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Heterogeneity of Defects in Mitochondrial Acetoacetyl-CoA Thiolase Biosynthesis in Fibroblasts from Four Patients with 3-Ketothiolase Deficiency

HIROYUKI NAGASAWA, SEIJI YAMAGUCHI, TADAO ORII, RUUD B. H. SCHUTGENS, LAWRENCE SWEETMAN, AND TAKASHI HASHIMOTO

Department of Pediatrics, Gifu University School of Medicine, Gifu, Japan [H.N., S.Y., T.O.], Department of Pediatrics, University Hospital Amsterdam, Amsterdam, The Netherlands [R.B.H.S.], Department of Pediatrics, University of California, San Diego, La Jolla, California [L.S.], and Department of Biochemistry, Shinshu University School of Medicine, Matsumoto, Japan [T.H.]

Deficient mitochondrial acetoacetyl-CoA thiolase in fibroblasts from four patients with 3-ketothiolase deficiency was studied using immunochemical methods. We also examined fibroblasts from two heterozygotes, the mother and the brother of the case 1 patient, identified on the basis of the results of the enzyme activity measurements, using 2-methylacetoacetyl-CoA as substrate. The results were as follows: *1*) in fibroblasts from all four patients, the thiolase activity using acetoacetyl-CoA was not activated by K^+, although that of the controls and the heterozygotes was activated about 2-fold. *2*) by immunoblot analyses, mitochondrial acetoacetyl-CoA thiolase was not detectable in fibroblasts from cases 2 and 3, although a very faint band was seen in tissues from cases 1 and 4. However, the band of mitochondrial 3-ketoacyl-CoA thiolase was clearly detected in all patients to the same extent as in the controls. *3*) mitochondrial acetoacetyl-CoA thiolase was observed after pulse labeling for 1-h and a 72-h chase period in three cell lines (cases 1, 2, and 4), but was fainter compared to the controls. In another cell line (case 3), a fluorographic band at the same position was detected following a 1-h pulse, but disappeared following a 6-h chase. These results demonstrate heterogeneity in the enzyme defect resulting in a deficiency of mitochondrial acetoacetyl-CoA thiolase in fibroblasts from patients with 3-ketothiolase deficiency. (*Pediatr Res.* 26: 145–149, 1989)

3-Ketothiolase deficiency (McKusick 20375) is a rare inborn error of isoleucine and ketone body catabolism (1–3). Four thiolases in mammalian tissues have been identified (4); mitochondrial acetoacetyl-CoA thiolase (EC 2.3.1.9) and 3-ketoacyl-CoA thiolase (EC 2.3.1.16), peroxisomal 3-ketoacyl-CoA thiolase (EC 2.3.1.16), and cytosolic acetoacetyl-CoA thiolase (EC 2.3.1.9). 3-Ketothiolase deficiency has been attributed to a deficiency of mitochondrial acetoacetyl-CoA thiolase, as deduced from the following observations. Among the four thiolases, only mitochondrial acetoacetyl-CoA thiolase is activated by K^+ (2–5), and activity of acetoacetyl-CoA thiolase in specimens from the patients is not affected by K^+ (2, 3). Yamaguchi *et al.* (6) reported a defect at the level of protein biosynthesis of mitochondrial acetoacetyl-CoA thiolase in fibroblasts from a Japanese patient with 3-ketothiolase deficiency. It remained to be determined whether all patients with the disease have the same defects in enzyme activity and whether the mutant enzyme is present in the tissues. Mitochondrial acetoacetyl-CoA thiolase is encoded by the nuclear genome and synthesized in the cytosol as a larger precursor with a 3 kD NH_2-terminal extension (signal or transit peptide) which mediates its post-translational translocation into mitochondria. A similar pathway for biosynthesis of most mitochondrial matrix enzymes has been documented (7). Thus, deficiency of acetoacetyl-CoA thiolase might be secondary to *1*) a large deletion in the gene causing lack of production of stable mRNA and lack of synthesis of the protein, *2*) synthesis of a labile enzyme rapidly degraded within mitochondria, *3*) synthesis of a stable enzyme which is inactive because the mutation inhibits substrate binding or alters the active site, or *4*) synthesis of a mutant precursor protein which cannot be incorporated into the mitochondrial matrix (8–10).

A wide range of clinical severity has also been noted in this genetic disorder (1, 2, 11–20). To address these questions, we investigated the defects of mitochondrial acetoacetyl-CoA thiolase biosynthesis in fibroblasts from four patients with this disease, using immunochemical methods. We obtained evidence for heterogeneity in the defects.

MATERIALS AND METHODS

Patients and fibroblasts. Fibroblast cell lines from four patients were studied: patients 1 and 2 were a boy and his father, respectively, described by Schutgens *et al.* (14); cases 3 and 4 were the unrelated patients described by Middleton *et al.* (18).

Patient 1 is a boy of nonconsanguineous patents, diagnosed at age 6.5 y by gas chromatographic analysis of urinary organic acids and the subsequent finding of a severe enzyme deficiency in cultured skin fibroblasts. The neonatal and infancy periods were normal and psychomotor development was average. He presented with vomiting, hypotonia, dehydration, and severe metabolic acidosis at 4.5 and 6.5 y of age (14). At present the patient is 14 y old and is doing well.

Patient 2, father of the first patient, is a healthy man. He became aware of having the defect after gas chromatographic analysis of organic acids in his urine and enzymatic determinations in his cultured skin fibroblasts were done because his son (patient 1) had the disease (14).

Received December 5, 1988; accepted April 16, 1989. Correspondence Hiroyuki Nagasawa, M.D., Department of Pediatrics, Gifu University School of Medicine, 40 Tsukasa-machi, Gifu 500, Japan.

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Patient 3 is a girl in whom development is retarded. Episodes of severe ketoacidosis were frequent. The disease was diagnosed at 7 y of age, after urinary organic acid analysis and the enzymatic determinations (18).

Patient 4 is a Laotian boy who was well nourished and well developed. From age 10 to 19 mo, he was admitted to hospital six times because of severe attacks of acidosis. The diagnosis of 3-ketothiolase deficiency was established when he was 19 mo of age (18).

The mother and the younger brother of patient 1 had no clinical abnormalities, but thiolase activity in the fibroblasts (2-methylacetoacetyl-CoA used as substrate) revealed intermediate values expected for heterozygotes (14).

The fibroblasts were grown in Eagle's minimum essential medium containing 10% FCS (vol/vol) (standard medium) and were routinely maintained at 37°C in a 5% CO_2/95% air atmosphere.

Materials. Acetoacetyl-CoA and 3-ketooctanoyl-CoA were prepared as described (4). The immunoblotting system was purchased from Promega Biotec, Madison, WI; Tran [^{35}S]-Label (>1000 Ci/mmol as [^{35}S]methionine) was from ICN Radiochemicals, Irvine, CA; EN^{3}HANCE was from Du Pont/NEN Research Products, Boston, MA; *Staphylococcus aureus* cells were from Sanraku Co., Tokyo, Japan, and anti-[human fibronectin] IgG (goat) was from CAPPEL Laboratories, West Chester, PA.

Preparation of enzymes and antibodies. Mitochondrial acetoacetyl-CoA thiolase and 3-ketoacyl-CoA thiolase were purified from rat liver and an autopsied human liver, as described (4). The antibodies against mitochondrial acetoacetyl-CoA thiolase and 3-ketoacyl-CoA thiolase from rat liver were raised in rabbits and partially purified by fractionation with ammonium sulfate and were dialyzed against 0.15 M NaCl/10 mM potassium phosphate (pH 7.5) (4).

Enzyme assay and immunoblot analysis. Fibroblasts from four patients, the mother and the brother of the patient 1 and from six controls were washed and harvested with 0.05% trypsin/0.01% EDTA. The cells were washed three times with 0.9% NaCl, centrifuged 500 × *g* for 5 min and the cell pellets stored at −80°C until enzyme assay. To measure the enzyme activity, cell pellets were suspended in 50 mM sodium phosphate, pH 7.5/1 mM dithiothreitol/0.5% Triton X-100. The suspension was sonicated and centrifuged at 10,000 × *g* for 10 min. The supernatant was used for the enzyme assay within 1 day and the remaining solution was preserved for protein determination and for immunoblot analysis.

Enzyme assay was performed as described (4). Thiolase activity, using acetoacetyl-CoA and 3-ketooctanoyl-CoA as substrate was determined. The acetoacetyl-CoA thiolase activity was measured in the presence and the absence of K^+ (50 mM KCl) and activity was expressed as nmol of the substrate used per min/mg protein. Protein concentration was determined by the method of Lowry *et al.* (21).

Immunoblot analysis was performed as described (6), according to Towbin *et al.* (22), using the immunoblotting system for color development with alkaline phosphatase after SDS-PAGE, according to Laemmli (23).

Pulse-chase experiment. Fibroblasts were grown in a 21-cm^2 dish, the cells were washed three times with methionine-depleted Eagle's minimum essential medium without FCS, and then incubated for 1 h in the methionine-depleted Eagle's minimum essential medium containing 5% dialyzed FCS (vol/vol).

The medium was replaced with 1 mL of the above medium to which had been added 100 μCi of [^{35}S]methionine. The cells were then pulse-labeled for 1 h. The labeling medium was removed and a chase for 24, 48, and 72 h with 3 mL of the standard medium performed. Additionally, in another set of experiments with fibroblasts from patient 3, 2-, 6-, and 24-h chase periods were used.

The labeled cells were washed three times with PBS and solubilized in 10 mM Tris-Cl (pH 7.4)/2 mM EDTA/0.1% SDS/0.1% Triton X-100/0.1% bovine serum albumin/0.02% NaN_3/10 mM unlabeled methionine, as described (6). The suspension was sonicated and centrifuged, and the supernatant was preserved for immunoprecipitation. To the supernatant was added 50 μL of 10% (wt/vol) *S. aureus* cell suspension, and the preparation shaken for 1 h. After centrifugation, the supernatant was incubated with 4 μL of anti-[human fibronectin]IgG (goat) at 25°C for 1 h and shaken with 50 μl of the *S. aureus* cell suspension for 1 h. The resulting supernatant was incubated with 2 μL of anti-[mitochondrial acetoacetyl-CoA thiolase]IgG (about 50 mg/ml of protein) at 25°C for 30 min. The antigen-antibody complex was recovered by adding 50 μL of the *S. aureus* cell suspension and shaking for 1 h, followed by centrifugation at 10,000 × *g* for 5 min. The pellets were washed three times with 1 mL of the same buffer. The washed immunoprecipitates were suspended in 40 μL of 125 mM Tris-Cl (pH 6.8)/5% SDS/20% glycerol/0.32 M 2-mercaptoethanol/0.005% Bromphenol blue and boiled for 3 min. The sample mixtures were centrifuged at 10,000 × *g* for 5 min and the supernatants were applied to SDS-PAGE. The gels were stained with Coomassie brilliant blue R 250, treated with EN^{3}HANCE, dried and fluorographed according to the supplier's direction.

RESULTS

Enzyme activity. The thiolase activities of the fibroblast extracts are summarized in Table 1. In the absence of K^+, the acetoacetyl-CoA thiolase activity in the four patients, ranging from 3.0 to 4.9, was nearly the same as the controls. In the presence of K^+, however, the acetoacetyl-CoA thiolase activity in all the patients was not increased (*i.e.* the ratio of $+K^+/-K^+$ was 1.0); although in the controls and in the mother and brother of patient 1, activity was approximately doubled (the ratio of $+K^+/-K^+$ ranging from 1.8 to 2.4). However, the thiolase activ-

Table 1. *Thiolase activity of skin fibroblasts**

Substrate	Acetoacetyl-CoA			3-Ketooctanoyl-CoA
	$+K^+$	$-K^+$	$(+K^+/-K^+)$	
Patient 1	3.0	3.0	(1.0)	10.1
Patient 2	3.0	3.1	(1.0)	9.4
Patient 3	3.5	3.5	(1.0)	9.2
Patient 4	4.7	4.9	(1.0)	10.8
Mother of patient 1	4.6	2.6	(1.8)	9.5
Brother of patient 1	6.8	3.5	(1.9)	9.6
Controls (n = 6)				
Mean	8.3	4.1	(2.0)	10.4
(Range)	(7.8–9.6)	(3.7–5.3)	(1.8–2.4)	(8.5–12.9)

* Thiolase activities were expressed as nmol of substrate used per min/mg protein. The acetoacetyl-CoA thiolase activity was determined in the presence of K^+ ($+K^+$) and the absence of K^+ ($-K^+$) and the ratio of these two activities ($+K^+/-K^+$) are listed.

ity with 3-ketooctanoyl-CoA in the patients' fibroblasts was much the same as that in the control fibroblasts. Because 3-ketooctanoyl-CoA thiolase activity is due to both mitochondrial and peroxisomal 3-ketoacyl-CoA thiolases, the above results show that the patients have normal activities of these thiolases, but lack the activity due to mitochondrial acetoacetyl-CoA thiolase. The residual thiolase activity with acetoacetyl-CoA in the patients' fibroblasts may be attributed to mitochondrial 3-ketoacyl-CoA thiolase and cytosolic acetoacetyl-CoA thiolase.

Immunoblot analysis. The results of immunoblot analysis of acetoacetyl-CoA thiolase are summarized in Figures 1 *A* and *B*. Fifty μg of protein of the fibroblast extracts and 10 ng of purified human enzyme were applied to each lane. No immunoreactive mitochondrial acetoacetyl-CoA thiolase protein was present in the patients' fibroblasts (Fig. 1*A*, *lanes 3*, *4*, *12*, and *13*). In contrast, in the controls (Fig. 1*A*, *lanes 2*, *7*, and *11*) and the mother and the brother of case 1 (Fig. 1*A*, *lanes 5* and *6*), immunoreactive protein was clearly detectable. The intensity of the signal of mitochondrial acetoacetyl-CoA thiolase protein in the heterozygotes (the mother and the brother of patient 1) was similar to that of the controls. After 200 μg of protein of the fibroblast extracts of the patients were applied to each lane, a very faint band was detected for patients 1 and 4, (Fig. 1*B*, *lanes 3* and *8*), but not patients 2 and 3 (Fig. 1*B*, *lanes 4* and *7*). Thus, fibroblasts from some patients with 3-ketothiolase deficiency contain cross-reactive protein with anti-[mitochondrial acetoacetyl-CoA thiolase] IgG. As a control, the band for mitochondrial 3-ketoacyl-CoA thiolase protein was identical in fibroblasts from the patients, the mother and the brother of patient 1, and the controls (Fig. 2).

Pulse chase experiment. Radiolabeled products after a 1-h pulse and 24-, 48-, and 72-h of chase were immunoprecipitated and fluorographed. The radiolabeled band for mitochondrial acetoacetyl-CoA thiolase in the control fibroblasts was observed after the 1-h pulse and after 24-, 48-, and 72-h chase (Fig. 3, *lanes 1*, *2*, *3*, and *4*, respectively). In the fibroblasts from the mother and the brother of patient 1, this band was also detected, to the same extent as in the controls (not shown). In patients 1, 2, and 4, the signal for mitochondrial acetoacetyl-CoA thiolase was also observed at the same position of the subunit of the

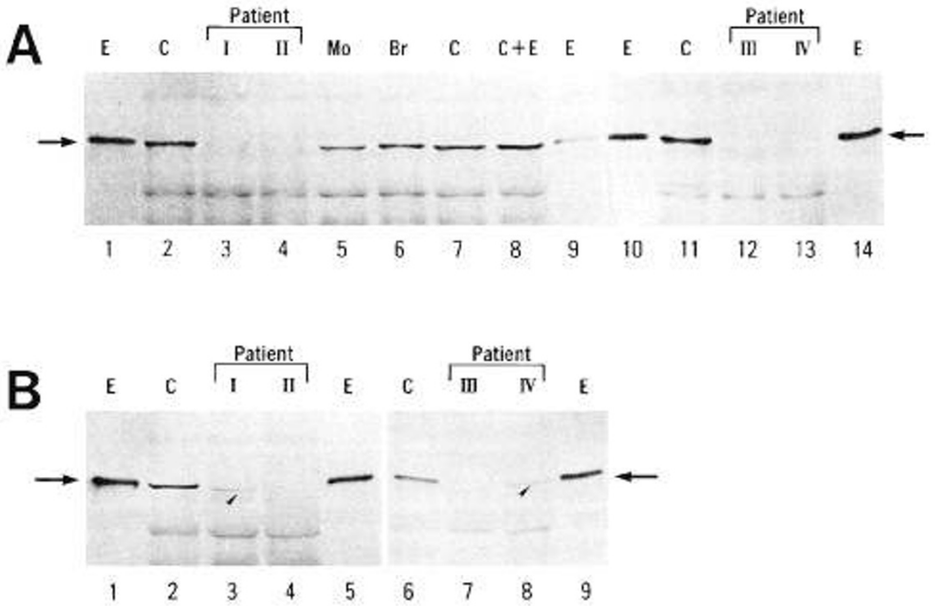

Fig. 1. Immunoblot analysis of mitochondrial acetoacetyl-CoA thiolase protein in cultured fibroblasts. *A*, 50 μg of protein of fibroblasts was applied to each lane. Abbreviations are as follows; *E*, purified enzyme from autopsied human liver; *C*, control fibroblasts; *I*, *II*, *III*, and *IV* are fibroblasts of patients 1, 2, 3 and 4, respectively; *Mo* and *Br* are fibroblasts of the mother and the brother of patient 1. *Lanes 1*, *9*, *10*, and *14*, purified human enzyme (10 ng of protein); *lanes 2*, *7*, and *11*, the control fibroblasts; *lanes 3*, *4*, *12*, and *13*, the fibroblasts of cases 1, 2, 3 and 4, respectively; *lane 8*, control fibroblasts plus human enzyme (10 ng). *Arrows* indicate the signals for human mitochondrial acetoacetyl-CoA thiolase. *B*, 200 μg of protein of the patients' fibroblasts were applied to each lane (control, 50 μg of protein). Abbreviations are the same as those in Figure 1*A*. *Lanes*, *1*, *5*, and *9*, purified human enzyme (10 ng of protein); *lanes 2* and *6*, the control fibroblasts (50 μg of protein applied) each; *lanes 3*, *4*, *7*, and *8*, the fibroblasts of cases 1, 2, 3 and 4, respectively (200 μg of protein applied).

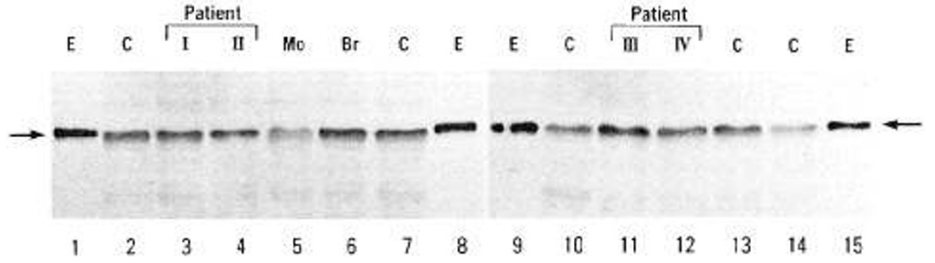

Fig. 2. Immunoblot analysis of mitochondrial 3-ketoacyl-CoA thiolase in cultured fibroblasts. Abbreviations are the same as those in Figure 1. *Lanes 1*, *8*, *9*, and *15*, human enzyme (5 ng of protein applied); *lanes 2*, *7*, *10*, *13*, and *14*, the control fibroblasts (50 μg of protein applied); *lanes 3*, *4*, *11*, and *12*, patients 1, 2, 3, and 4, respectively (50 μg of protein applied); *lanes 5* and *6*, the mother and the brother of patient 1, respectively (50 μg of protein applied.)

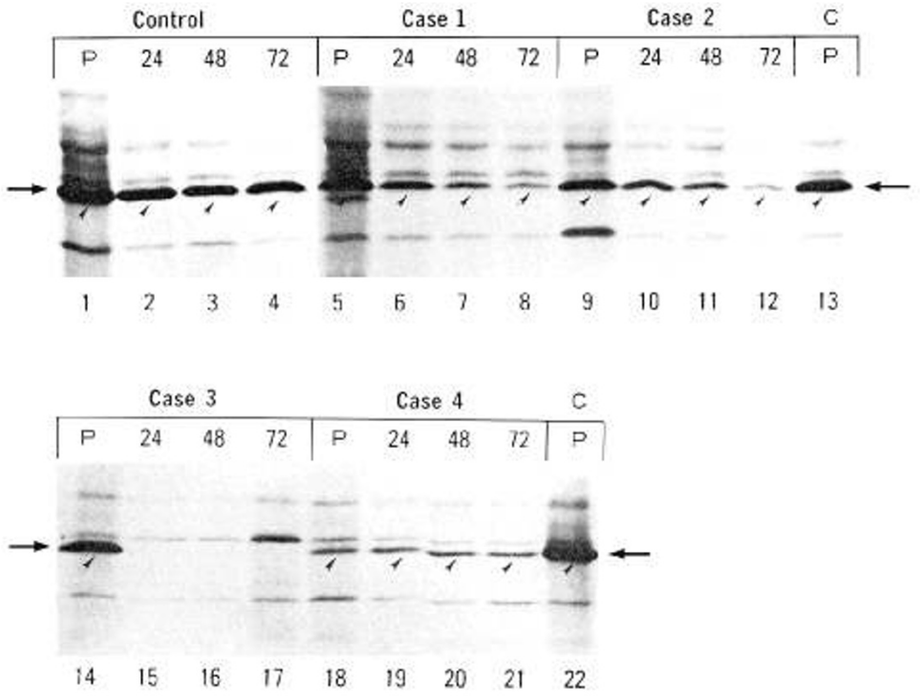

Fig. 3. Pulse-chase experiments of mitochondrial acetoacetyl-CoA thiolase. Abbreviations are as follows; *P*, *24*, *48*, and *72* are 1-h pulse, and 24-h chase, 48-h chase, and 72-h chase after 1-h pulse labeling, respectively. *C* is 1-h pulse-labeled products of the control fibroblasts. *Arrows* indicate the position of the subunit of the mature human enzyme.

mature enzyme from 1-h pulse to at least 72-h chase (Fig. 3, *lanes 5–8*, *9–12*, and *18–21*, respectively), but was fainter as compared to controls. In patient 3, however, the fluorographic band for mitochondrial acetoacetyl-CoA thiolase was detected after the 1-h pulse (Fig. 3, *lane 14*), but not at 24-, 48-, and 72-h chase (Fig. 3, *lanes 15*, *16*, and *17*, respectively). In addition, another experiment with the fibroblasts from patient 3 was performed with a 1-h pulse labeling, and 2-, 6-, and 24-h chase. The fluorographic band was observed at the position of the subunit of the mature enzyme at 1-h pulse and 2-h chase, but disappeared at 6-h chase, as shown in Figure 4. However, the fluorographic bands for mitochondrial 3-ketoacyl-CoA thiolase were identical in fibroblasts from all patients, the two heterozygotes and the controls (not shown).

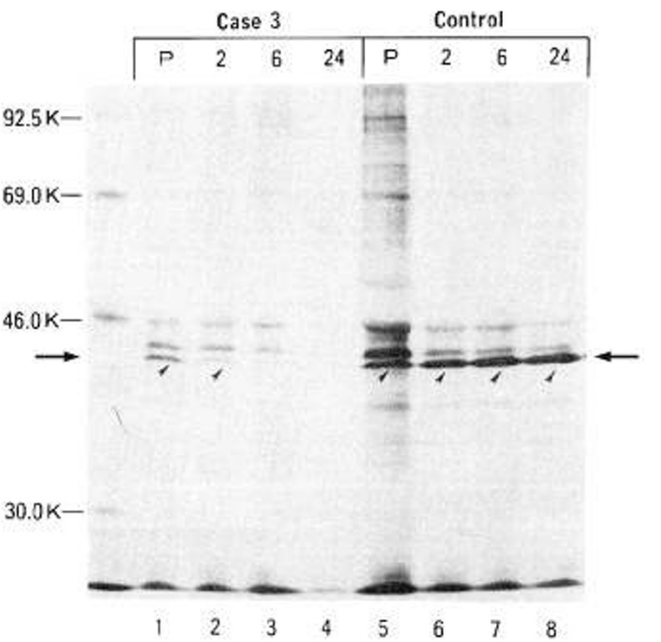

Fig. 4. Pulse-chase experiments of mitochondrial acetoacetyl-CoA thiolase in fibroblasts of case 3 with the shorter duration of chase. Abbreviations are the same as those in Figure 3; *P*, *2*, *6*, and *24* are 1-h pulse, and 2-h cases, 6-h chase, and 24-h chase after 1-h pulse labeling, respectively. *Arrows* indicate the position of the subunit of the mature human enzyme. [^{14}C]Methylated proteins used as molecular mass standards are phosphorylase-b (92.5 kD), BSA (69.0 kD), ovalbumin (46.0 kD), and carbonic anhydrase (30.0 kD), respectively.

DISCUSSION

Inasmuch as the first report by Daum *et al.* (1, 11), 10 or more cases of 3-ketothiolase deficiency have been documented (1, 2, 11–20). This disease has been regarded as being autosomal recessive. The patients have a wide variation of clinical features and most presented with recurrent episodes of severe ketoacidosis and characteristic organic aciduria including 2-methylacetoacetic and 2-methyl-3-hydroxybutyric acids and tiglylglycine.

Clinical heterogeneity was recognized in the four patients we studied. Although patients 1 and 2 are a boy and his father, respectively, classical symptoms such as episodic severe ketoacidosis were seen in the son, but the father had no symptoms. Patient 3 presented with repeated severe ketoacidosis, mental retardation, and characteristic organic aciduria. Patient 4 had milder symptoms and is a well-developed boy presenting with episodic ketoacidosis after infections and a minute amount of the characteristic organic acids in the urine. The fibroblasts from all the patients, however, were regarded as lacking the activity of mitochondrial acetoacetyl-CoA thiolase because K^+-dependency of the acetoacetyl-CoA thiolase activities was nil. However, there was no correlation between the clinical severities and the patterns of the enzyme protein defect, as demonstrated by immunoblot analyses and pulse-chase experiments. In the immunoblot analysis, a very faint band of mitochondrial acetoacetyl-CoA thiolase was detected in fibroblasts from patients 1 and 4, but not in those of the patient 2 with no symptoms, or in the severely

symptomatic patient 3. However, the results of pulse-chase experiments in patients 1, 2, and 4 were similar. The pulse-chase experiments revealed that the radiolabeled mitochondrial acetoacetyl-CoA thiolase migrates at the same position as the mature protein in the fibroblasts of all four patients after a 1-h pulse. The newly synthesized protein in the third patient was labile and disappeared during a 6-h chase. By contrast, in the others (patients 1, 2, and 4) the radiolabeled protein was stable and remained during the 72-h chase. One interpretation of these results is that in patients 1, 2, and 4, the mutant protein is normally translocated into mitochondria, and is thus stable; but is inactive because of inhibition of substrate binding or alteration within the active site. However, in patient 3, the mutation may alter the precursor peptide and block translocation into the mitochondria, resulting in rapid degradation in the cytosol. Alternatively, the case 3 mutant protein may be normally translocated, but have an altered conformation such that it is rapidly degraded by mitochondrial matrix proteases.

To determine the intracellular localization of precursors of these mutant enzymes, we attempted to do [^{35}S]methionine-labeling experiments in the presence of rhodamine 6G, a compound that inhibits mitochondrial transport of precursor proteins. To date, precursors of these mutant enzymes have not been detected.

Yamaguchi *et al.* (6) reported that no band was detected at the position of the mature form of the mitochondrial acetoacetyl-CoA thiolase in pulse-chase experiments of fibroblasts from a Japanese boy with this disease. Taken together, these results suggest that these four cell lines are capable of synthesizing a small amount of mutant enzyme protein which is mature-sized and that there is a heterogeneity of the defects in the enzyme biosynthesis in 3-ketothiolase deficiency. Further investigations on the precursor protein, localization of these proteins, or a genetic analysis should elucidate mechanisms of the enzyme defect.

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