Isovaleryl-CoA Dehydrogenase Activity in Isovaleric Acidemia Fibroblasts Using an Improved Tritium Release Assay

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ABSTRACT. Isovaleric acidemia is a disorder of leucine metabolism caused by a deficiency of isovaleryl-CoA dehydrogenase. At least two clinical subgroups of patients exist: a severe form, in which symptoms occur within the 1st wk of life, and a milder variant in which manifestations develop later in life. We developed a modified version of the tritium release assay to accurately measure residual isovaleryl-CoA dehydrogenase activity in fibroblasts from patients with both forms of isovaleric acidemia. In the modified assay, specific isovaleryl-CoA dehydrogenase-catalyzed tritium release from [2,3-³H]isovaleryl-CoA was determined by including an inhibitor of isovaleryl-CoA dehydrogenase, (methylenecyclopropyl)acetyl-CoA, in one of the tubes in paired assays, to determine the nonspecifically released ³H₂O. Residual activities of the nine isovaleric acidemia lines tested ranged from 0 to 0.67 pmol ³H₂O/min/mg protein (controls 19.4 ± 8.0). The three lines from mildly affected individuals all had no detectable activity, whereas the severe cases had a mean of 0.41 pmol ³H₂O/min/mg protein. Normal human fibroblast isovaleryl-CoA dehydrogenase had a K_m for isovaleryl-CoA of 22 μM, with a V_{max} of 51 pmol ³H₂O/min/mg protein. The K_i of isovaleryl-CoA dehydrogenase by (methylenecyclopropyl)acetyl-CoA was approximately 2 μM. (*Pediatr Res* 20: 59–61, 1986)

Abbreviation

MCPA-CoA, (methylenecyclopropyl)acetyl-CoA

Isovaleric acidemia is an inherited disorder of leucine metabolism caused by a deficiency of the activity of isovaleryl-CoA dehydrogenase (EC. 1.3.99.10) (1, 2). Isovaleric acidemia has generally been considered to have two clinical phenotypes (3). One form (severe type) manifests as severe vomiting, lethargy, and coma, which commences within the first 2 wk of life and often causes death in the neonatal period. The other form (mild type) is characterized by recurrent milder episodes of vomiting, lethargy, and ketoacidosis. These episodes tend to begin later in life, and they are often triggered by viral or bacterial infection.

However, this clinical classification is rather arbitrary. In those patients with the severe type who survive the first 2 wk of life with appropriate treatment, the subsequent course is not distinctly different from that of the mild type (3).

The enzyme defect in isovaleric acidemia had been assumed to be at the level of isovaleryl-CoA dehydrogenase because of the accumulation of isovaleric acid in blood (4) and isovalerylglycine in urine (5). Rhead and Tanaka (6) developed a tritium release assay for the determination of the activity of this enzyme, which measured ³H₂O formed through enzyme-catalyzed dehydrogenation of [2,3-³H]isovaleryl-CoA, and they demonstrated that residual isovaleryl-CoA dehydrogenase activity in cultured fibroblast mitochondria from five isovaleric acidemia patients was 13% of control activity (1). In contrast, the ability of isovaleric acidemia cells to oxidize [2-¹⁴C]leucine was only 1–2% of control values (7). The nature and magnitude of true residual isovaleryl-CoA dehydrogenase activity in isovaleric acidemia cells could not be accurately determined by the original tritium release assay.

Thus it was not possible to test whether difference in clinical severity can be related to the magnitude of the residual activity. Employing an inhibitor of several acyl-CoA dehydrogenases, MCPA-CoA (2, 8, 9), we developed an improved assay for isovaleryl-CoA dehydrogenase to accurately determine residual isovaleryl-CoA dehydrogenase activity in fibroblasts of patients with clinically severe and mild forms of isovaleric acidemia.

MATERIALS AND METHODS

Materials. [2,3-³H]isovaleric acid-CoA (10 mCi/mmol), custom synthesized by catalytic tritiation of 3-methylcrotonic acid, was purchased from New England Nuclear (Boston, MA). MCPA acetic acid was prepared from hypoglycin A [L-α-amino-β-(methylenecyclopropyl)propionic acid] using snake venom L-amino acid oxidase. The coenzyme A derivatives were prepared via mixed anhydride synthesis. The synthesized coenzyme A esters were purified using descending paper chromatography (9).

Tissue culture. Skin fibroblasts were grown in Eagle's minimal essential media with nonessential amino acids and glutamine (Flow Laboratories, McLean, VA) supplemented with 10% fetal calf serum, and containing kanamycin and phenol red.

Enzyme assay. Isovaleryl-CoA dehydrogenase activity was assayed as follows. The assay was done in two tubes: tube A contained a complete reaction mixture including 80 μM [2,3-³H]isovaleryl-CoA; 1 mM phenazine methosulfate; 0.1 mM FAD; and 30 mM potassium phosphate, pH 7.5. The final volume was 0.1 ml. Tube B contained 300 μM MCPA-CoA in addition to the complete reaction mixture. The reaction was started by the addition of cell homogenate. The activity detected in tube B, as a measure of nonspecific ³H₂O released, was subtracted from that detected in tube A. These conditions are essentially the same as those previously described for the determination of glutaryl-CoA dehydrogenase activity (9).

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RESULTS

Using a Lineweaver-Burke plot, the normal human fibroblast isovaleryl-CoA dehydrogenase had an apparent K_m for isovaleryl-CoA of 22 μM , with an apparent $V_{\max}$ of 51 pmol $^3\text{H}_2\text{O}/\text{min}/\text{mg}$ protein (Fig. 1). This K_m value is similar to that (33 μM) of the purified rat liver isovaleryl-CoA dehydrogenase (2). The K_i for MCPA-CoA could not be accurately measured by plotting reciprocal velocity *versus* inhibitor concentration (Dixon plot) as shown in Figure 2. This is because MCPA-CoA is a suicide inhibitor, a substrate analog which irreversibly inhibits the enzyme after it is modified by the enzyme action (10). The data in Figure 2 indicate, however, the K_i of MCPA-CoA was approximately 2 μM .

A total of nine isovaleric acidemia cell lines were tested, a subset of the 12 isovaleric acidemia lines (three other lines were extinct by the time of this study and not available) previously found not to exhibit evidence of genetic complementation after heterokaryon fusion (11). Of these lines, three were from non-related patients judged to have mild clinical manifestations. The remaining six lines were from patients with acute, severe clinical manifestations. Lines 765 and 766 were siblings, but the others were from different families. Information on these patients and references to published clinical data have previously been presented (11). Seven cell lines were used as controls, including three normals, one patient with ethylmalonic/adipic aciduria, one patient with multiple carboxylase deficiency, one patient with medium chain acyl-CoA dehydrogenase deficiency, and one patient with glutaric aciduria type II.

The activity in the controls ranged from 8.0 to 39 pmol $^3\text{H}_2\text{O}/$

Fig. 1. Lineweaver-Burke plot of normal human fibroblast isovaleryl-CoA dehydrogenase activity.

Fig. 2. Dixon plot of the reciprocals of human fibroblast isovaleryl-CoA dehydrogenase activity *versus* MCPA-CoA concentrations.

min/mg protein with a mean value of 19.4 ± 8.0 . All three cell lines from mildly affected isovaleric acidemia children had undetectable activity, as did one line from a severely affected patient. The remaining five lines showed activities varying from 0.42 ± 0.56 to 0.67 ± 0.08 pmol $^3\text{H}_2\text{O}/\text{min}/\text{mg}$ protein (Table 1). These activities correspond to 2.2 ± 2.9 to $3.5 \pm 0.4\%$ of control activity. An equal variance *t* test demonstrated a significant ($p < 0.05$) difference between the residual activities of the two clinical subtypes, with the mild form having the lower residual activity.

DISCUSSION

The long delay between the initial identification of isovaleric acidemia (4) and the unequivocal demonstration of a deficiency of isovaleryl-CoA dehydrogenase in patients with this disorder (1) was due to the difficulty in measuring isovaleryl-CoA dehydrogenase activity in crude tissue preparations. Assay of acyl-CoA dehydrogenase activity had previously been done by using changes in absorbance of electron-accepting dyes, such as the combination of phenazine methosulfate and dichloroindophenol. However, the method did not give adequate sensitivity or specificity in tissue homogenates, since compounds which nonspecifically reduced the dye overwhelmed the contribution of the acyl-CoA dehydrogenases.

The tritium release assay was developed in order to measure isovaleryl-CoA dehydrogenase activity in crude tissue preparations (1, 6). The kinetics of this method have been studied in detail using rat liver mitochondria and partially purified isovaleryl-CoA dehydrogenase as enzyme sources (6). The activity was linear with time and amount of enzyme, and it was saturable. This assay eliminated many of the problems associated with the dye-reduction assay, but tritium release due to microsomal ω -1 oxidation processes (12) and the formation of tritiated acyl-carnitines, which are not adsorbed by the ion-exchange column (13), caused significant interference in measuring low activity in crude cell homogenates. It was therefore necessary to isolate mitochondria from cultured fibroblasts for the study of isovaleric acidemia (1). Our use of an MCPA-CoA inhibited blank removes from consideration the effects of microsomal oxidation, acyl-carnitine formation, and nonenzymatic tritium exchange, making it possible to determine accurately isovaleryl-CoA dehydrogenase activity using whole cell homogenate.

There is less than 3.5% residual activity present in all isovaleric acidemia cell lines tested. However, a paradoxical correlation of residual activity and clinical severity was evident, which we are unable to explain. The low residual activity of isovaleryl-CoA dehydrogenating activity implies that significant amounts of isovaleryl-CoA are not metabolized by the related flavoprotein acyl-CoA dehydrogenases. Supporting this view is the observation that at 100 μM substrate concentration, neither 2-methyl branched-chain acyl-CoA dehydrogenase nor any of the three

Table 1. Residual activities of isovaleryl-CoA dehydrogenase in isovaleric acidemia cells

Cell line	Tritium release from [^3H]isovaleryl-CoA		Clinical course
	pmol $^3\text{H}_2\text{O}/\text{min}/\text{mg}$ protein	% of control	
262	0	0	Mild
502	0	0	Mild
778	0	0	Mild
834	-0.01 ± 0.25	0	Severe
766	0.42 ± 0.56	2.2 ± 2.9	Severe
763	0.44 ± 0.50	2.3 ± 2.6	Severe
501	0.52 ± 0.20	2.3 ± 1.0	Severe
765	0.58 ± 0.33	3.0 ± 1.7	Severe
747	0.68 ± 0.08	3.5 ± 0.4	Severe
Controls (7)*	19.4 ± 8.0	100	

* No. of control cell lines used.

straight chain acyl-CoA dehydrogenases, purified from rat liver, could oxidize isovaleryl-CoA (14–16).

In patients with isovaleric acidemia, detoxification of isovaleric acid is effectively performed by the enzyme glycine-N-acylase, which converts excess isovaleryl-CoA into the rapidly excreted and less toxic isovaleryl-N-glycine. This conjugation system is capable of handling 500–2000 mg of leucine per day in 3- to 5-yr-old children and is important in preventing ketoacidosis (5). Thus, it appears that this alternate route for disposal of isovaleryl-CoA is a more important factor in determining clinical severity than small differences in residual activities of the mutant isovaleryl-CoA dehydrogenases studied so far.

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