Activities of branched-chain 2-oxo acid dehydrogenase and its components in skin fibroblasts from normal and classical-maple-syrup-urine-disease subjects

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1. Comparisons of the activity and kinetics of the branched-chain 2-oxo acid dehydrogenase in cultured skin fibroblasts from normal and classical maple-syrup-urine disease (MSUD) subjects provide a kinetic explanation for the enzyme defect. 2. In the intact cell assays, normal fibroblasts demonstrated hyperbolic kinetics with 3-methyl-2-oxo[1-¹⁴C]butyrate as a substrate. Intact fibroblasts from four classical MSUD patients showed no decarboxylation over a substrate concentration range of 0.25 to 5.0 mM, and thiamin (4 mM) was without effect. 3. The overall reaction of the multienzyme complex was efficiently reconstituted by using a disrupted-cell system. Normals again showed typical hyperbolic kinetics at the 2-oxo acid concentrations of 0.1 to 5 mM. The $V_{\max}$ and apparent K_m values were 0.10 ± 0.02 m-unit/mg of protein and 0.05–0.1 mM respectively, with 3-methyl-2-oxobutyrate. In contrast, classical MSUD patients exhibited sigmoidal kinetics (Hill coefficient, 2.5) with activity approaching 40–60% of the normal value at 5 mM substrate. The $K_{0.5}$ values from the Hill plots for MSUD patients were 4–7 mM. 4. The E_1 (branched-chain 2-oxo acid decarboxylase) component of the multienzyme complex was measured in disrupted-particulate preparations. Normals again showed hyperbolic kinetics with the 2-oxo acid, whereas MSUD preparations exhibited sigmoidal kinetics with the activity of E_1 strictly dependent on substrate concentration. Apparent K_m or $K_{0.5}$ were 0.1 and 1.0 mM for normal and MSUD subjects respectively. 5. Measurements of E_2 (dihydrolipoyl transacylase) and E_3 (dihydrolipoyl dehydrogenase) in MSUD preparations showed them to be in the normal range. 6. The above data suggest a defect in the E_1 step of branched-chain 2-oxo acid dehydrogenase in classical MSUD patients.

MSUD is an autosomal recessive inborn error of metabolism in which the primary defect is that of oxidative decarboxylation of the branched-chain 2-oxo acids derived from the three branched-chain amino acids leucine, isoleucine and valine. MSUD has been classified into classical, variant and intermittent types, based on the rapidity of onset and severity of the disease as well as tolerance for dietary protein (Dancis *et al.*, 1967, 1972).

Dancis and his co-workers demonstrated that both the classical and variant forms of MSUD have a deficiency in the branched-chain 2-oxo acid

Abbreviations used: MSUD, maple-syrup-urine disease; TPP, thiamin pyrophosphate; E_1 , branched-chain 2-oxo acid decarboxylase; E_2 , dihydrolipoyl transacylase; E_3 , dihydrolipoyl dehydrogenase; $K_{0.5}$, concentration of substrate for half-maximal saturation.

dehydrogenase (EC 1.2.4.4) activity that is expressed both in peripheral leucocyte preparations (Dancis *et al.*, 1963) and in skin fibroblast cultures (Dancis *et al.*, 1969, 1972). By using fibroblast cultures and radioactive branched-chain amino acids labelled in the carboxy carbon atom, they showed a clear correlation between the degree of deficiency in decarboxylation of the branched-chain amino acid in the cell cultures from different patients with MSUD, and the clinical history and dietary protein tolerance (Dancis *et al.*, 1972). Other laboratories preferred to use radioactive branched-chain 2-oxo acid as substrate, thereby avoiding the interposed step of transamination (Seegmiller & Westall, 1967; Wendel *et al.*, 1973, 1975; Danner & Elsas, 1975). At low branched-chain 2-oxo acid concentration (0.5 mM), good correlation was found between the clinical

phenotype and decarboxylation of branched-chain 2-oxo acid (Wendel *et al.*, 1975; Dancis *et al.*, 1977), but at higher substrate concentrations (1.5–15.0 mM) most fibroblast cultures derived from MSUD patients showed considerable decarboxylation with overlap between normal and MSUD subjects.

A major controversy existed concerning the number of species of branched-chain 2-oxo acid dehydrogenase. Recently, Pettit *et al.* (1978) and Danner *et al.* (1979) have purified branched-chain 2-oxo acid dehydrogenase to homogeneity from bovine tissues, and demonstrated it to be a single multienzyme complex consisting of three components. The first component, E_1 , is a decarboxylase, the second, E_2 , is a dihydrolipoyl transacylase, and the third, E_3 , dihydrolipoyl dehydrogenase. In the present paper, the activity of the branched-chain 2-oxo acid dehydrogenase overall reaction is measured in intact and in disrupted skin fibroblasts derived from normal subjects and patients with the classical form of MSUD. These investigations clearly describe a kinetic basis for the enzyme deficiency in MSUD and clarify several ambiguities in the literature. Secondly, assays were developed to measure separately each of the three steps of the branched-chain 2-oxo acid dehydrogenase overall reaction. These assays are sensitive enough to be used with cultured fibroblasts. By using a particulate preparation derived from normal cells and from fibroblasts of two patients with classical MSUD, the enzymic lesion in these patients was demonstrated to involve the E_1 component.

Experimental

Cell culture

Normal fibroblasts were grown from skin biopsies in monolayer culture in Waymouth's medium containing 5% foetal- and 5% newborn-calf serum and antibiotics (penicillin, 50 units; streptomycin, 50 μ g/ml; kanamycin, 30 μ g/ml). For all but one (CoBa) classical MSUD patient, the fibroblasts had been maintained in liquid N_2 for several years. Both control and the patient's cells were grown to confluency and harvested approx. 1 week after the last medium change. The average number of passages of cells taken for assay was 12–16.

Source of fibroblasts

Patients DaMa and CoBa were referred by Dr. David L. Valle, Johns Hopkins University School of Medicine, Baltimore, MD, U.S.A. MSUD mutant GM-612 was received from the Genetic Mutant Cell Repository, Camden, NJ, U.S.A. Skin biopsy on patient MeCa was kindly provided by Dr. Selma Snyderman, Department of Pediatrics, New York University Medical Center, New York, NY, U.S.A.

All patients except CoBa have been previously reported (Dancis *et al.*, 1977) and defined as having classical MSUD by clinical history and enzyme assay using branched-chain amino acid as substrate. CoBa was diagnosed as a classical patient by measuring plasma branched-chain amino acid concentrations. Fibroblasts from the normal subjects were selected to provide an age range comparable with the patients.

Radioactive substrate

3-Methyl-2-oxo[1- 14 C]butyrate was prepared from [1- 14 C]L-valine (sp. radioactivity 52.9 Ci/mol; New England Nuclear, Boston, MA, U.S.A.) by oxidation with L-amino oxidase (Meister, 1953). The radioactive purity of the 2-oxo (1- 14 C)-labelled acid was found to be >99% by ascending t.l.c. (Benson *et al.*, 1950). The labelled 2-oxo acid was maintained at pH 4 and stored at -20°C .

Protein determination

Fibroblasts were precipitated with 7.5% trichloroacetic acid and dissolved in 0.6 M-NaOH containing 2% sodium desoxycholate, and protein was determined by the Lowry method (Lowry *et al.*, 1951), with bovine serum albumin as standard.

Intact cell assay

Fibroblasts were harvested with a medium containing 0.02% EDTA and 0.04% trypsin, in Puck's saline A without glucose (Marcus *et al.*, 1956). The harvested cells were washed by centrifugation (650 g) once with a Krebs–Ringer phosphate buffer (Krebs, 1933) containing 20% foetal-calf serum and once with the buffer only. The washed cells (1×10^6 , equivalent to 0.3 mg of protein, in 0.05 ml of Krebs buffer) were suspended in 0.35 ml of medium containing 0.25 ml of the Krebs–Ringer phosphate buffer, pH 7.4, 0.005 ml of thiamin hydrochloride (100 mg/ml) and 3-methyl-2-oxo[1- 14 C]butyrate (sp. radioactivity 172 c.p.m./nmol). Incubation medium containing equivalent numbers of boiled normal cells were used as a blank. The blank value was within 10–20% of the total sample counts that ranged from 600 to 1200 c.p.m. with normal fibroblasts depending on substrate concentrations. The assay was carried out in a Kontes pear-shaped flask (10 ml volume) capped with a serum stopper. Incubations were for up to 80 min at 35°C with $^{14}\text{CO}_2$ collected in a centre well containing 0.1 ml of 2 M-NaOH. After stopping the reaction with 0.05 ml of 15% trichloroacetic acid, the incubation was allowed to continue for 1 h to recover residual $^{14}\text{CO}_2$ from the incubation medium. At the end of the incubation, the NaOH solution was transferred to 10 ml of scintillation cocktail to determine radioactivity. The scintillation cocktail contained in 1 litre: 640 ml of methanol, 320 ml of toluene, 20 ml of phenethyl-

amine, 20 ml of glycerol, 6 g of methylbenzethonium chloride, 6 g of 2,5-diphenyloxazole and 120 mg of 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene. Activity was expressed as nmol of $^{14}\text{CO}_2$ evolved/min per mg of cell protein (equivalent to m-units/mg of cell protein).

Assay for branched-chain 2-oxo acid dehydrogenase

The overall reaction catalysed by the multi-enzyme complex was assayed by using disrupted fibroblast suspensions. The assay was essentially that described by Danner & Elsas (1975), except for the way the cells were disrupted and the inclusion of calf serum in the assay to protect the enzyme from proteolysis. The fibroblasts were harvested and washed as previously described and suspended in the Krebs-Ringer buffer ($1 \times 10^6/0.05$ ml). The cell suspension was rapidly frozen in a Revco refrigerator (-75°C) and kept for 15 h or longer and then thawed for assays. Fibroblasts thus treated were fully disrupted as observed under a phase-contrast microscope. The activity of the frozen cells was stable for at least 2 months at -75°C . The assay mixture contained 50 mM-Tris/HCl, pH 7.5, 0.2 mM-EDTA, 0.35 mM-MgCl₂, 0.2 mM-CoA, 0.2 mM-NAD⁺, 0.2 mM-TPP, 1.4% foetal-calf serum, the disrupted cell suspension (1×10^6 cells equivalent to 0.3 mg protein) in 0.05 ml of Krebs buffer and 3-methyl-2-oxo[1- ^{14}C]butyrate (sp. radioactivity 172 c.p.m./nmol) in a final volume of 0.37 ml. The blank value with Krebs buffer was within 10–20% of the total sample counts, which ranged from 300 to 800 c.p.m. with normal fibroblasts, depending on substrate concentrations. The incubation and remaining procedure were the same as in the intact cell assay.

Preparation of particulate fraction

Particulate fraction of skin fibroblasts was prepared by a modification (Mackall & Lane, 1977) of the digitonin-treatment method (Zuurendonk & Tager, 1974). Cultured fibroblasts were harvested as described above, resuspended in an iso-osmotic buffer (0.22 M-mannitol/0.07 M-sucrose/0.005 M-4-morpholinepropanesulphonic acid, pH 7.5) containing 1 mg of digitonin/ml (B. L. Lemke and Co., Lodi, NJ, U.S.A.) at 0°C for exactly 60 s. The cells were rapidly pelleted with an Eppendorf micro-centrifuge 5412 at 12800 g for 60 s. The pellet was washed three times with the above iso-osmotic buffer without digitonin, resuspended in the same medium (2 mg of protein/ml) and stored at -75°C .

Assay for the E_1 component

The radiochemical assay was modified from a previous spectrophotometric procedure with $\text{K}_3\text{Fe}(\text{CN})_6$ as an electron acceptor (Gubler, 1961; Sullivan *et al.*, 1976). The reaction mixture contained: 20 mM-phosphate buffer, pH 7.4; 0.54 mM-

EDTA; 1 mM-MgCl₂; 3.4 mM-K₃Fe(CN)₆; 0.2 mM-TPP; 1.4% foetal-calf serum; 3-methyl-2-oxo[1- ^{14}C]butyrate (sp. radioactivity 423 c.p.m./nmol) and frozen and thawed particulate suspension (0.05 ml) in a final volume of 0.37 ml. The particulate fraction from 1×10^6 cells contained approximately 0.1 mg of protein. The remaining procedure was the same as with intact cells. Activity was expressed as nmol of $^{14}\text{CO}_2$ evolved/min per mg of protein (equivalent to m-units/mg of protein).

Assay for the E_2 component

Activity of the E_2 component was measured by using a model reaction similar to the one for the transacetylase of pyruvate dehydrogenase complex (Butterworth *et al.*, 1975). In this model reaction exogenous dihydrolipoamide substituted for the protein-bound dihydrolipoyl moiety and 1- ^{14}C -labelled acyl-CoA was used as a substrate. The assay mixture contained 50 mM-potassium phosphate, pH 7.4, 2 mM-dihydrolipoamide, 0.4 mM-2-methyl[1- ^{14}C]propionyl-CoA (sp. radioactivity 400 c.p.m./nmol) and frozen-and-thawed fibroblasts (in 0.05 ml of Krebs buffer), in a final volume of 0.37 ml. The mixture was incubated at 35°C for up to 20 min. To stop the reaction, 0.75 ml of benzene was added and the mixture was vortex-mixed for 10 s to extract the labelled *S*-(2-methylpropionyl)dihydrolipoamide. A 0.2 ml portion of the benzene layer was withdrawn and the radioactivity was measured in a Beckman liquid-scintillation spectrometer. The values were approximately 250 c.p.m. after a 20 min incubation. Dihydrolipoamide was omitted from the control tubes (blank value approx. 60 c.p.m.). Activity was expressed as nmol of *S*-(2-methylpropionyl)dihydrolipoamide formed per min/mg of protein (equivalent to m-units/mg of protein).

Assay for the E_3 component

The E_3 component was assayed in the direction of lipoamide reduction as described previously (Stumpf & Parks, 1978). The assay mixture contained 50 mM-potassium phosphate, pH 6.5, 1.5 mM-EDTA, 1 mM-DL-lipoamide, 0.2 mM-NADH, 0.15% Triton X-100 and cell suspension [$(1-5) \times 10^5$ cells]. The reaction was followed spectrophotometrically at 37°C and 340 nm. Blanks without lipoamide were used. Activity was expressed as nmol of NADH oxidized per min/mg of protein (m-units/mg of protein).

Assays for pyruvate and 2-oxoglutarate dehydrogenase complexes

Activity of dichloroacetate-stimulated pyruvate dehydrogenase complex was assayed by the method of Sheu *et al.* (1981). The assay for 2-oxoglutarate dehydrogenase complex was the same as that for

branched-chain 2-oxo acid dehydrogenase except that 0.5 mM-2-oxo[1- 14 C]glutarate (sp. radioactivity 1100 c.p.m./nmol) was used as a substrate and 2.7 mM- CaCl_2 and 2.7 mM- MgCl_2 were also included in the assay mixture. Incubation for the latter assay was at 35°C for 10 min.

Miscellaneous

Dihydrolipoamide was prepared from DL-lipoamide by the procedure of Reed *et al.* (1958). 2-Methyl[1- 14 C]propionyl-CoA was synthesized enzymically by using medium-chain fatty acid:CoA ligase (AMP-forming) (EC 6.2.1.2) from bovine liver (D. T. Chuang, W.-L. Niu & R. P. Cox, unpublished work). Lactate dehydrogenase was assayed as described by Kornberg (1955). All assays were linear with time and the amount of protein added. All kinetic data were at least duplicates and all experiments were repeated at least twice to ensure reproducibility.

Results

Intact cell assay

Fig. 1 shows the rate of decarboxylation of 3-methyl-2-oxo[1- 14 C]butyrate by intact fibroblasts derived from two normal subjects (V-58 and Pa) and from four patients with the classical forms of MSUD (DaMa, CoBa, GM-612 and MeCa). The normal cell strains demonstrated typical hyperbolic kinetics for the 2-oxo acid over a substrate con-

Fig. 1. Rate of decarboxylation of 3-methyl-2-oxo[1- 14 C]butyrate by normal and classical MSUD intact fibroblasts at various substrate concentrations

The intact cell assays were carried out in the presence of 4 mM-thiamin as described in the Experimental section with the 2-oxo (1- 14 C)-labelled acid (sp. radioactivity 172 c.p.m./nmol) as the variable substrate. The incubation was at 35°C for 80 min. The protein added to each assay was approx. 0.33 mg or equivalent to 1×10^6 cells. The viability of intact cells used for the assay was 95% or greater by the Trypan Blue stain. V-58 and Pa are fibroblast strains from normals and DaMa, CoBa, GM-612 and MeCa are those from classical MSUD patients.

centration range of 0.25 to 5.0 mM. The $K_{0.5}$ value and $V_{\max}$ were 0.3 mM and 0.25–0.30 m-unit/mg of protein for the normal fibroblasts when assayed in the presence of 4 mM-thiamin/HCl. Under the same conditions, all four classical MSUD patients showed no decarboxylation.

Disrupted-cell assay

To minimize variables, for example membrane effects and pre-existing substrate pools, as well as to allow a more direct measurement of enzyme activity, a disrupted cell assay was developed. This approach permitted an evaluation of kinetic parameters of the overall branched-chain 2-oxo acid dehydrogenase reaction in normal and in MSUD subjects. By using the reconstituted system and conditions of assay described in the Experimental section, a rate of decarboxylation of approx. 40–60% of that measured in intact cells was achieved (Fig. 2). In both intact and disrupted cells, decarboxylation rates were linear for 80 min at 35°C. The bottom line in Fig. 2 shows that disrupted-cell suspensions incubated with 2-oxo (1- 14 C)-labelled acid alone, without the other components of the reconstituted

Fig. 2. Comparison of decarboxylation rates for 3-methyl-2-oxo[1- 14 C]butyrate in intact and disrupted normal fibroblasts

All assays were carried out at 2 mM-2-oxo (1- 14 C)-labelled acid and 35°C using the same cell suspension. Branched-chain 2-oxo acid dehydrogenase activity in intact cells (●) was measured as described in Fig. 1. Branched-chain 2-oxo acid dehydrogenase activity in disrupted cells (▲) was reconstituted as described in the Experimental section. The ○ represents activity in disrupted cells incubated with the 2-oxo (1- 14 C)-labelled acid and 4 mM-thiamin/HCl in Krebs–Ringer phosphate buffer but without added cofactors and metal ions.

system, have no measurable branched-chain 2-oxo acid dehydrogenase activity. These findings suggest that mitochondria, where the enzyme resides, are leaky or ruptured during the preparation of disrupted cells by freezing and thawing.

Kinetics of the overall branched-chain 2-oxo acid dehydrogenase reaction

Disrupted normal fibroblasts (Tu and Pa) showed hyperbolic Michaelis–Menten kinetics over the substrate range 0.1–5.0 mM similar to those observed with the intact cell assay. The $V_{\max}$ and apparent K_m values were 0.10 ± 0.02 m-unit/mg of cell protein and 0.05–0.1 mM respectively with 3-methyl-2-oxo-[1- 14 C]butyrate as substrate (Fig. 3). In contrast, disrupted fibroblast preparations from mutant cells (DaMa and GM-612; Fig. 3) revealed sigmoidal kinetics with essentially no activity in the 0 to 1 mM range, but approaching 40–60% of the normal value at 5 mM concentration of 2-oxo acid. Hill plots (Fig. 3 insert) gave $K_{0.5}$ values of 4 and 7 mM for MSUD patients DaMa and GM-612 respectively. Hill coefficients (h) were 2.5 for both patients according to the same plots, suggesting strong

positive co-operativity. Similar results with hyperbolic and sigmoidal kinetics for normal and MSUD patients respectively were obtained when disrupted particulate preparations were substituted for the broken cells (results not shown). Decarboxylation by normal and mutant (GM-612) disrupted fibroblasts were both stimulated by TPP. However, TPP at 0.2 mM did not change the sigmoidal kinetics of the classical MSUD (results not shown). Moreover, activities of other TPP-dependent multienzymes were measured as controls. Disrupted cells of MSUD subjects showed activities of 2.32 ± 0.02 and 2.91 ± 0.12 m-units/mg of protein for 2-oxoglutarate and dichloroacetate-activated pyruvate dehydrogenase complexes respectively. These values were similar to those measured in normal fibroblasts.

E_1 activity in particulate fractions isolated from normal and MSUD fibroblasts

To determine a possible defect in the E_1 component, a modified method using digitonin treatment was employed to prepare a particulate fraction containing mitochondria from normal and MSUD skin fibroblasts. Table 1 shows that the particulate

Fig. 3. Activity of branched-chain 2-oxo acid dehydrogenase in disrupted fibroblasts from normals and classical MSUD. Rate of the overall reaction catalysed by the multienzyme complex was measured in the presence of 0.2 mM-TPP as described in the Experimental section with 3-methyl-2-oxo[1- 14 C]butyrate (sp. radioactivity 172 c.p.m./nmol) as the variable substrate. The incubation was at 35°C for 80 min. The disrupted-cell suspension added per assay was approx. 0.33 mg of protein or equivalent to 1×10^6 cells. Tu and Pa are fibroblasts derived from normals. DaMa and GM-612 are those from classical MSUD patients. The insert shows the Hill plots.

Table 1. *Cellular distribution of lactate dehydrogenase, dihydrolipoyl dehydrogenase and branched-chain 2-oxo acid dehydrogenase in cultured skin fibroblasts*

Harvested human skin fibroblasts (15×10^6 cells) were treated with digitonin (1 mg/ml), and the particulate fraction was separated from the cytosol by centrifugation (see the Experimental section for details). Lactate dehydrogenase was assayed as described by Kornberg (1955). Dihydrolipoyl dehydrogenase (E_3) and branched-chain 2-oxo acid dehydrogenase (overall reaction) activities were measured as described in the Experimental section.

Enzyme	Activity (m-units)		Distribution (%)	
	Cytosolic	Particulate	Cytosolic	Particulate
Lactate dehydrogenase	7380	321	95.48	4.52
Dihydrolipoyl dehydrogenase	0	223	0	100
Branched-chain 2-oxo acid dehydrogenase	0	0.11	0	100

Fig. 4. *Kinetics of the E_1 component in particulate preparations from normal and classical MSUD fibroblasts* Radiochemical ferricyanide assays were carried out as described in the Experimental section in the presence of 0.2 mM-TPP. Specific activities of 3-methyl-2-oxo[1- 14 C]butyrate were 400 c.p.m./nmol for the 0.1–0.5 mM range and 91 c.p.m./nmol for 1 mM or higher concentrations. To each assay mixture, 0.1 mg of frozen-and-thawed particulate protein was added. Pa is a normal, and DaMa and GM-612 are classical MSUD subjects. The insert shows the Hill plots.

fraction was free of cytosol using lactate dehydrogenase as a marker and that virtually all the E_3 component and the branched-chain 2-oxo acid dehydrogenase were located in this particulate fraction.

Determination of E_1 activity and the kinetics of the reaction in particulate fractions prepared from normal fibroblasts (Pa) and from two strains of MSUD fibroblasts (DaMa and GM-612) is shown in Fig. 4. Kinetic patterns of the normal and the two MSUD patients are quite similar to those observed with the overall reaction. That is, particulate fractions from a normal (Pa) subject (Fig. 4) show hyperbolic kinetics over the substrate concentration range 0.1–5.0 mM, whereas both mutant cell strains

exhibit sigmoidal kinetics with the decarboxylase activity within the normal range at high substrate concentrations (5 mM). Hill plots (Fig. 4 insert) yielded a $K_{0.5}$ value of approx. 1.0 mM for patients DaMa and GM-612, which was 10-fold higher than the apparent K_m value of 0.1 mM for the normal subject. Hill coefficients of 1.5–1.7 for MSUD subjects were calculated from the plots.

E_2 and E_3 activities

To determine if the enzymic lesion in a classical MSUD patient was confined to the first step, E_1 of the multienzyme complex, the activities of the other two components, E_2 and E_3 were measured. Table 2 (middle column) shows that E_2 activity

Table 2. Activities of the E_2 and E_3 components in skin fibroblasts from normal and classical MSUD patients

The assay mixture for E_2 contained 50 mM-potassium phosphate, pH 7.4, 2 mM-dihydrolipoamide, 0.4 mM-2-methyl[1- 14 C]propionyl-CoA (sp. radioactivity 400 c.p.m./nmol), and frozen-and-thawed fibroblast suspension (0.325 mg), in a final volume of 0.37 ml. The incubation was at 35°C for up to 20 min. To terminate the reaction, 0.75 ml of benzene was added and the mixture was vortex-mixed to extract the labelled S-(2-methylpropionyl)dihydrolipoamide. A 0.2 ml portion of the benzene layer was withdrawn and the radioactivity was counted. Complete assay mixture omitting dihydrolipoamide was used as a blank. To assay for E_3 activity, the reaction mixture contained, in 1 ml, 50 mM-potassium phosphate, pH 6.5, 1.5 mM-EDTA, 0.2 mM-NADH, 0.15% Triton X-100, 1 mM-DL-lipoamide and frozen-and-thawed fibroblast suspension (0.03–0.16 mg of protein). The oxidation of NADH at 37°C was monitored spectrophotometrically at 340 nm. Complete assay mixture minus lipoamide served as a blank. Results are means $\pm$ s.d.

Cell lines	Specific activity (m-units/mg of cell protein)	
	E_2	E_3
Normals	0.17 ± 0.02 ($n = 6$)	23.9 ± 4.0 ($n = 6$)
Patient DaMa	0.15 ± 0.01 ($n = 4$)	23.1 ± 5.9 ($n = 4$)
Patient GM-612	0.16 ± 0.01 ($n = 4$)	26.8 ± 1.8 ($n = 4$)

in disrupted cells derived from normal and MSUD patients DaMa and GM-612 were similar. The E_2 activity was exclusively located in the particulate fraction (results not shown). Table 2 (right column) also shows that in two fibroblast strains derived from patients with classical MSUD (DaMa and GM-612), the activity of the E_3 component was in the normal range. The activity of the E_3 component tends to fluctuate from experiment to experiment as indicated by the magnitude of the standard deviation.

Discussion

A central question in defining the biochemical basis of MSUD has been the number of branched-chain 2-oxo acid dehydrogenases involved in the oxidative decarboxylation of the three branched-chain 2-oxo acids. The concordant decrease of activity for all three substrates in fibroblasts from patients with classical (grade I), variant (grade II) and intermittent (grade III) forms of the disease argues for a single enzyme complex (Dancis *et al.*, 1972; Danner & Elsas, 1975). Recently, Pettit *et al.* (1978) and Danner *et al.* (1979) have purified the branched-chain 2-oxo acid dehydrogenase from bovine kidney and liver respectively to homogeneity and have shown unequivocally that a single mitochondrial multienzyme complex oxidatively

decarboxylates all three 2-oxo acids in bovine tissues. The activity ratios among the three substrates were essentially constant for both enzyme preparations throughout purification and K_m values in the 30–50 μ M range were determined. Further, the three 2-oxo acids inhibit one another competitively with K_i values similar to the respective K_m values (Danner *et al.*, 1978).

On the basis of the single multienzyme complex model, we approached the enzyme defect in MSUD by several complementary methods. The results of certain of our studies differ from previous publications. Measurement of the decarboxylation rates for 3-methyl-2-oxobutyrate in intact normal and mutant fibroblasts showed that normals exhibit monophasic hyperbolic kinetics up to 5 mM concentrations of the 2-oxo acid and is consistent with a single multienzyme complex model. Wendel *et al.* (1975) previously reported biphasic decarboxylation kinetics for the 2-oxo acids with the high-affinity component defective in MSUD. Our results with intact MSUD fibroblasts from four patients showed no activity at substrate concentrations up to 10 mM (data for 10 mM concentration not shown in Fig. 1).

Our second approach to characterize the enzyme deficiency in MSUD was to develop a disrupted-cell system where relatively high activity of branched-chain 2-oxo acid dehydrogenase was reconstituted. Purified enzyme from fibroblasts is not available, because of low activity in cultured mutant cells and difficulties in obtaining sufficient quantities of cells to purify an intact multienzyme complex. Nonetheless, by using the disrupted-cell system, normal cells exhibited hyperbolic kinetics, and its apparent K_m of 0.05–0.10 mM was in the range of highly purified bovine branched-chain 2-oxo acid dehydrogenase (Pettit *et al.*, 1978; Danner *et al.*, 1979). In contrast, the sigmoidal kinetics and $K_{0.5}$ values of 4 and 7 mM for patients DaMa and GM-612 respectively suggest a significant decrease in the enzyme's affinity for substrate.

The striking differences in decarboxylation of 2-oxo acid by MSUD fibroblasts between the intact and broken cell assays are probably expected. Intact mutant cells with high $K_{0.5}$ values for substrate combined with putative membrane effects that limit availability of substrate in mitochondria probably account for the absence of branched-chain 2-oxo acid decarboxylation. The close correlations between the patient's phenotype and tolerance for dietary protein with the degree of enzyme deficiency observed in the intact cell assay (Wendel *et al.*, 1975; Dancis *et al.*, 1977) suggest that this system approximates the situation *in vivo*. On the other hand, the reconstituted disrupted-cell assay provides a more direct measurement of enzyme activities and the opportunity to study the substrate-dependent kinetics of mutant enzymes.

Our third approach was to separate the activity of the overall branched-chain 2-oxo acid dehydrogenase reaction into its individual steps and measure these in fibroblasts from normal subjects and patients with classical MSUD. The availability of these assays may be useful in defining more precisely genetic heterogeneity in MSUD (Lyons *et al.*, 1973). The ferricyanide electron-acceptor method (Gubler, 1961; Sullivan *et al.*, 1976) permitted a functional isolation of the first E_1 step from the overall reaction, and the use of radioactive substrate in the present study provided the sensitivity necessary to assay fibroblasts. The validity of the ferricyanide assay was demonstrated by its insensitivity to arsenite (1.5-mM) inhibition (Sullivan *et al.*, 1976) and the low apparent K_m of 0.026 mM for 3-methyl-2-oxobutyrate with disrupted rat liver mitochondria (results not shown). This K_m value for E_1 reaction is in good agreement with those of the overall reaction using the homogeneous bovine enzyme (Pettit *et al.*, 1978; Danner *et al.*, 1979). The ferricyanide assay of E_1 activity in classical MSUD fibroblasts showed altered kinetics similar to the overall reaction, suggesting that the enzymic abnormality is associated with the E_1 step.

The E_2 assays appeared to be specific in that the reaction was sensitive to arsenite inhibition (Sanadi *et al.*, 1959) and was not catalysed by purified pyruvate dehydrogenase complex (results not shown). Results using this assay with disrupted fibroblasts showed similar E_2 activity, in both control and classical patients. Activities of the E_3 components were also measured and were in the normal range in both classical patients. The E_3 component is believed to be a common moiety shared by pyruvate, 2-oxoglutarate and branched-chain 2-oxo acid dehydrogenase complexes (Sakurai *et al.*, 1970; Koike & Koike, 1976). A defect in this component has been reported to result in deficiencies in all three multienzymes (Robinson & Taylor, 1979).

In summary, the present study provides a kinetic explanation for the enzyme deficiency in MSUD, and suggests that the enzymic lesion most likely involves the E_1 component of branched-chain 2-oxo acid dehydrogenase. The molecular basis for the altered kinetics observed with patients' fibroblasts is unknown. An $R \rightleftharpoons T$ equilibrium (Monod *et al.*, 1965) with a shift towards the T state, resulting from a structural defect in the E_1 component, is compatible with the positive co-operativity with substrate exhibited by the mutant enzyme. However, aberrations in enzyme regulation, for example, a defect in the end-product inhibition (Pettit *et al.*, 1978; Bremer & Davis, 1978; Parker & Randle, 1978), or in the proposed phosphorylation-dephosphorylation cycle (Odessey, 1980a,b; Parker & Randle, 1980; Lau *et al.*, 1981) could also favour a

conformation with decreased substrate affinity. Further investigations are required to differentiate among the above possibilities.

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