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Biochemistry

Isovaleryl-CoA dehydrogenase: Demonstration in rat liver mitochondria by ion exchange chromatography and isoelectric focusing

(leucine metabolism/isovaleric acidemia/acyl-CoA dehydrogenase/glutaryl-CoA dehydrogenase)

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ABSTRACT **There has been ambiguity concerning the specificity of the enzymes that dehydrogenate short branched-chain acyl-CoAs. It previously had been assumed that isovaleryl-CoA is dehydrogenated by *n*-butyryl-CoA dehydrogenase [butyryl-CoA:(acceptor) oxidoreductase, EC 1.3.99.2]. To solve this problem, we fractionated five short-chain acyl-CoA dehydrogenases (isovaleryl-CoA, *n*-butyryl-CoA, isobutyryl-CoA, *n*-octanoyl-CoA, and glutaryl-CoA dehydrogenases) from rat liver mitochondria by isoelectric focusing and DEAE-cellulose column chromatography. The isovaleryl-CoA dehydrogenase [isovaleryl-CoA:(acceptor) oxidoreductase, EC 1.3.99.10] peak was almost completely separated from the peaks of *n*-butyryl-CoA- and *n*-octanoyl-CoA dehydrogenases by isoelectric focusing, and it was well separated from glutaryl-CoA dehydrogenase [glutaryl-CoA:(acceptor) oxidoreductase (decarboxylating), EC 1.3.99.7] and *n*-octanoyl-CoA dehydrogenase by DEAE-cellulose column chromatography. The isovaleryl-CoA dehydrogenase peak partly overlapped that of *n*-butyryl-CoA and isobutyryl-CoA dehydrogenases in the latter procedure. These results unequivocally demonstrate that isovaleryl-CoA is oxidized by a specific isovaleryl-CoA dehydrogenase. The other dehydrogenase peaks also demonstrated activity toward a single substrate, except that isobutyryl-CoA dehydrogenase activity could not be clearly resolved from *n*-butyryl-CoA dehydrogenase activity.**

In the mid-1950s it was shown that there are three fatty acyl-CoA dehydrogenases that dehydrogenate straight-chain acyl-CoAs of different chain lengths. Butyryl-CoA dehydrogenase [butyryl-CoA:(acceptor) oxidoreductase, EC 1.3.99.2], which is also called short-chain acyl-CoA dehydrogenase or green acyl-CoA dehydrogenase (G), dehydrogenates C_4–C_8 fatty acyl-CoA (1, 2); another enzyme, general acyl-CoA dehydrogenase (Y_1) [acyl-CoA:(acceptor) oxidoreductase, EC 1.3.99.3] utilizes C_4–C_{16} fatty acyl-CoA derivatives as substrate (3); and the third one, long-chain acyl-CoA dehydrogenase (or Y_2), oxidizes C_6–C_{18} fatty acyl-CoA derivatives (4). The best substrates for these dehydrogenases are *n*-butyryl-CoA, *n*-octanoyl-CoA, and myristoyl-CoA, respectively. These three acyl-CoA dehydrogenases which oxidize straight chain acyl CoAs have been extensively characterized in the past 2 decades (5–8).

The enzymes that dehydrogenate the three short branched-chain acyl-CoAs, such as isobutyryl-CoA, isovaleryl-CoA, and 2-methylbutyryl-CoA, intermediates in the metabolism of valine, leucine, and isoleucine, respectively, have not been rigorously studied. Dehydrogenation of isovaleryl-CoA to 3-methylcrotonyl-CoA was considered to be analogous to that of butyryl-CoA to crotonyl-CoA by butyryl-CoA dehydrogenase. Bachhawat *et al.* (9) have shown that isovaleryl-CoA was dehydrogenated by dialyzed rat liver extract but it was not known whether this reaction was catalyzed by butyryl-CoA dehydrogenase or by a specific isovaleryl-CoA dehydrogenase [isovaleryl-CoA:(acceptor) oxidoreductase, EC 1.3.99.10].

Previous studies of isovaleric acidemia, an inborn error of leucine metabolism, suggested that isovaleryl-CoA may be dehydrogenated by a specific isovaleryl-CoA dehydrogenase and that isovaleric acidemia is due to a deficiency of this enzyme (10–12). Isovaleric acid and its glycine conjugate (13) accumulate in blood and urine in patients with this disease but the amounts of other short-chain fatty acids, such as *n*-butyric, isobutyric, 2-methylbutyric, and *n*-hexanoic acids, do not increase significantly in body fluids of these patients. However, the demonstration of isovaleryl-CoA dehydrogenase deficiency in homogenates of leukocytes or skin fibroblasts from patients with isovaleric acidemia was unsuccessful due to difficulties in assay of this enzyme. These difficulties were due in part to the insensitivity of the conventional dye-reduction assay methods and in part to a strong nonspecific reduction of the artificial electron acceptors utilized in this assay by an endogenous reducing substance (14).

Recently, Rhead and Tanaka (15, 16) developed a sensitive acyl-CoA dehydrogenase assay (tritium release assay) that utilized [2,3-^{3}H]acyl-CoA as substrate. With this method, acyl-CoA dehydrogenase activity can be measured by determining the rate of ^{3}H release from substrate in the presence of phenazine methosulfate as an electron acceptor. The ^{3}H transferred enzymatically from the labeled substrate to the acceptor is readily released to water by nonenzymatic exchange. Using this method as well as the dye-reduction assay, they demonstrated that the activity of mitochondrial isovaleryl-CoA dehydrogenase in isovaleric acidemia fibroblasts was only 13% of that of controls, whereas the activity of butyryl-CoA dehydrogenase was maintained at a normal level (16). This result gave further support for the hypothesis that isovaleryl-CoA is oxidized by a specific isovaleryl-CoA dehydrogenase and that patients with isovaleric acidemia are deficient in this enzyme activity. To date, isovaleryl-CoA dehydrogenase has not been identified in normal mammals enzymologically.

We report here results of fractionation of five acyl-CoA dehydrogenases from rat liver mitochondria by ion exchange column chromatography and isoelectric focusing. These results unequivocally demonstrate the existence of a specific isovaleryl-CoA dehydrogenase that is clearly separated from *n*-butyryl-CoA, isobutyryl-CoA, *n*-octanoyl-CoA, and glutaryl-

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CoA dehydrogenases [glutaryl-CoA:(acceptor) oxidoreductase (decarboxylating), EC 1.3.99.7].

MATERIALS AND METHODS

Materials. CoA lithium salt and unlabeled acyl-CoAs were procured from P-L Biochemicals or Sigma. DEAE-cellulose (Whatman DE-52) and carrier ampholyte (40%, wt/vol) were obtained from Whatman (Kent, England) and LKB (Stockholm, Sweden), respectively. [2,3-^{3}H]Isovaleric acid (10 mCi/mmol; 1 Ci = 3.7×10^{10} becquerels), [2,3-^{3}H]butyric acid (10 mCi/mol), and [2,3-^{3}H]glutaric acid (10 mCi/mmol) were synthesized by New England Nuclear via catalytic tritiation of 3-methylcrotonic, crotonic, and glutaconic acids, respectively. [1,5-^{14}C]Glutaric acid (17.9 mCi/mmol) was purchased from ICN (Irvine, CA). A thioesters of [2,3-^{3}H]isovaleric and [2,3-^{3}H]butyric acid were synthesized by the mixed anhydride method (17, 18). [2,3-^{3}H]Glutaryl- and [1,5-^{14}C]glutaryl-CoAs were synthesized via glutaric anhydride (19) according to the method of Simon and Shemin (20).

Preparation of Rat Liver Mitochondria and Solubilization of Acyl-CoA Dehydrogenases. Rat liver mitochondria were prepared by the method of Loewenstein *et al.* (21), with omission of digitonin treatment. A buffer containing 250 mM sucrose, 3.4 mM Tris·HCl, and 1 mM ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid at pH 7.4 was used throughout for homogenization, suspension, and washing of mitochondria. The final mitochondrial pellets were suspended in an appropriate buffer as indicated and were sonicated for 2 min in a Branson sonifier. The sonicated mitochondria were centrifuged at 34,800 × *g* for 40 min and the supernatant was used for enzyme fractionation.

Isoelectric Focusing. The mitochondria from one rat liver were suspended in 10 ml of 2.5 mM potassium phosphate at pH 7.2 and were sonicated as described above. The 34,800 × *g* supernatant was acidified to pH 5.0 with 1 M sodium acetate (pH 3.2). The precipitate was removed by centrifuging at 25,000 × *g* for 10 min. The yellow supernatant was neutralized with 1 M K_2HPO_4. The supernatant, containing 40–80 mg of protein, was used for isoelectric focusing. The density gradient containing 1% carrier ampholyte was prepared according to the method of Vesterberg (22) by using a LKB gradient mixer. A 110-ml column (LKB) was used. Focusing was carried out for 48 hr at 4°C, 1200 V, and 2–3 mA. The eluate was collected in 1-ml fractions and the pH of each fraction was measured at 4°C.

DEAE-Cellulose Chromatography. The mitochondrial pellets prepared from rat livers (about 30 g) were suspended in 20 ml of 5 mM potassium phosphate (pH 7.2) and sonicated as described above. The 34,800 × *g* supernatant was applied to a DEAE-cellulose column (2.2 × 20 cm) that was equilibrated with 5 mM potassium phosphate (pH 7.2). The column was washed with 120 ml of the loading buffer. The adsorbed proteins were eluted with 800 ml potassium phosphate buffer (pH 7.2) with a linear concentration gradient (5–150 mM).

Assay of Acyl-CoA Dehydrogenases. The tritium release assays for butyryl-CoA and isovaleryl-CoA dehydrogenases were done by using appropriate [2,3-^{3}H]acyl-CoAs as substrate according to the method of Rhead and Tanaka (16) with the following modifications: total volume of assay mixture, 120 μl; potassium phosphate at 83 mM and pH 7.5; phenazine methosulfate, 550 μM; FAD, 83 μM; [2,3-^{3}H]acyl-CoA, 83 μM. After incubation at 37°C for 30 min, the reaction was stopped by adding 10 μl of 0.5 M HCl, and an aliquot of the resulting mixture (100 μl) was applied to a small column packed with 0.5 ml of AG-1 anion exchange resin, acetate form (Bio-Rad), to trap the unreacted substrate. The reaction product, tritiated water, was eluted by washing the column with 1 ml of deionized water, and radioactivity in the eluate was measured by liquid scintillation using Biofluor (New England Nuclear).

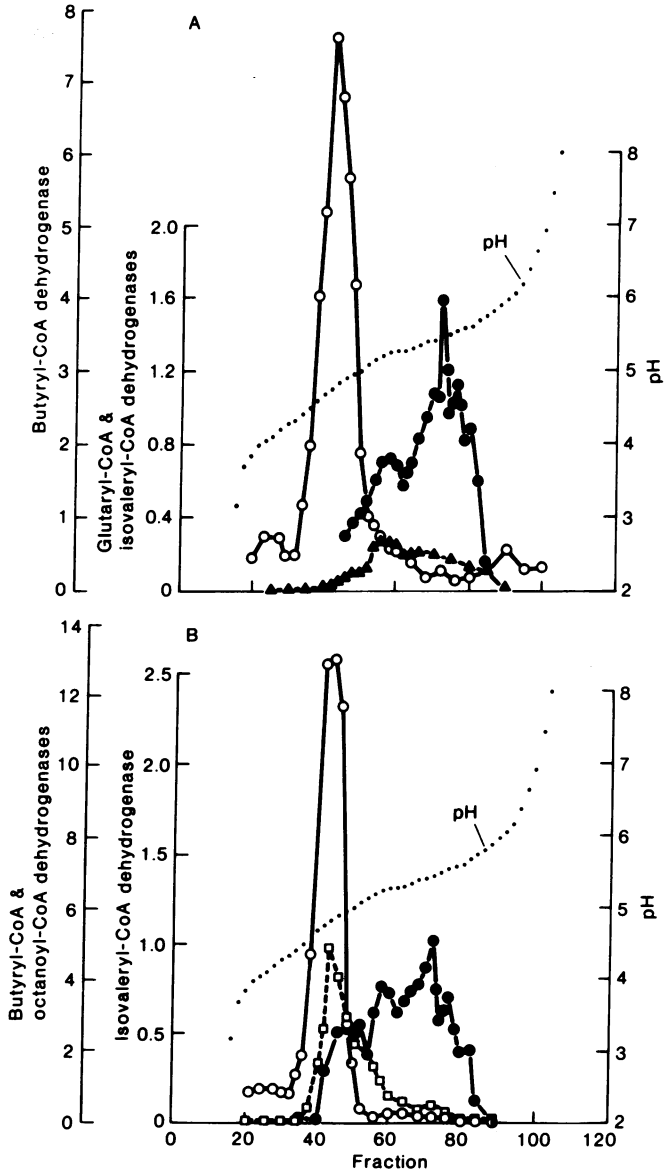

FIG. 1. Fractionation of acyl-CoA dehydrogenases from rat liver mitochondria by isoelectric focusing. (*A*) By tritium release assays; results are shown as cpm × 10^{-4}/30 min per 100 μl. (*B*) By dye-reduction assays; results are shown as $\Delta A_{600} \times 10^2$/min per 100 μl. ○, *n*-Butyryl-CoA dehydrogenase; ●, isovaleryl-CoA dehydrogenase; ▲, glutaryl-CoA dehydrogenase; □, *n*-octanoyl-CoA dehydrogenase.

The dye-reduction assays for isovaleryl-CoA, isobutyryl-CoA, *n*-butyryl-CoA, and *n*-octanoyl-CoA dehydrogenases were done spectrophotometrically by using phenazine methosulfate and 2,6-dichlorophenolindophenol as electron acceptors as described by Hall (14) with minor modifications.

Glutaryl-CoA dehydrogenase activity was measured by a tritium release assay using [2,3-^{3}H]glutaryl-CoA[§] in a manner similar to that for isovaleryl-CoA and butyryl-CoA dehydrogenases as described above. The overall activity of two sequential reactions involving glutaryl-CoA—namely, glutaryl-CoA dehydrogenation and glutaconyl-CoA decarboxyl-

[§] This is, in fact, a mixture of [2,3-^{3}H]- and [3,4-^{3}H]glutaryl-CoA.

ation—was assayed by quantitation of $^{14}CO_2$ released from [1,5-^{14}C]glutaryl-CoA according to the method of Besrat *et al.* (23) with minor modifications.

RESULTS

Fractionation of Mitochondrial Acyl-CoA Dehydrogenases by Isoelectric Focusing. In a preliminary experiment, we electrofocused the supernatant of rat liver mitochondrial sonicate by using a carrier empholyte with a wide pH range (3–10). Dehydrogenases for isovaleryl-CoA, *n*-butyryl-CoA, and *n*-octanoyl-CoA were all detected in fractions within a pH range of 4–6 (data not shown). Therefore, further analyses were carried out using a carrier ampholyte with this narrower pH range and acyl-CoA dehydrogenases were assayed by the tritium release assay. Isovaleryl-CoA and *n*-butyryl-CoA dehydrogenases were well separated although the activity of the former was detected over a relatively wide pH range. The highest activity of isovaleryl-CoA dehydrogenase was found at pH 5.4–5.5 whereas butyryl-CoA was at 4.7–4.8 (Fig. 1*A*). The isovaleryl-CoA dehydrogenase and butyryl-CoA dehydrogenase peaks were further verified by the conventional dye-reduction assays (Fig. 1*B*). The major activity of octanoyl-CoA dehydrogenase was detected in the fractions that also exhibited butyryl-CoA dehydrogenase activity but a small shoulder of octanoyl-CoA dehydrogenase activity was detected on the descending slope in the pH 5.1–5.2 region. Glutaryl-CoA dehydrogenase activity mostly precipitated with the acid treatment prior to isoelectric focusing. A low residual activity of this enzyme was detected at pH 5.2 by the tritium release assay and the $^{14}CO_2$ release assay (data not shown).

The yield of each enzyme from isoelectric focusing differed from experiment to experiment depending on the power used and also on the length of time of focusing. In general, isovaleryl-CoA and octanoyl-CoA dehydrogenases were more labile than butyryl-CoA dehydrogenase; in a typical experiment, in which focusing was carried out with 2–3 W for 48 hr, 34% of the butyryl-CoA dehydrogenase activity was recovered whereas 19 and 23% of isovaleryl-CoA and octanoyl-CoA dehydrogenase activities were recovered, respectively, as assayed by the dye-reduction assays.

Fractionation of Mitochondrial Acyl-CoA Dehydrogenases by DEAE-Cellulose Chromatography. Rhead and Tanaka reported (15) briefly that isovaleryl-CoA dehydrogenase could be partially separated from butyryl-CoA dehydrogenase by DEAE-cellulose chromatography as monitored by the tritium release assays. We have expanded this study. An elution pattern of four acyl-CoA dehydrogenases (isovaleryl-CoA, *n*-butyryl-CoA, *n*-octanoyl-CoA, and glutaryl-CoA dehydrogenases) from a DEAE-cellulose column is shown in Fig. 2. A KP_i buffer (pH 7.2) with a concentration gradient from 5 to 150 mM was used for elution. All of the activities were assayed by the tritium release method except that octanoyl-CoA dehydrogenase was assayed by the dye-reduction method. Isovaleryl-CoA dehydrogenase eluted toward the end of the gradient and was closely preceded by the peaks of octanoyl-CoA dehydrogenase and butyryl-CoA dehydrogenase. The butyryl-CoA dehydrogenase peak partly overlapped with octanoyl-CoA dehydrogenase in the front and isovaleryl-CoA dehydrogenase in its descending slope. The isovaleryl-CoA dehydrogenase peak was completely separated from that of octanoyl-CoA dehydrogenase. Glutaryl-CoA dehydrogenase was eluted at a low potassium phosphate concentration and was widely separated from the butyryl-CoA, octanoyl-CoA, and isovaleryl-CoA dehydrogenase peaks. The activities of two sequential reactions of glutaryl CoA—namely, glutaryl-CoA dehydrogenation (tritium release) and glutaconyl-CoA decarboxylation ($^{14}CO_2$

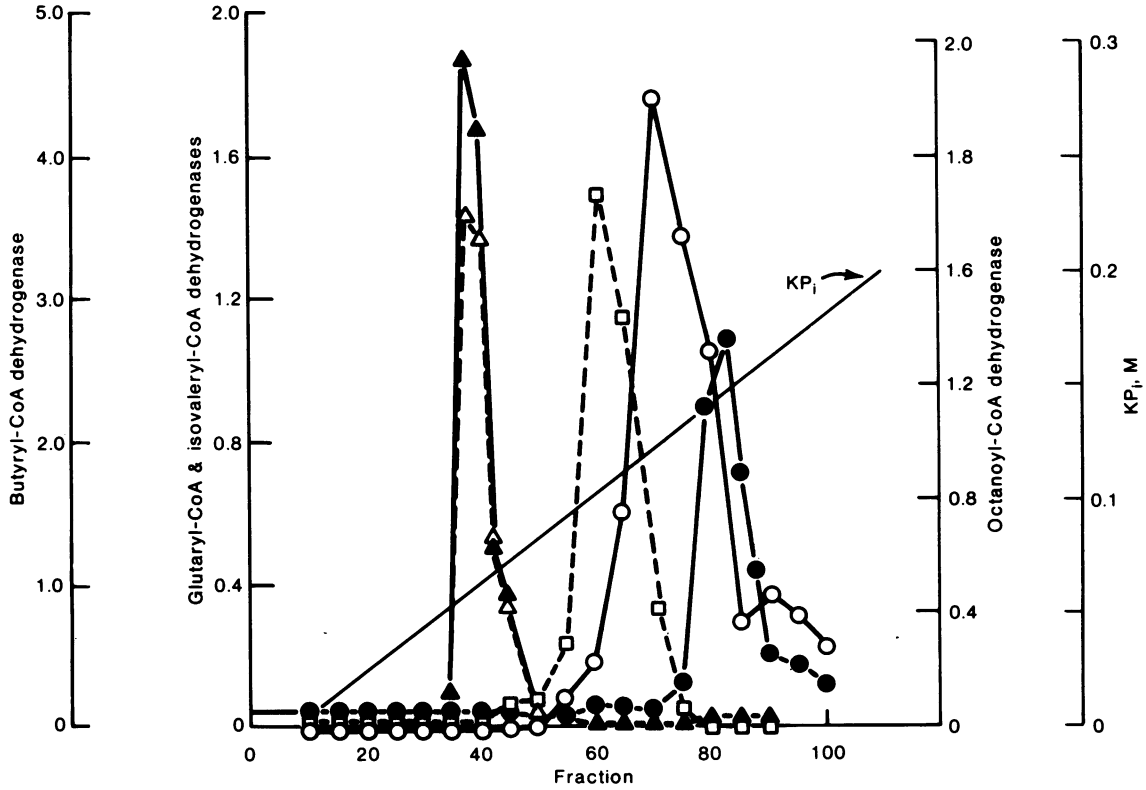

FIG. 2. Fractionation of acyl-CoA dehydrogenases from rat liver mitochondria by DEAE-cellulose column chromatography as assayed by tritium release assays; results are shown as cpm × 10^{-4}/30 min per 100 μl. ○, *n*-Butyryl-CoA dehydrogenase; ●, isovaleryl-CoA dehydrogenase; ▲, glutaryl-CoA dehydrogenase (by ^{3}H release); △, glutaryl-CoA dehydrogenase (by $^{14}CO_2$ release); □, *n*-octanoyl-CoA dehydrogenase (by dye-reduction assay; results are $\Delta A_{600} \times 10^2$/min per 100 μl).

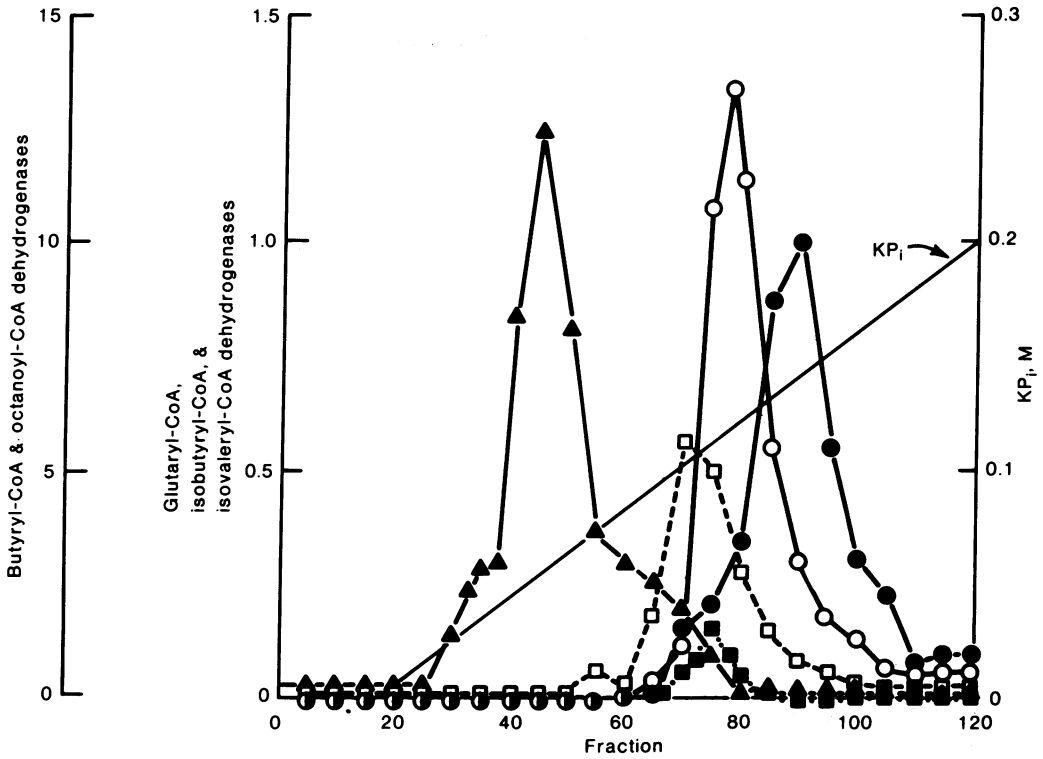

FIG. 3. Fractionation of acyl-CoA dehydrogenases from rat liver mitochondria by DEAE-cellulose column chromatography as assayed by dye reduction assays; results are shown as $\Delta A_{600} \times 10^2$/min per 100 μl. ○, *n*-Butyryl-CoA dehydrogenase; ■, isobutyryl-CoA dehydrogenase; ●, isovaleryl-CoA dehydrogenase; ▲, glutaryl-CoA dehydrogenase; □, *n*-octanoyl-CoA dehydrogenase.

release)—were not separable by DEAE-cellulose chromatography.

In a separate run, five acyl-CoA dehydrogenases including the four enzymes mentioned above plus isobutyryl-CoA dehydrogenase were assayed by the dye reduction assay (Fig. 3). The separation of isovaleryl-CoA dehydrogenase from the other enzymes was confirmed by this method. Isobutyryl-CoA dehydrogenase activity was barely detectable between the peaks of *n*-butyryl-CoA and octanoyl-CoA dehydrogenases. The tip of the isobutyryl-CoA dehydrogenase peak was consistently a few tubes ahead of that of *n*-butyryl-CoA dehydrogenase, possibly indicating a heterogeneity of these two enzymes although the separation of these two peaks was small.

DISCUSSION

Dehydrogenation of branched-chain acyl-CoAs, such as isovaleryl-CoA, 2-methylbutyryl-CoA, and isobutyryl-CoA, has not been extensively studied. Identity of enzymes that catalyze dehydrogenation of the branched-chain acyl-CoAs has become an important subject since isovaleric acidemia was identified in 1966 (10–12). Subsequently, glutaric aciduria (24), glutaric aciduria type II (25), and ethylmalonic-adipic aciduria (26) have been found. In patients with isovaleric acidemia or glutaric aciduria, dehydrogenation of a single substrate appeared to be deficient whereas in patients with glutaric aciduria type II or ethylmalonic-adipic aciduria, dehydrogenation of multiple acyl-CoA derivatives was inhibited.[¶] Thus, it is important to elucidate whether dehydrogenation of the short-chain acyl-CoAs, straight or branched, is catalyzed by a single common enzyme or by multiple specific enzymes. The results of the studies on the latter two diseases are consistent with a deficiency of a factor in the electron transport chain linking acyl-CoA dehydrogenases to coenzyme Q (27).

When butyryl-CoA dehydrogenase was first isolated from bovine liver, Crane *et al.* (3) observed that this enzyme also catalyzed dehydrogenation of isobutyryl-CoA. It was not certain, however, whether isobutyryl-CoA was dehydrogenated by butyryl CoA dehydrogenase or by a specific isobutyryl-CoA dehydrogenase that coexisted in the preparation. Beinert (28) also mentioned briefly that isobutyryl-CoA, isovaleryl-CoA, and 2-methylbutyryl-CoA may be oxidized by butyryl-CoA dehydrogenase purified from beef heart, but no experimental detail was given. More recently, Osmundsen *et al.* (29) measured activities of butyryl-CoA and isovaleryl-CoA dehydrogenases in four different extracts of a rat liver mitochondrial preparation and observed widely differing ratios of these two activities. They suggested that these two reactions were catalyzed by two separate enzymes. However, it was still a possibility that their observation may be due to a different degree of inactivation of the two dehydrogenases by different extraction procedures. Their attempts to purify isovaleryl-CoA dehydrogenase further failed because of the loss of activity during ion exchange or gel chromatography.

We present here unequivocal evidence for a specific isovaleryl-CoA dehydrogenase that is distinct from butyryl-CoA, isobutyryl-CoA, general acyl-CoA, and glutaryl-CoA dehydrogenases. This enzyme did not catalyze oxidation of butyryl-CoA, isobutyryl-CoA, octanoyl-CoA, or glutaryl-CoA to a significant degree, judging from the elution profiles. Our results also show that the other four acyl-CoA dehydrogenases studied here did not dehydrogenate isovaleryl-CoA, indicating strict substrate specificity of these acyl-CoA dehydrogenases. It is interesting to note that, recently, Hall[‖] briefly described the

[¶] Because these four diseases are due to a genetic deficiency of activity of different acyl-CoA dehydrogenases, they may be classified as "human acyl-CoA dehydrogenase mutant diseases."

[‖] Hall, C., in *Abstracts of the Eleventh International Congress of Biochemistry* (Toronto, 1979), p. 434.

isolation of an ETF-linked flavoprotein dehydrogenase from pig liver mitochondria. This was 75–80% pure as judged by disc polyacrylamide gel electrophoresis. It was equally active with isovaleryl-CoA and octanoyl-CoA as substrate but showed only 10% of that activity when *n*-butyryl-CoA was used. This was in sharp contrast to the findings on general acyl-CoA dehydrogenase which exhibited 22 times higher activity with octanoyl-CoA than with isovaleryl-CoA.

The results of the present study, together with our recent demonstration of deficient isovaleryl-CoA dehydrogenase and normal butyryl-CoA dehydrogenase activities in isovaleric acidemia cells, verify our previous hypothesis that isovaleric acidemia is due to a genetic alteration of a specific isovaleryl-CoA dehydrogenase (10, 13). These observations completely rule out the possibility, proposed by Engel (30), that butyryl-CoA dehydrogenase also catalyzes isovaleryl-CoA dehydrogenation and that the metabolic derangement in isovaleric acidemia may be explained by a point mutation that rendered butyryl-CoA dehydrogenase incapable of oxidizing isovaleryl-CoA without affecting its activity toward its other substrate(s).

The isovaleryl-CoA dehydrogenase peak in isoelectric focusing was always broad although it was well separated from the other acyl-CoA dehydrogenases. A shoulder consistently was found on its ascending slope at pH 5.2. At present it is not known whether this peak shape indicates the presence of isozymes of isovaleryl-CoA dehydrogenase or resulted from an interference by precipitated proteins. Partial alteration of enzyme during isoelectric focusing is also a possibility.

In the present study, 2-methylbutyryl-CoA dehydrogenase was not assayed because there are two enantiomers of 2-methylbutyrate. It has recently been shown that *S* and *R* enantiomers of 2-methylbutyrate-CoA are metabolized by two different pathways (31, 32), and it is likely that *S*- and *R*-2-methylbutyryl-CoA may be dehydrogenated by two different dehydrogenases. For instance, isobutyryl-CoA dehydrogenation occurs stereospecifically by subtraction of a proton each from (2-proS)methyl and α-methine groups, respectively (33). We judged that resolution of the *S*- and *R*-enantiomers of 2-methylbutyrate and characterization of the dehydrogenases for the CoA ester of each enantiomer were beyond the scope of the present study.

We would like to point out that the characterization of isovaleryl-CoA dehydrogenase and other acyl-CoA dehydrogenases by isoelectric focusing and ion exchange chromatography in the present study were greatly facilitated by the use of sensitive tritium release assays. Traditionally, the bands of the different acyl-CoA dehydrogenases have been separated visually during isolation on the basis of the yellow-green color due to enzyme-bound flavin (14). The activities present in each band were then assayed using different acyl-CoAs as substrates because the conventional dye reduction assays are time-consuming (14). Therefore, when a purified enzyme fraction catalyzed oxidation of more than one substrate, there always remained uncertainty as to whether more than one acyl-CoA dehydrogenase was present in the purified fraction or there was only a single enzyme that oxidized multiple substrates. The simplicity and the high sensitivity of the tritium release assays made it possible to carry out numerous assays of serial fractions from column chromatography or electrophoresis with high accuracy to follow delicate separations of closely related acyl-CoA dehydrogenases. The further characterization and confirmation of the identity of the dehydrogenase thus separated was accomplished by the dye reduction assay.

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