoA. As a matter of fact, both properties are related; a high mitochondrial methylmalonyl-CoA decarboxylase activity would cause a futile cycle with propionyl-CoA carboxylase. This also means, however, that metabolite repair of ethylmalonyl-CoA essentially occurs in the cytosol, mainly compensating for the lack of specificity of acetyl-CoA carboxylase (Fig. 11). Furthermore, the enzyme is also able to eliminate cytosolic methylmalonyl-CoA, which is potentially formed from cytosolic propionyl-CoA by acetyl-CoA carboxylase.

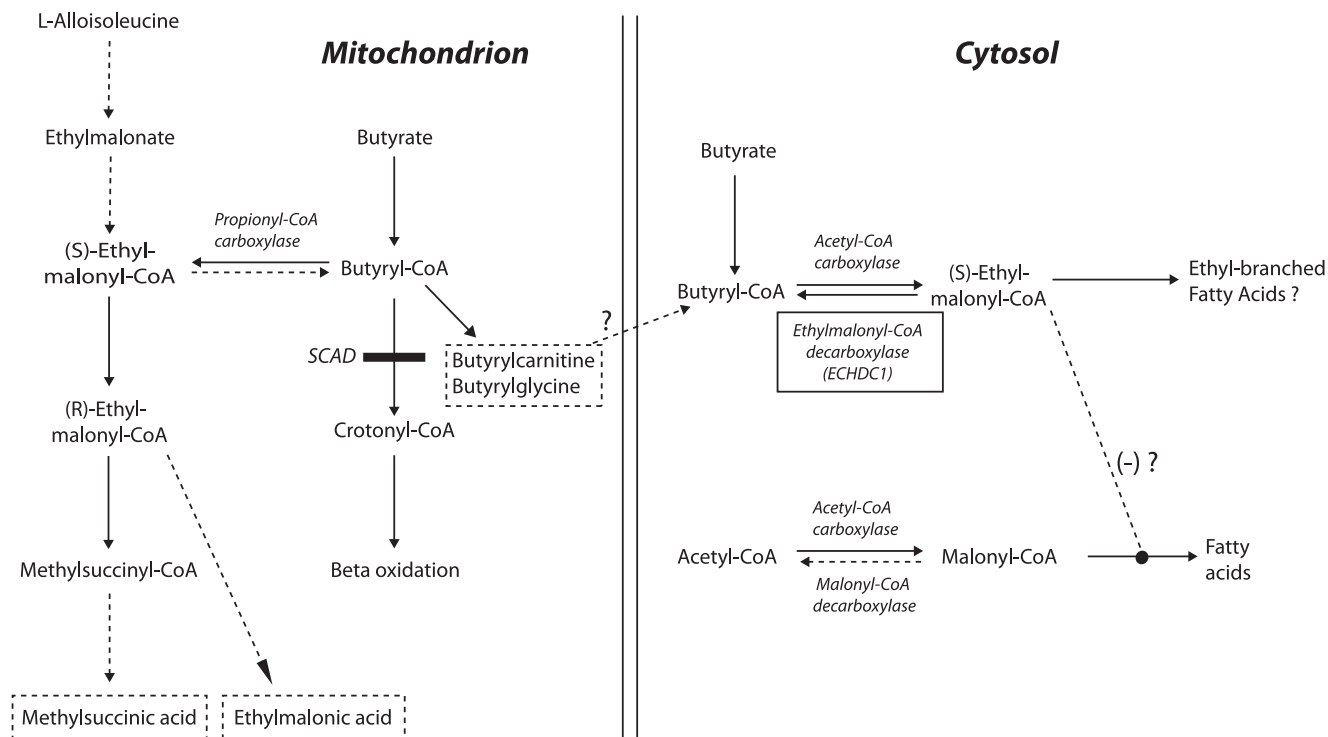
The presence of ethylmalonyl-CoA decarboxylase in the cytosol may prevent perturbation of lipid synthesis by fatty acid synthase (Fig. 11). Ethylmalonyl-CoA and methylmalonyl-CoA could perturb this process merely by trapping CoA, by inhibiting fatty acid synthase due to their structural similarity with malonyl-CoA, or even by being used by fatty acid synthase, leading then to the formation of fatty acids with ethyl or methyl branches. The presence of fatty acids with methyl and ethyl

branches has been described on the feathers of birds (38) and in the fleece of cashmere goats (39), respectively. The formation of methyl-branched fatty acids from methylmalonyl-CoA by fatty acid synthase has been demonstrated in goose uropygial gland extracts (40). The formation of branched fatty acids by fatty acid synthase is favored in these glands by the presence of a highly active malonyl-CoA decarboxylase, which does not act on methylmalonyl-CoA and thereby specifically depletes the malonyl-CoA pool (41). Ethylmalonyl-CoA decarboxylase might reciprocally prevent the formation of methyl or ethyl branches by depleting the cytosolic pool of (m)ethylmalonyl-CoA.

**Phenotype Expected in Ethylmalonyl-CoA Decarboxylase Deficiency?**—Absence of ethylmalonyl-CoA decarboxylase is expected to cause an increase in ethylmalonic acid concentration and to be therefore a cause of ethylmalonic aciduria. Two different enzymatic defects are presently known to cause abnormal excretion of ethylmalonic acid. The first one is a defect in short chain acyl-CoA dehydrogenase (SCAD) (42), an enzyme that serves to metabolize butyryl-CoA (Fig. 11). The abnormal excretion of ethylmalonate is easily explained in the following manner. The block in the oxidation of butyryl-CoA causes a rise in its concentration and thereby stimulates its “illegitimate” carboxylation by acetyl-CoA carboxylase and propionyl-CoA carboxylase.

This explanation is certainly partially true, and many patients with ethylmalonic aciduria have been found to have mutations in the *SCAD* gene that cause marked alterations in the kinetic properties of this enzyme. However, two of the frequent “mutations” that have been found in this disorder have minimal effects on the kinetic properties of the enzyme and are largely present in the normal population. 6% of normal people are

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**FIGURE 11. Potential role of ethylmalonyl-CoA decarboxylase in eliminating ethylmalonyl-CoA made by carboxylases.** Ethylmalonyl-CoA can be formed from butyryl-CoA by propionyl-CoA carboxylase in the mitochondria and by acetyl-CoA carboxylase in the cytosol. It is efficiently broken down by ethylmalonyl-CoA decarboxylase in the cytosol but much less so in the mitochondria, where it is converted to methylsuccinyl-CoA by the enzymes involved in methylmalonyl-CoA metabolism. SCAD deficiency enhances the formation of butyrylcarnitine and butyrylglycine, which are both found in urines. It also enhances the formation of ethylmalonyl-CoA and methylsuccinyl-CoA, leading to the appearance of ethylmalonic acid and methylsuccinic acid in urine. Ethylmalonyl-CoA decarboxylase deficiency is likely to exacerbate ethylmalonic aciduria and interfere with fatty acid synthesis.

homozygous for the mutation that has been most frequently found in ethylmalonic aciduria (42). One potential explanation for this discrepancy could be that ethylmalonic aciduria develops in patients with these hypoactive variants of SCAD only if these variants are combined with a defect in ethylmalonyl-CoA decarboxylase. It would be most interesting to search for mutations in the *ECHDC1* gene in such patients.

A second important cause of ethylmalonic aciduria is known as ethylmalonic encephalopathy. This more severe condition is due to a defect in an enzyme (*ETHE1*) involved in sulfur metabolism. The accumulation of  $\text{HS}^-$  leads to multiple metabolic alterations (43), including defects in complex IV and in short chain acyl-CoA dehydrogenase. The latter is thought to be responsible for the accumulation of ethylmalonate, as described above for the genetic alterations in the *SCAD* gene. We have checked if ethylmalonyl-CoA decarboxylase is inhibited by  $\text{HS}^-$  and found no effect.<sup>3</sup>

Isolated ethylmalonyl-CoA decarboxylase deficiency could lead to a third form of ethylmalonic aciduria. It is expected that in this case, no enhanced excretion of butyrylcarnitine or butyrylglycine would be observed. One expectation is, however, that this disorder might be accompanied by the accumulation of abnormal, ethyl-branched fatty acids. Whether this would be an isolated biochemical anomaly or whether it would lead to major pathophysiological perturbations is difficult to predict.

In conclusion, ethylmalonyl-CoA decarboxylase is a new enzyme, which we postulated to exist based on the metabolite proofreading hypothesis. We provide evidence for the fact that one of the roles of this enzyme, maybe its exclusive role, is

indeed to “correct” ethylmalonyl-CoA formed by acetyl-CoA carboxylase. This enzyme is likely to be mutated in an as yet undescribed form of ethylmalonic aciduria, which would therefore qualify as a new disorder of metabolite proofreading.

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