Purification and Characterization of 2-Methyl-branched Chain Acyl Coenzyme A Dehydrogenase, an Enzyme Involved in the Isoleucine and Valine Metabolism, from Rat Liver Mitochondria*

Yasuyuki Ikeda and Kay Tanaka

From the Department of Human Genetics, Yale University School of Medicine, New Haven, Connecticut 06510

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2-Methyl-branched chain acyl-CoA dehydrogenase was purified to homogeneity from rat liver mitochondria. The native molecular weight of the enzyme was estimated to be 170,000 by gel filtration. On sodium dodecyl sulfate-polyacrylamide gel electrophoresis both with and without 2-mercaptoethanol, the enzyme showed a single protein band with Mₚ = 41,500, suggesting that this enzyme is composed of four subunits of equal size. Its isoelectric point was 5.50 ± 0.2, and Aᵦ₂₈₀ nm was 12.5. This enzyme contained protein-bound FAD.

The purified enzyme dehydrogenated S-2-methylbutyryl-CoA and isobutyryl-CoA with equal activity. The activities with each of these compounds were co-purified throughout the entire purification procedure. This enzyme also dehydrogenated R-2-methylbutyryl-CoA, but the specific activity was considerably lower (22%) than that for the S-enantiomer. The enzyme did not dehydrogenate other acyl-CoAs, including isovaleryl-CoA, propionyl-CoA, butyryl-CoA, octanoyl-CoA, and palmitoyl-CoA, at any significant rate. Apparent Kᵦ and Vₚₐₜₐₐₐₐ values for S-2-methylbutyryl-CoA were 20 μM and 2.2 μmol min⁻¹ mg⁻¹, respectively, while those for isobutyryl-CoA were 89 μM and 2.0 μmol min⁻¹ mg⁻¹ using phenazine methosulfate as an artificial electron acceptor. The enzyme was also active with electron transfer flavoprotein. Tiglyl-CoA and methylacrylyl-CoA were identified as the reaction products from S-2-methylbutyryl-CoA and isobutyryl-CoA, respectively. 2-Ethylacrylyl-CoA was produced from R-2-methylbutyryl-CoA. Tiglyl-CoA competitively inhibited the activity with both S-2-methylbutyryl-CoA and isobutyryl-CoA with a similar Kᵦ. The enzyme activity was also severely inhibited by several organic sulfhydryl reagents such as N-ethylmaleimide, p-hydroxymercuribenzoate, and methyl mercury iodide. The pattern and degree of inhibition were essentially identical for both substrates. The purified 2-methylbranched chain acyl-CoA dehydrogenase was immuno-logically distinct from isovaleryl-CoA-, short chain acyl-CoA-, medium chain acyl-CoA-, or long chain acyl-CoA dehydrogenase.

It has been well established that there are three acyl-CoA dehydrogenases which catalyze dehydrogenation of straight chain acyl-CoAs of different chain lengths at the first step of the β-oxidation cycle. These are butyryl-CoA- (EC 1.3.99.2), general acyl-CoA- (EC 1.3.99.3), and long chain acyl-CoA dehydrogenases; they are most active with butyryl-CoA, octanoyl-CoA, and palmitoyl-CoA, respectively, as substrate. These enzymes are localized in the mitochondria of various tissues in mammals. However, the enzymes which dehydrogenate branched chain acyl-CoAs such as isovaleryl-CoA, 2-methylbutyryl-CoA, and isobutyryl-CoA have not been extensively studied until recently. These branched chain acyl-CoAs are produced as intermediates in the metabolism of the branched chain amino acids, leucine, isoleucine, and valine, respectively. Previously, butyryl-CoA dehydrogenase (short chain acyl-CoA dehydrogenase) had been thought to catalyze the dehydrogenation of all short branched chain acyl-CoAs (1, 2). However, several years ago, our biochemical observations on patients with isovaleric acidemia, an inborn error of leucine metabolism, suggested the existence of a dehydrogenase which is specific for isovaleryl-CoA (3–5). Using rat liver mitochondria as an enzyme source, we subsequently demonstrated that a specific isovaleryl-CoA dehydrogenase indeed exists and that it is distinct from butyryl-CoA dehydrogenase (6–8). Furthermore, we have recently reported the purification and characterization of isovaleryl-CoA dehydrogenase (8, 9). This enzyme is biochemically and immuno-logically distinct from butyryl-CoA dehydrogenase (9).

In the course of these studies, we demonstrated for the first time that 2-methylbutyryl-CoA and isobutyryl-CoA were not dehydrogenated by either isovaleryl-CoA dehydrogenase or butyryl-CoA dehydrogenase. Instead, these two 2-methyl-substituted acyl-CoAs were dehydrogenated by an enzyme which was distinct from the other four acyl-CoA dehydrogenases (8). We have recently reported partial purification of this enzyme from rat liver mitochondria by a sequence of DEAE-Sephadex and hydroxyapatite column chromatographies and isoelectric focusing, and designated it 2-methyl-branched chain acyl-CoA dehydrogenase (8). In the present paper, we report the purification to homogeneity of 2-methyl-branched chain acyl-CoA dehydrogenase from rat liver mitochondria. We also describe here the molecular characteristics, kinetic parameters, requirement for ETF, susceptibility to various types of inhibitors, and immunological properties of this enzyme.

EXPERIMENTAL PROCEDURES

Materials

DCIP, FAD, FMN, l-isoleucine, and l-allo-isoleucine were obtained from Sigma Chemical Co. (St. Louis, MO). Isovaleric acid and

1The abbreviations used are: ETF, electron transfer flavoprotein; DCIP, 2,6-dichlorophenolindophenol; PMS, phenazine methosulfate; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate.
Purification of 2-Methyl-branched Chain Acyl-CoA Dehydrogenase

PM2S were purchased from Eastman Kodak Co. (Rochester, NY). Coenzyme A (lithium salt), all acyl-CoA esters, and agarse-hexanoyl-CoA (type I) were obtained from P-L Biochemicals (Milwaukee, WI) unless otherwise mentioned. Matrex Gel Blue A and Diaflow membrane PM-30 were purchased from Amicon Co. (Lexington, MA). Bio-Gel A-0.5m and the molecular weight kit for SDS-polyacrylamide gel electrophoresis were from Bio-Rad (Richmond, CA).

Methods

Synthesis of Coenzyme A Thioesters of S- and R-2-Methylbutyryl Acids, and Isovaleric Acid—S- and R-2-methylbutyric acids were prepared from L-isoleucine and L- allo-isoleucine, respectively. The reaction mixture was composed of 0.1 M potassium phosphate buffer (pH 8.0) and 80% acetic acid. The reaction was carried out at 32°C and the reaction was started by the addition of the substrate. The reaction was monitored by the decrease in absorbance at 270 nm. The extinction coefficient of DCIP (21 mM^-1 cm^-1) at 600 nm was used as the basis for calculation of the amount of DCIP reduced.

Preparation of Rat Liver Mitochondria and the First Four Steps of Purification—In a typical experiment, 100 adult male Charles River CD rats, weighing 200 to 280 g, were killed by decapitation and the liver mitochondria were isolated by the method of de Duve et al. (11). The first four steps of the purification are the same as those utilized to isolate crude 2-methyl-branched chain acyl-CoA dehydrogenase as described previously (8, 9). The purification was followed by the addition of 0.05 ml of PMS (0.8 mg of protein in 1.5 ml) from step 6 was applied to a Bio-Gel A-0.5m column (1.5 x 100 cm) equilibrated with 10 mM KPO4 buffer, pH 8.0 containing 0.1 M NaCl. The column was eluted at a flow rate of 0.16 ml/min. The proteins used as standards to calibrate the native molecular weight were as follows: catalase (M, = 240,000), ovalbumin (M, = 45,000), bovine serum albumin (M, = 68,000), and ovalbumin (M, = 45,000).

Identification of Reaction Product—The reaction conditions for this purpose were similar to those utilized in the dye reduction assay except for the following modifications: the reaction mixture contained 1% albumin, 10% glycerol, 3 mM PMS, 0.1 mM FAD, and 0.1 mM acyl-CoA unless otherwise mentioned. The final volume was 1 ml. The enzyme reaction was carried out at 32°C and the reaction was started by the addition of the substrate. The mixture was then incubated at 37°C for 2 h. The reaction was monitored by the decrease in absorbance at 270 nm. The extinction coefficient of DCIP (21 mM^-1 cm^-1) at 600 nm was used as the basis for calculation of the amount of DCIP reduced.

Preparation of Electron Transfer Flavoprotein—ETF was purified to homogeneity from rat liver mitochondria according to the method described in our previous papers (8, 9). The final preparation was used to obtain the pure enzyme preparation was done by the method of Bradford (14). Determination of the protein concentrations was done by the method of Lowry et al. (15). The final preparation was used as standard in all of the assay methods.

Protein Determinations—Protein concentrations were determined by the method of Bradford (14). Determination of the protein concentrations of the pure enzyme preparation was done by the method of Lowry et al. (15). The final preparation was used as a standard in all of the assay methods.

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Preparation of Bio-Gel A-0.5m Chromatography (Step 7) The enzyme preparation was again assayed for monitoring DCIP bleaching. The purity of the purified enzyme was determined by SDS-PAGE in both the absence and presence of 2-mercaptoethanol. These subunit M, = 30,000 and 35,500, in close agreement with those reported by Furuta et al. (12). The ratios of absorbance at the maxima, 270:375:456:460 nm, were 6:9:0:41:0:10:0, as described previously (9).

Protein Determinations—Protein concentrations were determined by the method of Lowry et al. (15) unless otherwise indicated. Because the protein concentration from the Matrex Gel Blue A column contained a large amount of FAD, protein concentrations were determined by the method of Bradford (14). Determination of the protein concentrations of the pure enzyme preparation was done by the method of Lowry et al. (15). Bovine serum albumin was used as a standard in all of the assay methods.

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Purification of 2-Methyl-branched Chain Acyl-CoA Dehydrogenase

| Purification | Total Protein | 14\(_c\) CoA | S-2-MeC\(_4\) CoA | 14\(_c\) CoA | S-2-MeC\(_4\) CoA | Purification | Yield |
|--------------|--------------|--------------|------------------|--------------|------------------|--------------|-------|
| 1. Sonication Supernatant | 35,000 | mmol.min\(^{-1}\)d | mmol.min\(^{-1}\).mg\(^{-1}\) | -fold | percent |
| 2. Ammonium Sulfate (60-80%) | 14,400 | -c | -c | - | - |
| 3. DEAE-Sephadex A-50 (Preparation B)\(^a\) | 1,500 | 40,400 | 40,500 | 26.9 | 27.0 | 1 | 1 | 100 | 100 |
| 4. Hydroxyapatite (Preparation E)\(^a\) | 108 | 12,900 | 13,600 | 119 | 126 | 4.4 | 4.7 | 32 | 34 |
| 5. Matrex Gel Blue A | 5.0 | 6,200 | 6,500 | 1,740 | 1,300 | 46 | 48 | 15 | 16 |
| 6. Agarose-Hexane-CoA | 0.8 | 1,570 | 1,710 | 1,963 | 2,138 | 73 | 79 | 3.9 | 4.2 |
| 7. Bio Gel A-0.5m | 0.42 | 985 | 1,040 | 2,345 | 2,476 | 87 | 92 | 2.4 | 2.6 |

\(^a\) Typical column chromatographic patterns are presented in reference (8).
\(^b\) mmol of DCIP reduced.

\(^c\) Activities could not be determined due to interference of the dye-reduction assay by non-specific reductants.

RESULTS

Purification of 2-Methyl-branched Chain Acyl-CoA Dehydrogenase—The entire purification procedure is summarized in Table I. Technical details are described under "Experimental Procedures." The first four steps of the purification are the same as previously described (8). Rat liver mitochondria were solubilized by sonication (step 1) and the supernatant was fractionated by the sequence of ammonium sulfate precipitation (40-80%) (step 2), DEAE-Sephadex A-50 (step 3), and hydroxyapatite (step 4) chromatography. Fractionation patterns at steps 3 and 4 are shown in our previous publication (8). Preparation E, which was obtained from hydroxyapatite chromatography, contained other acyl-CoA dehydrogenase activities in addition to those dehydrogenating S-2-methylbutyryl-CoA and isobutyryl-CoA. In particular, butyryl-CoA dehydrogenase activity was very high while isovaleryl-CoA dehydrogenase activity was undetectable. Relative specific activities using S-2-methylbutyryl-CoA, isobutyryl-CoA, isovaleryl-CoA, n-butyryl-CoA, n-octanoyl, and palmitoyl-CoA as substrates in preparation E were 1.0, 0.95, 0, 9.4, 0.68, and 1.5, respectively. The octanoyl-CoA-dehydrogenating activity in preparation E was due to the co-existing long chain acyl-CoA dehydrogenase; medium chain acyl-CoA dehydrogenase was not present in this preparation. In order to separate 2-methyl-branched chain acyl-CoA dehydrogenase from other acyl-CoA dehydrogenases, preparation E (108 mg of protein in 25 ml) was applied to a Matrex Gel Blue A column (step 5). When elution was done with a linear FAD gradient (0-7 mM), S-2-methylbutyryl-CoA- and isobutyryl-CoA-dehydrogenating activities were co-eluted as a sharp single peak at 1-2 mM FAD, while only a small amount of n-butyryl-CoA-dehydrogenating activity was eluted in this region; no significant activities for n-octanoyl-CoA and palmitoyl-CoA were detectable. Most of the butyryl-CoA-and long chain acyl-CoA dehydrogenases were eluted as very broad peaks at FAD concentrations higher than 3 mM. When the column was further eluted with 10 mM KPO\(_4\), buffer (pH 8.0) containing 0.8 M NaCl and 3 mM FAD, the butyryl-CoA- and long chain acyl-CoA dehydrogenases which still remained in the column were eluted as a sharp peak (Fig. 1). Fractions 18 to 35, containing both S-2-methylbutyryl-CoA- and isobutyryl-CoA-dehydrogenating activities, were pooled together. Relative specific activities with S-2-methylbutyryl-CoA, isobutyryl-CoA, n-butyryl-CoA, n-octanoyl-CoA, and palmitoyl-
CoA in the pooled fraction were 1.0, 0.92, 0.23, 0, and 0, respectively. After concentration, the sample preparation (5 mg of protein in 7 ml) was applied to an agarose-hexane-CoA column (step 6).

When the agarose-hexane-CoA column was eluted with a linear NaCl gradient (0-0.4 M), both S-2-methylbutyryl-CoA- and isobutyryl-CoA-dehydrogenating activities were again co-eluted as a single peak in fractions 25 to 45; these activities were well separated from butyryl-CoA-dehydrogenase (Fig. 2). After concentration, the sample solution (0.8 mg of protein in 1.5 ml) from step 6 was applied to a Bio-Gel A-0.5m column (step 7). As shown in Fig. 3, both S-2-methylbutyryl-CoA- and isobutyryl-CoA-dehydrogenating activities were still co-eluted as a single peak in fractions 50 to 70. These fractions were combined and then concentrated; this sample represents the final preparation. The specific activities of the final preparation for S-2-methylbutyryl-CoA and isobutyryl-CoA were 2.4 and 2.3 μmol min⁻¹ mg⁻¹, respectively. The overall yield of the enzyme was 2.5%. An identical value was obtained when the recoveries of either of the activities were utilized for this computation. The purified enzyme was stable for at least 30 days when stored in 50% glycerol at −20 °C.

Purity, Molecular Weight, and Subunit Structure—The purity of the final 2-methyl-branched chain acyl-CoA dehydrogenase preparation was determined by PAGE with and without SDS. When boiled, the purified enzyme gave a single protein band with Mᵣ = 41,500 in SDS-PAGE in both the absence and presence of 2-mercaptoethanol (Fig. 4, B and C). When the sample was subjected to SDS-PAGE without boiling, the enzyme gave an apparent single protein band with Mᵣ = 85,000, both in the presence and absence of 2-mercaptoethanol, indicating a dimeric form of the protein (Fig. 4A).

In PAGE without SDS, the purified enzyme also gave a single protein band in 5.0% gel (Rf value of 0.55) (Fig. 4E). The native molecular weight of the enzyme was estimated to be 170,000 by gel filtration on Bio-Gel A-0.5m chromatography (Fig. 3). These data indicated that the enzyme is composed of four subunits of identical size.

Isoelectric Point—The isolectric point of the purified enzyme was 5.5 ± 0.2 as determined by sucrose discontinuous isoelectric focusing (LKB) using a 1:4 mixture of pH 3.5-10 and 4-6 ampholytes. The pH of each fraction was determined using a pH meter at 0 °C. This pH value was almost identical with our previous results using a crude 2-methyl-branched chain acyl-CoA dehydrogenase preparation from the hydroxyapatite chromatography step (8). Chromatofocusing (Pharmacia) was also carried out using PBE 94 and Polybuffer 74. This enzyme was eluted from the chromatofocusing at pH 5.1 ± 0.2.

Amino Acid Composition—The amino acid composition is shown in Table II. The number of cysteine residues was estimated to be 5/subunit. The subunit molecular weight of the enzyme was calculated to be 42,400 from the amino acid composition, in close agreement with the value (41,500) determined by SDS-PAGE. The specific volume of the enzyme was 0.72 as computed from the amino acid composition.

Absorption and Fluorescence Spectra, and Prosthetic Group—The visible and ultraviolet absorption spectrum of the purified enzyme is shown in Fig. 5. The major absorption maxima were found at 275, 340, and 435 nm. The ratios of absorbance at 275, 340, and 435 nm were 10:31:3:1:0. The fluorescence emission spectrum of the purified enzyme excited at 450 nm showed a peak at 520 nm as in the case of authentic FAD; its intensity was 28% of the equivalent amount of authentic FAD, indicating quenching due to FAD-protein interaction. The excitation spectrum of the enzyme as monitored with emission at 530 nm showed two maxima at 375 and 465 nm as in the case of authentic FAD. Its fluorescence intensity was 25% of that of authentic FAD. These results on absorption spectrum and fluorescence spectra indicate that 2-methyl-branched chain acyl-CoA dehydrogenase contains FAD as a prosthetic group. The FAD content was estimated to be 0.5 mol/mol of subunit as calculated from the observed absorption at 450 nm. The absorption coefficient of FAD (450 nm) at 20°C in 1.0 M K-phosphate buffer (pH 7.0) was 10.3 μmol·cm⁻¹·M⁻¹. The absorption coefficient of FAD at 20°C in 1.0 M K-phosphate buffer (pH 7.0) was 10.3 μmol·cm⁻¹·M⁻¹.
FIG. 2. Separation of 2-methyl-branched chain acyl-CoA dehydrogenase from the residual butyryl-CoA dehydrogenase on agarose-hexane-CoA column chromatography. The sample (5.0 mg of protein in 7.3 ml) obtained from Matrex Gel Blue A chromatography was applied to an agarose-hexane-CoA column (1 X 7 cm) equilibrated with 10 mM K-phosphate buffer (pH 8.0) containing 0.5 mM EDTA and 10% glycerol. The adsorbed proteins were eluted with a linear gradient of the starting buffer and the same buffer (pH 8.0) containing 0.5 M NaCl. The shaded rectangle indicates the fractions combined. Abbreviations of substrates are the same as in Fig. 1. G, indicates the point at which the gradient was started.

FIG. 3. The final step of S-methyl-branched chain acyl-CoA dehydrogenase purification by Bio-Gel A-0.5m column chromatography. The sample (0.8 mg of protein in 1.5 ml) obtained from agarose-hexane-CoA chromatography was applied to a Bio-Gel A-0.5m column (1.5 X 100 cm) equilibrated with 10 mM K-phosphate buffer (pH 8.0) containing 0.2 M NaCl. The shaded rectangle indicates the fractions combined as the final preparation. The inset shows the calibration curve for the molecular weights of the protein. The standard proteins used are as follows: I, catalase ($M_r$ = 240,000); 2, aldolase ($M_r$ = 158,000); 3, bovine serum albumin ($M_r$ = 68,000); and 4, ovalbumin ($M_r$ = 45,000). The arrow in the inset indicates the molecular weight of 2-methyl-branched chain acyl-CoA dehydrogenase (2-meBCAD). Other abbreviations are as in Fig. 1.

Substrate Specificity, Reaction Products, and Kinetic Analysis—Using either S-2-methylbutyryl-CoA or isobutyryl-CoA as a substrate, the optimum pH for the purified enzyme was 8.0. The substrate specificity of the enzyme is summarized in Table I. The enzyme exhibited its highest activity with either S-2-methylbutyryl-CoA or isobutyryl-CoA as a substrate. When R-2-methylbutyryl-CoA was used as a substrate, its activity was 22% of that observed with S-2-methylbutyryl-CoA. In contrast, the activity was extremely low or not detectable when the following compounds were used as a substrate: isovaleryl-CoA, propionyl-CoA, n-butyryl-CoA, n-valeryl-CoA, n-hexanoyl-CoA, n-octanoyl-CoA, palmitoyl-CoA, glutaryl-CoA, and sarcosine. The apparent $V_{max}$ and $K_m$ values for isobutyryl-CoA were 2.0 $\mu$mol min$^{-1}$ mg$^{-1}$ and 89 $\mu$M, respectively, and the apparent $V_{max}$ and $K_m$ values for 2-methylbutyryl-CoA were 2.2 $\mu$mol min$^{-1}$ mg$^{-1}$ and 20 $\mu$M, respectively (Tables III and VI).

The reaction products were identified by gas chromatographic analysis as shown in Fig. 6. Under the conditions employed, isobutyric and 2-methylbutyric acids, and the two 2,3-unsaturated products from these acids (methacrylic and tiglic acids), were all well separated from each other as shown in Fig. 6d. The S- and R-enantiomers of 2-methylbutyric acid were not separated. The reaction product of the purified enzyme with isobutyryl-CoA was identified as methacryl-CoA by detection of its hydrolysis product, methacrylic acid (Fig. 6a). The reaction product of the same enzyme with S-2-methylbutyryl-CoA was identified as tiglyl-CoA (Fig. 6b). When R-2-methylbutyryl-CoA was used as a substrate, a compound which had a considerably shorter retention time (7.6 min) than that of tiglic acid (9.8 min) was detected (Fig. 6c). After conversion to a methyl ester, this compound was identified as ethylacrylic acid using mass spectroscopy by the identity of its mass spectrum to that of the authentic standard (23).

The reaction rates as assessed by measuring the amount of product in the first 5 min using gas chromatography were 12.8 and 12.2 $\mu$mol/min/0.5 ml of reaction mixture for methacryl-CoA and tiglyl-CoA, respectively. These values were in good agreement with the values (13 $\mu$mol of DCIP reduced/
Purification of 2-Methyl-branched Chain Acyl-CoA Dehydrogenase

The reaction mixture was incubated at 37 °C for 2, 5, 10, and 20 min. The reaction products from isobutyryl-CoA and S-2-methylbutyryl-CoA were produced linearly with time for at least 5 min, but the reaction rate diminished after this point. The decrease of isobutyryl-CoA and the increase of methacryl-CoA both plateaued, revealing an equilibrium after 20 min. A similar time course was observed with S-2-methylbutyryl-CoA as a substrate. The apparent \( K_m \) was determined as the ratio of the product concentration to the substrate concentration at the equilibrium. The apparent \( K_m \) for these two substrates differed greatly: \( K_m \) for isobutyryl-CoA was 1.0 while that for S-2-methylbutyryl-CoA was 4.0.

The inhibitory effects of various acyl-CoAs on 2-methyl-branched chain acyl-CoA dehydrogenase activity were investigated by using isobutyryl-CoA or S-2-methylbutyryl-CoA as substrates. The results are summarized in Table IV. No difference between the two substrates was observed. The most notable finding was that tiglyl-CoA strongly inhibited both the isobutyryl-CoA- and S-2-methylbutyryl-CoA-dehydrogenating activities, while crotonyl-CoA (100 \( \mu \)M) inhibited both of the dehydrogenating activities by 50%. 3-Methylcro-

![Figure 4](image1.png)

**Figure 4.** Polyacrylamide gel electrophoresis with and without sodium dodecyl sulfate of the purified 2-methyl-branched chain acyl-CoA dehydrogenase. The purified enzyme (10 \( \mu \)g of protein each) was applied to SDS-PAGE as follows: A, without 2-mercaptoethanol and with boiling; B, with 2-mercaptoethanol and without boiling the enzyme preparation; C, with 2-mercaptoethanol and with boiling. D represents the authentic proteins described under “Experimental Procedures” as analyzed with 2-mercaptoethanol as calibration standards. The purified enzyme protein (10 \( \mu \)g of protein) was also subjected to 5.0% polyacrylamide gel without SDS as shown in E. The migration is from top to bottom.

**Table II**

| Amino acid composition of the purified 2-methyl-branched chain acyl-CoA dehydrogenase |
|---------------------------------|
| The number of residues per subunit was estimated from values for each amino acid detected after 24-h hydrolysis. The values are based on a subunit \( M_s = 41,500.0 \) |

| Amino acid | Residues/subunit |
|------------|------------------|
| Asx        | 29               |
| Thr        | 19               |
| Ser        | 40               |
| Gin        | 54               |
| Pro        | 12               |
| Gly        | 67               |
| Ala        | 38               |
| \( \frac{1}{2}-\text{Cys} \) | 5*              |
| Val        | 23               |
| Met        | 5                |
| Ile        | 19               |
| Leu        | 26               |
| Tyr        | 7                |
| Phe        | 16               |
| His        | 17               |
| Lys        | 20               |
| Arg        | 11               |
| Trp        | 2*               |

Total amino acid residues 410

* Determined after performic acid oxidation (20).
* Determined using 3 \( N \) mercaptoethanesulfonic acid hydrolysis (21).

![Figure 5](image2.png)

**Figure 5.** Absorption spectrum of the purified 2-methyl-branched chain acyl-CoA dehydrogenase. The enzyme preparation \( (A_{380} = 0.390, A_{450} = 0.058) \) in 0.01 M potassium phosphate buffer, pH 7.8, at 25 °C, was used for the determination. The absorbance was scanned from 700 to 300 nm on the 0 to 0.1 scale and from 300 to 220 nm on the 0 to 0.5 scale.

**Table III**

| Substrate Specific activity Relative activity nmol DCIP reduced min\(^{-1}\) mg\(^{-1}\) % |
|---------------------------------|-----------------|-----------------|
| Isobutyryl-CoA                   | 2024            | 92              |
| S-2-Methylbutyryl-CoA            | 2200            | 100             |
| \( R-2 \)-Methylbutyryl-CoA      | 484             | 22              |
| Isovaleryl-CoA                   | 0               | 0               |
| Propionyl-CoA                    | 0               | 0               |
| Butyryl-CoA                      | 110             | 5               |
| \( \alpha \)-Valeryl-CoA          | 0               | 0               |
| \( \alpha \)-Hexanoyl-CoA         | 0               | 0               |
| Palmitoyl-CoA                    | 0               | 0               |
| Glutaryl-CoA                     | 0               | 0               |
| Sarcosine                        | 0               | 0               |
Tiglyl-CoA was determined using isobutyryl-CoA or S-2-methylbutyryl-CoA, and isobutyryl-CoA-dehydrogenating activities were competitively inhibited by tiglyl-CoA. The apparent \( K_i \) values for tiglyl-CoA were 7 \( \mu \)M when S-2-methylbutyryl-CoA was the substrate and 3 \( \mu \)M when isobutyryl-CoA was the substrate. These results indicate that tiglyl-CoA, the product from S-2-methylbutyryl-CoA, competitively inhibits the dehydrogenation of both S-2-methylbutyryl-CoA and isobutyryl-CoA with similar \( K_i \) values.

**ETF as Electron Acceptor**—The purified ETF preparation (2.4 mg of protein/ml) was used in the following experiments. The basic assay media contained 100 mM K-phosphate buffer (pH 6.0), 100 \( \mu \)M S-2-methylbutyryl-CoA or 200 \( \mu \)M isobutyryl-CoA, 48 \( \mu \)M DCIP, and 10 \( \mu \)g of purified 2-methyl-branched chain acyl-CoA dehydrogenase, unless otherwise mentioned. The purified enzyme did not exhibit any activity unless PMS or ETF was added to the assay media. When 1.5 mM PMS was added to the media in the absence of FAD, specific activities of 0.87 and 0.83 \( \mu \)mol min\(^{-1}\) mg\(^{-1}\) were observed with S-2-methylbutyryl-CoA and isobutyryl-CoA, respectively. The enzyme activity was further enhanced 2.3-fold by the addition of 100 \( \mu \)M FAD. Such an enhancement was not observed with 100 \( \mu \)M FMN. When ETF was added to the assay media in amounts ranging from 50 to 200 \( \mu \)g replacing PMS, both S-2-methylbutyryl-CoA- and isobutyryl-CoA-dehydrogenating activities were increased linearly with the amount of ETF. The purified ETF preparation per se did not exhibit any S-2-methylbutyryl-CoA- or isobutyryl-CoA-dehydrogenating activity without addition of 2-methyl-branched chain acyl-CoA dehydrogenase. The specific activity with S-2-methylbutyryl-CoA in the presence of 100 \( \mu \)g of ETF was 0.37 \( \mu \)mol min\(^{-1}\) mg\(^{-1}\) and that of isobutyryl-CoA in the presence of 200 \( \mu \)g of ETF was 0.39 \( \mu \)mol min\(^{-1}\) mg\(^{-1}\). When isobutyryl-CoA was the substrate, the amount of ETF needed was twice as large as that required to obtain an approximately equal specific activity with S-2-methylbutyryl-CoA. In these experiments, we could not determine an apparent \( K_m \) for ETF, because saturation was not observed with the amounts of ETF employed.

**Effects of Various Inhibitors**—The effects of various inhibitors on both S-2-methylbutyryl-CoA- and isobutyryl-CoA-dehydrogenating activities of the purified enzyme are shown in Table V. Essentially identical inhibitory effects on these two activities were observed using various inhibitors. These dehydrogenating activities were severely inhibited by sulfhy-

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**Table IV**

**Inhibitory effects of various acyl-CoAs on 2-methyl-branched chain acyl-CoA dehydrogenase activity**

The purified 2-methyl-branched chain acyl-CoA dehydrogenase (10 \( \mu \)g of protein) was first incubated with each acyl-CoA (100 \( \mu \)M) for 2 min at 25 °C in the presence of 100 \( \mu \)M FAD. The dehydrogenating activity was then determined by the dye reduction assay using isobutyryl-CoA (200 \( \mu \)M) or S-2-methylbutyryl-CoA (100 \( \mu \)M) as substrate as described under "Experimental Procedures.'

| Preincubated with          | Residual activity assayed with |
|----------------------------|-------------------------------|
|                            | Isobutyryl-CoA | S-2-Methylbutyryl-CoA |
|                            | \%              | \%                  |
| None                       | 100             | 100                 |
| Acetyl-CoA                  | 107             | 93                  |
| Propionyl-CoA               | 107             | 100                 |
| Butyryl-CoA                 | 50              | 42                  |
| Valeryl-CoA                 | 50              | 48                  |
| Hexanoyl-CoA                | 81              | 100                 |
| Isovaleryl-CoA              | 107             | 106                 |
| Crotonyl-CoA                | 47              | 68                  |
| Tiglyl-CoA                  | <5              | <10                 |
| 3-Methylcrotonyl-CoA        | 89              | 90                  |

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**Fig. 6. Identification of the reaction products of 2-methyl-branched chain acyl-CoA dehydrogenase by gas chromatography.** The purified enzyme was reacted for 10 min at 37 °C with a standard mixture which contained isobutyryl (iBu), methacrylic (ma), n-butyric (Bu), crotonic (Cro), isovaleric (iC\(_5\)), 2-methylbutyric (2-meBu), 3-methylcrotonic (3-me), tiglic (tg), and hexanoic acids (hC\(_6\)).
Purification of 2-Methyl-branched Chain Acyl-CoA Dehydrogenase

The effects of various compounds on 2-methyl-branched chain acyl-CoA dehydrogenase activity

The purified enzyme (10 μg of protein) was preincubated with each compound at the concentration indicated for 5 min at 32 °C. The dehydrogenating activity was determined by the dye reduction assay in the presence of 100 μM FAD. 2-met-CoA, 2-methylbutyryl-CoA, iC4CoA, isobutyryl-CoA.

| Preincubated with          | Residual activity with  |
|---------------------------|-------------------------|
|                           | Final concentration | 2-met-CoA | iC4CoA | % |
| None                      | 100                      | 7         | 100    |
| N-Ethylmaleimide          | 100                      | 7         | 73     |
| p-Hydroxymercurobenzoate  | 88                       | 48        | 92     |
| Iodoacetamide             | 50                       | 0         | 0      |
| Methyl mercury iodide     | 44                       | 0         | 0      |
| HgCl2                     | 18                       | 0         | 0      |
| CuCl2                     | 18                       | 0         | 0      |
| AgNO3                     | 18                       | 0         | 0      |

—, not determined.

dry reagents such as 2 mM N-ethylmaleimide and 0.1 mM p-hydroxymercurobenzoate. Methyl mercury iodide (0.1 mM) inhibited the activity by 50%, but iodoacetamide (2 mM) did not significantly inhibit the activity. The enzyme activity was completely inhibited by heavy metals such as Hg2+, Cu2+, and Ag+ (0.1 mM each) which are known to affect thiol groups in proteins. Ca2+, Zn2+, Pb2+, and Fe3+ did not inhibit this enzyme activity at all.

Immunological Properties—In both immunoprecipitation and Ouchterlony double diffusion experiments, the purified 2-methyl-branched chain acyl-CoA dehydrogenase (10 μg of protein; specific activity = 2.2 μmol min⁻¹ mg⁻¹ for S-2-methylbutyryl-CoA) was reacted with individual antisera raised against isovaleryl-CoA, short chain acyl-CoA-, medium chain acyl-CoA-, or long chain acyl-CoA-dehydrogenase, respectively. The 2-methyl-branched chain acyl-CoA dehydrogenase did not exhibit any cross-reaction with these four antibodies in Ouchterlony double diffusion experiments; its enzyme activity was not precipitated by the four antibodies with either isobutyryl-CoA or S-2-methylbutyryl-CoA as a substrate (data not shown). These results indicate that 2-methyl-branched chain acyl-CoA dehydrogenase is immunologically distinct from the four other acyl-CoA dehydrogenases and that the final preparation was not contaminated by the other enzymes.

DISCUSSION

In the present study, we purified 2-methyl-branched chain acyl-CoA dehydrogenase from rat liver mitochondria to homogeneity in seven steps including affinity chromatographies with Matrex Gel Blue A and agarose-hexane-CoA, which were used at the fifth and sixth steps, respectively. The activity to dehydrogenate S-2-methylbutyryl-CoA and that to dehydrogenate isobutyryl-CoA were co-purified throughout the entire seven steps of purification (Table I). The specific activity of the final preparation was enriched 90-fold over that of the preparation obtained after the DEAE-Sephadex step. The activities in the crude preparations such as mitochondrial sonic supernatant and (NH₄)₂SO₄ precipitates could not be accurately measured due to interference by nonspecific reductants. The tritium release assay which is free of such interference was not available for these activities. In our previous study (9), the specific activity of isovaleryl-CoA dehydrogenase preparation after the DEAE-Sephadex stage was enriched approximately 20 times over that of the mitochondrial sonic supernatant as measured by the tritium release assay, and a similar degree of purification can be expected for 2-methyl-branched chain acyl-CoA dehydrogenase at these steps. Thus, the final preparation of 2-methyl-branched chain acyl-CoA dehydrogenase is probably enriched nearly 1800-fold over that in the mitochondrial sonic supernatant.

The fact that isobutyryl-CoA- and S-2-methylbutyryl-CoA-dehydrogenating activities co-purified throughout all steps of purification suggests that a single enzyme catalyzes the dehydrogenation of both isobutyryl-CoA and S-2-methylbutyryl-CoA. We have also shown in this report that both isobutyryl-CoA- and S-2-methylbutyryl-CoA-dehydrogenating activities of this enzyme were competitively inhibited by tiglyl-CoA, the product from S-2-methylbutyryl-CoA (Tables IV and VI). In our previous observation, there was no additive effect on the Vₘₐₓ of the enzyme when 2-methylbutyryl-CoA and isobutyryl-CoA were added together in concentrations well above their respective Kₘ values (8). These observations together unequivocally indicate that a single enzyme catalyzes the dehydrogenation of both S-2-methylbutyryl-CoA and isobutyryl-CoA.

The purified enzyme exhibited a high substrate specificity (Table III). It dehydrogenated isobutyryl-CoA and S-2-methylbutyryl-CoA with high specific activities. The rates for these two substrates were approximately equal. This enzyme also dehydrogenated the R-enantiomer of 2-methylbutyryl-CoA, but the rate of the reaction with this substrate was only 22% of that with the S-enantiomer. The reaction products from isobutyryl-CoA and S-2- and R-2-methylbutyryl-CoA by this enzyme were identified as methacryl-CoA, tiglyl-CoA, and ethylcacyl-CoA, respectively, by the detection of their hydrolysis products (Fig. 6). In contrast, this enzyme did not dehydrogenate any other straight chain acyl-CoAs, or a branched one, isovaleryl-CoA, at any significant reaction rate. This substrate specificity is very narrowly limited to those substrates with a methyl substitution at the α-carbon. Among the substrates with an α-methyl substitution, S-2-methylbutyryl-CoA and isobutyryl-CoA were dehydrogenated with high efficiencies while R-2-methylbutyryl-CoA was dehydrogenated at a considerably slower rate. These results on the substrate specificity and the identification of the products suggest that the reaction of this enzyme proceeds by elimination of one hydrogen each, respectively, from the α-methylene and the β-methylene (methyl) group taking the a position as illustrated in Fig. 7. Whether the substitution on the a position is a methyl or an ethyl does not significantly affect the rate of reaction. In contrast, when the substitution on the c position is an ethyl, the rate of reaction was significantly slower than that when it was a methyl. This suggests that the size of the substitution directed to the c position, although it does not participate in the dehydrogenase reaction, is important in defining the fitness of the substrate to the conformation of this enzyme at the active site.

The product inhibition by tiglyl-CoA was also specific. 2-Methyl-branched chain acyl-CoA dehydrogenase activity was inhibited by neither 3-methylcrotonyl-CoA nor isovaleryl-CoA. However, it was moderately inhibited by n-butyl-CoA, n-valeryl-CoA, or crotonyl-CoA. This suggests that the enzyme can bind n-butyryl-CoA, valeryl-CoA, or crotonyl-CoA as substrate analogs, although the enzyme does not dehydrogenate them at a significant rate (Table III).

The substrate specificity of 2-methyl-branched chain acyl-CoA dehydrogenase and the inhibition of this enzyme by tiglyl-CoA are of particular interest in view of the regulation of the branched chain amino acid metabolism. The three
### Purification of 2-Methyl-branched Chain Acyl-CoA Dehydrogenase

**TABLE VI**

Properties of 2-methyl-branched chain acyl-CoA dehydrogenase in comparison with isovaleryl-CoA- and short chain acyl-CoA dehydrogenases

Three enzymes were purified to homogeneity from rat liver mitochondria. 2-meC₄CoA, 2-methylbutyryl-CoA; iC₄CoA, isobutyryl-CoA; n-C₄CoA, n-butyryl-CoA; iC₅-CoA, isovaleryl-CoA; 2-ME, 2-mercaptoethanol.

| Property                                      | 2-methyl-branched chain acyl-CoA dehydrogenase (2-meBCAD) | Isovaleryl-CoA (IVD) | Short chain acyl-CoA dehydrogenase (SC-AD) |
|-----------------------------------------------|------------------------------------------------------------|----------------------|---------------------------------------------|
| Native Mr:                                    | 170,000                                                    | 175,000              | 160,000                                     |
| Subunit Mr: with 2-ME                         | 41,500                                                     | 43,000               | 41,000                                      |
| Subunit Mr: without 2-ME                      | 41,500                                                     | 43,000               | 41,000                                      |
| Isoelectric point 5.5±0.2                      | 5.6±0.2                                                    | 4.9±0.2              |
| Spectral maxima (nm)                          | 275:340:435                                                | 275:325:375:445      | 275:365:450                                |
| Emission peak 530 nm                          | 530 nm                                                     | 530 nm               | 530 nm                                      |
| Excitation peak 380 and 465 nm                | 370 and 465 nm                                             | 370 and 465 nm       |
| Intensity                                     | 28% of FAD                                                 | 24% of FAD           | 2% of FAD                                   |
| Kinetic Parameters for the respective best substrate: | | | |
| V_max for 2-meC₆CoA                            | 2.2μmol.min⁻¹.mg⁻¹                                       | 0                    | 0                                           |
| for iC₄CoA                                    | 0                                                         | 0                    | 0                                           |
| for n-C₄CoA                                   | 0                                                         | 2.6μmol.min⁻¹.mg⁻¹   | 0                                           |
| for n-C₅CoA                                   | 0                                                         | 0                    | 7.5μmol.min⁻¹.mg⁻¹                          |
| Kᵢ for 2-meC₆CoA                              | 20μM                                                       | ---                  | ---                                         |
| for iC₄CoA                                    | 89μM                                                       | ---                  | ---                                         |
| for n-C₄CoA                                   | ---                                                       | 33μM                 | ---                                         |
| for n-C₅CoA                                   | ---                                                       | ---                  | 18μM                                        |
| Competitive Inhibition by Product:            | | | |
| Kᵢ for Tiglyl-CoA                             | 2-meC₆CoA as substrate 7μM                                 | ---                  | ---                                         |
| iC₄CoA as substrate                           | 3μM                                                       | e                    | f                                           |
| Kᵢ for 3-methylcrotonyl-CoA                   | ---                                                       | 20μM                 | ---                                         |

**Immunological properties:**

| Antibody to IVD                                | no-cross reaction | positive reaction | no-cross reaction |
| Antibody to SC-AD                              | no-cross reaction | no-cross reaction | positive reaction |

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*a* Described in detail elsewhere (9).

*b* Unpublished data (Y. Ikeda, K.O. Ikeda and K. Tanaka).

*c* These values were determined spectrophotometrically on the purified enzyme preparations. FAD might have been partially lost in the purification procedures. The FAD content per subunit of both enzymes in the native form is estimated to be 1 mol per subunit because activities of the final preparations were enhanced 1.5-2.5 times by the addition of exogenous FAD.

*d* Emission spectra were monitored with excitation of 450nm, and excitation spectra were taken with emission at 530nm.

*e* The activity of isovaleryl-CoA dehydrogenase was not inhibited by tiglyl-CoA.

*f* The activity of short chain acyl-CoA dehydrogenase was not inhibited by tiglyl-CoA.
branched chain amino acids, leucine, isoleucine, and valine, are first transaminated to the corresponding 2-oxo acids. These three 2-oxo analogs are then oxidatively decarboxylated to isovaleryl-CoA, S-2-methylbutyryl-CoA, and isobutyryl-CoA, respectively, by a single common enzyme, branched chain 2-oxo acid dehydrogenase. This enzyme is subject to inhibition by any of the three branched chain acyl-CoAs (24).

Thus, the branched chain 2-oxo acid dehydrogenase step has been considered to be the site for metabolic regulation which is common for the three branched chain amino acids. We have shown in the previous report that isovaleryl-CoA dehydrogenase is specifically dehydrogenated by isovaleryl-CoA dehydrogenase and this reaction is specifically inhibited by 3-methylcrotonyl-CoA (9). Isoleucy-CoA and S-2-methylbutyryl-CoA were dehydrogenated commonly by 2-methyl-branched chain acyl-CoA dehydrogenase, which is subject to the inhibition by tiglyl-CoA as shown in the present paper. Thus, after the 2-oxo acid decarboxylation, the metabolism of isoleucine and valine may be commonly regulated while the leucine metabolism is independently controlled.

The ability of this enzyme to dehydrogenate R-2-methylbutyryl-CoA may be of more than a theoretical interest. It has previously been shown that when experimental animals were given RS-2-methylbutyric acid labeled with stable isotopes, they excreted labeled 2-ethyl-3-hydroxypropanoic acid (2-ethylhydracrylic acid) in their urine (25–26). The results from detailed mass spectrometric analyses of the urinary metabolites indicated that ethylhydracrylic acid was further oxidized by a pathway (R-pathway) which is analogous to the valine pathway (25). It was hypothesized that R-2-methylbutyryl-CoA was dehydrogenated on the shorter acyl chain producing 2-ethylacrylyl-CoA which was then hydrated to 2-ethylhydracrylyl-CoA while S-2-methylbutyryl-CoA was dehydrogenated on the longer chain producing tiglyl-CoA (Fig. 7). The data presented in this report represent the first scientific evidence that the two enantiomers of 2-methylbutyryl-CoA are, in fact, stereospecifically dehydrogenated.

The properties of 2-methyl-branched chain acyl-CoA dehydrogenase are summarized in Table VI, along with those of isovaleryl-CoA- and short chain acyl-CoA dehydrogenases. These three enzymes are similar in molecular size, prosthetic group, and basic mode of enzyme reaction, but they differ significantly from each other in catalytic and immunological properties. The native molecular weight of 2-methyl-branched chain acyl-CoA dehydrogenase is 170,000 as determined by gel filtration (Fig. 3). Its molecular weight is slightly larger than that of short chain acyl-CoA dehydrogenase. The subunit molecular weight of 2-methyl-branched chain acyl-CoA dehydrogenase was 41,500 on SDS-PAGE in the presence and absence of 2-mercaptoethanol (Fig. 4). These data indicate that the enzyme consists of four equal size subunits as in the case of isovaleryl-CoA- and short chain acyl-CoA dehydrogenase and that the binding between subunits is not through a disulfide linkage. However, unlike these other two enzymes which readily dissociate into four subunits in SDS-PAGE, 2-methyl-branched chain acyl-CoA dehydrogenase gave a single protein band with $M_r = 85,000$ when analyzed without boiling the enzyme preparation (Fig. 4). This finding may suggest that the binding forces for four subunits are not equal and that the force between two subunits in a dimer is stronger than that which binds two dimers.

The absorption spectrum and fluorescence emission and excitation spectra of 2-methyl-branched chain acyl-CoA dehydrogenase are typical for FAD, indicating that this enzyme contains FAD as a prosthetic group. The FAD content was calculated to be 0.5 mol/subunit from the absorption spectrum. This FAD content is not a whole number, probably due to a partial loss of FAD in the purification process judging from the observation that the activity of the purified enzyme is enhanced 2.5-fold by the addition of 100 $\mu$M FAD. These results suggest that 2-methyl-branched chain acyl-CoA dehydrogenase originally contained 1 mol of FAD/mol of subunit in native form, as in the case of isovaleryl-CoA dehydrogenase (Table VI). In contrast, the activity of the purified short chain acyl-CoA dehydrogenase was not enhanced at all by the addition of FAD: its $A_{275}/A_{450}$ ratio in the absorption spectrum was 6.3, a typical value for an acyl-CoA dehydrogenase fully saturated with FAD (Table VI), indicating that the final short chain acyl-CoA dehydrogenase preparation contains 1 mol of FAD/mol of subunit. In catalytic properties, these three enzymes distinctly differ from each other. 2-Methyl-branched chain acyl-CoA dehydrogenase is specific for isovaleryl-CoA and S-2-methylbutyryl-CoA, isovaleryl-CoA dehydrogenase is for isovaleryl-CoA (9), and short chain acyl-CoA dehydrogenase is for n-butyryl-CoA and n-valeryl-CoA (8). There is essentially no cross-reactivity in these enzyme-substrate combinations except for n-valeryl-CoA, which is dehydrogenated by both short chain acyl-CoA and isovaleryl-CoA dehydrogenases. The high degree of substrate specificities of these three acyl-CoA dehydrogenases for short chain acyl-CoA inimic enzymes is indicative of finely defined information surrounding the active and substrate-binding sites of these enzyme. These three enzymes are also immunologically distinct from each other (Table VI) (9).

The activity of 2-methyl-branched chain acyl-CoA dehydrogenase for either substrate is inhibited by low concentrations of organic sulfhydryl reagents such as N-ethylmaleimide, $p$-hydroxymercurobenzoate, and methyl mercury iodide (Table V). The degrees of inhibition were essentially equal for the two substrates. The enzyme activity was severely inhibited by heavy metal ions such as $\text{Hg}^+$, $\text{Cu}^{++}$, and $\text{Ag}^+$ which are known to interact with sulfhydryl groups in proteins. These results suggest the existence of an essential cysteine residue at the active site. Similar inhibitory effects by organic sulfhydryl reagents have been observed by isovaleryl-CoA dehydrogenase (9) and apo-medium chain acyl-CoA dehydrogenase (22).

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