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# Two Mitochondrial 3-Hydroxyacyl-CoA Dehydrogenases in Bovine Liver[1]

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**3-Hydroxyacyl-CoA dehydrogenase catalyzes the third reaction of fatty acid β-oxidation spiral. There are three enzymes catalyzing the 3-hydroxyacyl-CoA dehydrogenase reaction: mitochondrial monofunctional 3-hydroxyacly-CoA dehydrogenase, mitochondrial enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase/3-ketoacyl-CoA thiolase trifunctional protein, and peroxisomal enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase bifunctional protein. The presence of isozymes of monofunctional 3-hydroxyacyl-CoA dehydrogenase was not known. In the present study, two monofunctional mitochondrial 3-hydroxyacyl-CoA dehydrogenases were purified from bovine liver. Type I enzyme was composed of two identical subunits with molecular mass of 35 kDa, and type II enzyme was a homotetramer of a 28 kDa polypeptide. In respect to the molecular structures, immunochemical properties, and carbon chain length specificities of acyl-CoA substrates, type I enzyme was the same as the well-known classical enzyme purified from various tissues, but type II enzyme was concluded to be a new enzyme. Type I enzyme was ubiquitous, but type II enzyme was rich in bovine and sheep, of several animal livers so far examined.**

**Key words: bovine, 3-hydroxyacyl-CoA dehydrogenase, isozyme, mitochondria.**

There are two fatty acid β-oxidation systems consisting of different enzymes in animal cells: one is located in mitochondria and the other in peroxisomes. There are several enzymes involved in the third reaction of the β-oxidation spiral, that is, 3-hydroxyacyl-CoA dehydrogenase reaction. 3-Hydroxyacyl-CoA dehydrogenase (HADH) [(*S*)-3-hydroxyacyl-CoA:$NAD^+$ oxidoreductase] [EC 1.1.1.35] (*1*) and enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase/3-ketoacyl-CoA thiolase trifunctional protein (*2*) are located in mitochondria, and enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase bifunctional protein (*3*) is in peroxisomes.

In a survey of human liver HADH, we noticed separate activity peaks different from known multifunctional enzymes by phosphocellulose column chromatography. This finding prompted us to study whether isozymes of HADH are present. We happened to use bovine liver for this purpose, and purified two separate HADHs having different carbon chain length specificities. Both enzymes were present in mitochondria. One of these enzymes was confirmed to be the same enzyme as the well-characterized classical one, but the other was concluded to be a new enzyme.

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Abbreviations: HADH, 3-hydroxyacyl-CoA dehydrogenase; SDS-PAGE, SDS-polyacrylamide gel electrophoresis.

## MATERIALS AND METHODS

*Materials*—Phosphocellulose was P11 obtained from Whatman (Maidstone, England). DEAE-cellulose was from Serva (Heidelberg, Germany). Sephadex G-25, G-100, and G-150 were from Pharmacia LKB Biotechnology AB (Uppsala, Sweden). AF-Blue Toyopearl 650 was from Tosoh (Tokyo). Calcium phosphate gel/cellulose was prepared according to the method of Koike and Hamada (*4*). Acyl-CoA oxidase from *Arthrobacter* sp. was obtained from Toyobo (Osaka). Fatty acid oxidation multienzyme complex purified from *Pseudomonas fragi* (*5*) was a kind gift from Nippon Shoji Kaisha (Osaka). Proteins and enzymes were purchased from Boehringer (Mannheim, Germany), Bio-Rad Laboratories (Richmond, CA), and Sigma (St. Louis, MO). Pyridine nucleotide coenzymes and coenzyme A were obtained from Kyowa Hakko (Tokyo).

All other chemicals were of analytical grade.

*Preparation of Substrates*—Crotonyl-CoA was prepared by the method of Steinman and Hill (*6*). Other enoyl-CoAs were synthesized by the mixed anhydride method (*7*). 2-Tetradecenoic acid was not commercially available. Therefore, tetradecanoyl-CoA was synthesized by the mixed anhydride method, and then converted to tetradecenoyl-CoA by the acyl-CoA oxidase reaction (*5*). In this reaction, $O_2$ was supplied by addition of $H_2O_2$ and catalase instead of air bubbling. The enoyl-CoA esters were purified by DEAE-cellulose column chromatography with a linear gradient elution from 0 to 0.3 M NaCl in 5 mM HCl. The CoA esters with carbon chain lengths of 12 and more were chromatographed with the same system except that solu-

tions were made with a mixture of water/ethanol/1-butanol (1 : 1.5 : 0.4) instead of water. Enoyl-CoAs were determined by the method described below. The fractions containing enoyl-CoAs eluted with aqueous solution were pooled, adjusted to pH 5.5-6.0 and stored at −20°C. The fractions containing dodecenoyl-CoA were pooled, adjusted to pH 5.5-6.0, and evaporated to dryness in a vacuum. The dried material was dissolved in water and stored at −20°C. The fractions containing CoA esters with longer carbon chain length were diluted 3-fold with water, then the CoA esters were precipitated by addition of $HClO_4$ to give final concentrations of 1% (w/v) or more. The precipitates were washed with 1% (w/v) $HClO_4$, dissolved in water, and stored at −20°C after pH of the solutions had been adjusted to 5.5-6.0.

Acetoacetyl-CoA was synthesized from diketene and CoA (*8*). Enoyl-CoAs were converted to the corresponding 3-ketoacyl-CoAs using *P. fragi* multienzyme complex and rabbit muscle lactate dehydrogenase according to the method of Imamura *et al.* (*5*), except that a lower concentration of Tris-Cl buffer and a higher concentration of pyruvate were used. When shorter carbon chain compounds were converted, bovine serum albumin was omitted. Conversion of enoyl-CoA to 3-ketoacyl-CoA was quantified by the method described below. After the reaction was completed, the mixture was directly applied onto a DEAE-cellulose column for purification of short- and medium-chain 3-ketoacyl-CoAs. For purification of longer chain 3-ketoacyl-CoAs, $HClO_4$ was added to the reaction mixture to give a pH of about 1. The precipitate was washed once with water, suspended in water, and dissolved by addition of Trisma base solution. Then the CoA esters were purified by DEAE-cellulose column chromatography.

L-(+)-3-Hydroxyacyl-CoAs were prepared from 3-ketoacyl-CoAs by the method of Imamura *et al.* (*5*) using bovine liver type I HADH. D-(−)-3-Hydroxyoctanoyl-CoA was synthesized by the mixed anhydride method (*7*) with D-(−)-3-hydroxyoctanoic acid, which was separated from the racemic mixture, DL-3-hydroxyoctanoic acid, by repeating crystallization with *S*-α-phenylethylamine according to a procedure used by Stoffel *et al.* (*9*). These 3-hydroxyacyl-CoAs were purified by the same procedures as the corresponding 3-ketoacyl-CoAs.

Acyl-CoA derivatives were determined by the following methods. CoA derivatives were determined with the use of 5,5′-dithiobis(2-nitrobenzoate) after hydrolysis by acyl-CoA hydrolase (*10*). Fractions containing enoyl-CoAs were screened by the use of crotonase, which was purified from bovine liver (*6*). Enoyl-CoA preparations were determined by conversion to the corresponding 3-ketoacyl-CoA. The reaction mixture contained 0.1 M Tris-HCl, pH 8.3, 25 mM $MgCl_2$, 50 mM KCl, 0.1% Tween 20, 0.1 mM $NAD^+$, 1 mM pyruvate, 1 unit of *P. fragi* multienzyme complex, and 1 unit of lactate dehydrogenase. The reaction was followed by measurement of increase in absorbance at 303 nm. The basic reaction mixture was the one used for determination of 3-ketoacyl-CoA thiolase (*11*). 3-Hydroxyacyl-CoAs were determined as described for the determination of enoyl-CoAs. 3-Ketoacyl-CoAs were determined by the HADH reaction (*1*).

Synthesized acyl-CoAs contained some impurities, but the purities of the final preparations after DEAE-cellulose column chromatography were about 90%.

*Enzyme Assay*—The HADH activity in the reverse reaction was assayed by 3-ketoacyl-CoA-dependent NADH oxidation in a mixture of 50 mM potassium phosphate, pH 7.5/0.1 mM NADH/20 μM 3-ketoacyl-CoA (*1*). The activity in the forward reaction was assayed by 3-hydroxyacyl-CoA-dependent $NAD^+$ reduction in 0.1 M Tris-0.1 M KCl, pH 10/1 mM $NAD^+$/40 μM 3-hydroxyacyl-CoA (*1*). The reaction was carried out at 30°C. One unit of the enzyme was defined as the amount of the enzyme which catalyzed the oxidation of 1 μmol of NADH or the reduction of 1 μmol of $NAD^+$ per min.

*Purification of HADH Isozymes from Bovine Liver*—The enzyme activity was assayed in the reverse reaction with acetoacetyl-CoA and 3-ketooctanoyl-CoA during purification, and separation of two types of the enzyme was confirmed by ratios of the activities with these two substrates.

*Extraction of the enzymes:* Frozen bovine liver (100 g) was homogenized with 400 ml of 10 mM $K_3PO_4$/1 mM benzamidine hydrochloride/1 mM phenylmethylsulfonyl fluoride/1 mM EDTA/2 mM 2-mercaptoethanol. The latter two reagents were included in all buffers and water used for dilution of the enzyme fractions, and all procedures were carried out at 4°C. The homogenate was centrifuged at 100,000×*g* for 1 h.

*Phosphocellulose column chromatography:* The supernatant was applied onto a phosphocellulose column (70 ml, equilibrated with 20 mM potassium phosphate, pH 7.5). The column was washed with a column volume of 50 mM potassium phosphate, pH 7.5, and the enzyme was eluted with a linear gradient system composed of 200 ml each of 50 mM and 250 mM potassium phosphate, pH 7.5.

*Calcium phosphate gel/cellulose column chromatography:* Active fractions were pooled and diluted 4-fold with water, and applied onto a calcium phosphate gel/cellulose column (40 ml). The column was washed with 40 ml of 50 mM potassium phosphate, pH 7.5. The enzymes were eluted with a linear gradient system composed of 100 ml each of 50 mM and 300 mM potassium phosphate, pH 7.5.

*Separation of type I and type II enzymes:* Separation of type I and type II enzymes was attempted by ammonium sulfate fractionation, because these two enzymes were poorly separated by various column chromatographic procedures. Solid ammonium sulfate (200 g per liter) was added to the enzyme solution. After centrifugation, solid ammonium sulfate (400 g/liter of the original volume) was added to the supernatant. The precipitate was collected by centrifugation and sequentially extracted with 5 ml each of 50 mM potassium phosphate, pH 7.5 containing the following concentrations of ammonium sulfate: 2.6, 2.5, 2.4, 2.3, 2.2, 2.1, 2.0, 1.9, and 1.4 M. The ratio of the activity with acetoacetyl-CoA to that with 3-ketooctanoyl-CoA was determined for each extract. Fractions with higher ratios than 1.9 were pooled as crude type I enzyme fraction and those with lower ratios were pooled as crude fraction of type II enzyme. Both fractions were treated with solid ammonium sulfate to precipitate the enzymes, and the precipitates were dissolved in a minimal volume of 100 mM potassium phosphate, pH 7.5. Type II enzyme was purified first, by the following procedures, because this enzyme was relatively labile under the conditions.

*Purification of type II enzyme:* The enzyme preparation was desalted with the use of Sephadex G-25 equilibrated

with 5 mM potassium phosphate, pH 8.0, and then passed through a DEAE-cellulose column (7.5 ml), which had been equilibrated with 5 mM potassium phosphate, pH 8.0. The percolated fractions were applied onto a column of AF-Blue Toyopearl 650 (2.5 ml). The column was washed with 5 ml of 20 mM potassium phosphate, pH 7.5, and then eluted with a linear gradient from 20 to 150 mM potassium phosphate, pH 7.5 in a total volume of 25 ml. Type I enzyme was eluted in the front part of a relatively broad activity peak, and type II enzyme was recovered in the latter part. Type II enzyme fractions containing a small amount of type I enzyme were diluted 4-fold with water and applied onto a column of AF-Blue Toyopearl 650 (1 ml). After the column had been washed with 2 ml of 40 mM potassium phosphate, pH 7.5, the enzyme was eluted with the same buffer containing 1 mM NADH. The enzyme fractions were directly applied onto a calcium phosphate gel/cellulose column (2.5 ml, equilibrated with 20 mM potassium phosphate, pH 7.5), and eluted with a linear gradient elution system composed of 12 ml each of 50 and 300 mM potassium phosphate, pH 7.5. Type II enzyme was eluted as the main activity peak, but a small amount of type I enzyme was found in the tail of the peak. Type II enzyme fractions that were free of type I enzyme were confirmed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

*Purification of type I enzyme:* Type I enzyme fraction concentrated by ammonium sulfate precipitation after the sequential extraction with ammonium sulfate solutions was stored overnight at 4°C. Type I enzyme was stable but type II enzyme lost most of its activity during storage. Type I enzyme was desalted with Sephadex G-25, then passed through a DEAE-cellulose column as described for purification of type II enzyme. The enzyme fraction was applied onto a column of AF-Blue Toyopearl 650 (2 ml). The column was washed with 6 ml of 10 mM potassium phosphate, pH 7.5, and eluted with a linear gradient from 10 to 100 mM potassium phosphate, pH 7.5 in a total volume of 20 ml. The enzyme fraction was diluted 4-fold with water and applied onto a column of AF-Blue Toyopearl 650 (1 ml). After the column had been washed with 2 ml of 10 mM potassium phosphate, pH 7.5, the enzyme was eluted with the same buffer containing 1 mM NADH. The active fractions were pooled and applied onto a phosphocellulose column (2 ml, equilibrated with 20 mM potassium phosphate, pH 7.5), and eluted with a linear gradient elution system composed of 10 ml each of 20 and 200 mM potassium phosphate, pH 7.5. Purity of type I enzyme was checked by SDS-PAGE.

*Purification of Bovine Heart Type I Enzyme*—Heart type I enzyme was purified by similar procedures for purification of liver type I enzyme. The sizes of all columns used for chromatography were reduced to about 2/3. The enzyme collected by precipitation with ammonium sulfate after calcium phosphate gel/cellulose column chromatography was desalted and subjected to DEAE-cellulose column chromatography without sequential extraction of the enzyme with ammonium sulfate solutions. Subsequent procedures were the same as those described for purification of liver type I enzyme.

*Preparation of Antibodies*—Antibodies against rat liver enzyme and bovine liver type II enzyme were raised in rabbits, and crude IgG fractions were obtained by ammonium sulfate precipitation as described previously (*1*).

*Other Methods*—Protein concentration was determined with the use of Coomassie Protein Assay Reagent (Pierce, Rockford, IL). SDS-PAGE was performed according to the method of Laemmli (*12*). Immunoblot analysis was done according to the method of Towbin *et al.* (*13*).

## RESULTS

*Purification of the Enzymes*—Tables I and II summarize typical results of purification of the enzymes from bovine liver and heart. Two distinct HADHs with different carbon chain length specificities were isolated from bovine liver. The classical enzyme was named type I enzyme, and the other enzyme was named type II enzyme. Bovine heart contained both type I and type II enzymes, but the content of type II enzyme was lower. Therefore, only type I enzyme was purified.

The activities of crotonase (*3*) and 3-ketoacyl-CoA thiolase (*14*) of these purified enzyme preparations were measured by the cited procedures with crotonyl-CoA and acetoacetyl-CoA, respectively. These activities were less than 0.07% of the HADH activity of the reverse reaction

TABLE I. **Purification of bovine type I and type II HADHs.**[a]

| Step | Total activity[b] (units) | Total protein (mg) | Specific activity (units/mg) | Activity ratio[c] |
|---|---|---|---|---|
| Extract | 7,650 | 13,500 | 0.57 | 1.7 |
| Phosphocellulose | 5,540 | 270 | 21 | 2.0 |
| Calcium phosphate gel/cellulose | 4,520 | 138 | 33 | 1.6 |
| Type I HADH | | | | |
| Ammonium sulfate | 2,520 | 32.4 | 78 | 5.0 |
| DEAE-cellulose | 1,520 | 11.3 | 135 | 10 |
| 1st Blue Toyopearl | 1,280 | 6.2 | 206 | 20 |
| 2nd Blue Toyopearl | 876 | 4.4 | 199 | 21 |
| 2nd Phosphocellulose | 679 | 3.1 | 219 | 21 |
| Type II HADH | | | | |
| Ammonium sulfate | 927 | 42.5 | 22 | 1.2 |
| DEAE-cellulose | 848 | 25.6 | 33 | 1.2 |
| 1st Blue Toyopearl | 622 | 8.6 | 72 | 1.1 |
| 2nd Blue Toyopearl | 591 | 3.6 | 164 | 1.3 |
| 2nd Calcium phosphate gel/cellulose | 298 | 1.9 | 157 | 1.1 |

[a]The enzymes were purified from 100 g of bovine liver. [b]The values are activities determined by the reverse reaction with acetoacetyl-CoA under the standard conditions. [c]The values are ratios of the activities with acetoacetyl-CoA to those with 3-ketooctanoyl-CoA.

TABLE II. **Purification of bovine heart type I HADH.**[a]

| Step | Total activity[b] (units) | Total protein (mg) | Specific activity (units/mg) | Activity ratio[c] |
|---|---|---|---|---|
| Extract | 2,200 | 4,590 | 0.48 | 8.0 |
| Phosphocellulose | 2,000 | 105 | 19 | 14 |
| Calcium phosphate gel/cellulose | 1,540 | 19.2 | 80 | 16 |
| Ammonium sulfate | 1,400 | 14.0 | 100 | 18 |
| DEAE-cellulose | 1,050 | 7.5 | 140 | 19 |
| 1st Blue Toyopearl | 754 | 5.0 | 151 | 19 |
| 2nd Blue Toyopearl | 500 | 3.1 | 161 | 19 |
| 2nd Phosphocellulose | 440 | 2.6 | 169 | 18 |

[a]The enzyme was purified from 100 g of heart. [b]The values are activities by the reverse reaction with acetoacetyl-CoA under the standard assay conditions. [c]The values are ratios of the activities with acetoacetyl-CoA to those with 3-ketooctanoyl-CoA.

with acetoacetyl-CoA. Immunoblot signals for other mitochondrial and peroxisomal fatty acid $\beta$-oxidation enzymes were not observed for the purified HADH preparations even when relatively large amounts of samples were used (data not shown). The data suggest that the both preparations are practically free from not only crotonase and monofunctional 3-ketoacyl-CoA thiolases but also other enzymes involved in the $\beta$-oxidation spiral, including peroxisomal bifunctional protein and mitochondrial trifunctional protein. The data also suggest that type I and type II enzymes are monofunctional.

These final preparations were dialyzed against 50% (v/v) glycerol/100 mM potassium phosphate, pH 7.5/2 mM 2-mercaptoethanol/1 mM EDTA, and could be stored at −20°C without detectable loss of activity for several months.

The purified preparations of heart type I, and liver type I and type II enzymes each gave one polypeptide band by SDS-PAGE (Fig. 1, lanes 1, 2, and 4). The preparation of lane 3 was purified by a slightly different procedure from that described under "MATERIALS AND METHODS." The liver extract was acidified to give a pH of 5.5 to remove a large amount of proteins. By this treatment, sequential extraction with ammonium sulfate solutions was omitted, because type I and type II enzymes were efficiently separated by AF-Blue Toyopearl 650 column chromatography. It was confirmed that doublet polypeptide bands were also found for the liver type I enzyme preparation if the pH of the extract was about 7 or lower, even with addition of protease inhibitors to the extraction buffer as described. Proteolytic modification of liver type II and heart type I enzymes was not detected by lowering the pH of the extract.

*Molecular Properties*—The molecular masses of the subunits of liver type I and type II enzymes were estimated to be 35 and 28 kDa, respectively, by SDS-PAGE (Fig. 1). Molecular massess of the liver enzymes were estimated by molecular sieve with Sephadex G-100 and G-150 columns. Values of 72 and 125 kDa were obtained for type I and type II enzymes, respectively (Fig. 2). The value for heart type I enzyme was also calculated to be 72 kDa (data not shown). These results indicate that liver and heart type I enzymes are homodimers, and type II enzyme is a homotetramer.

Liver and heart type I enzymes were titrated by the IgG preparation against rat liver enzyme (Fig. 3). The equivalence amounts to titrate 100 milliunits of these enzymes were around 50 $\mu$g of IgG. When 100 milliunits of type II enzyme was used, however, the enzyme activity was not changed at all even with the use of 5-fold excess amount of this IgG Type II enzyme was titrated by the IgG raised against this enzyme. The equivalence amount was about 170 $\mu$g to titrate 100 milliunits of the enzyme (Fig. 3). Both liver and heart type I enzymes were not titrated even by the use of 5-fold excess amount of the anti-type II enzyme IgG.

The antibody against rat liver enzyme also titrated commercially obtained pig heart and bovine liver enzyme

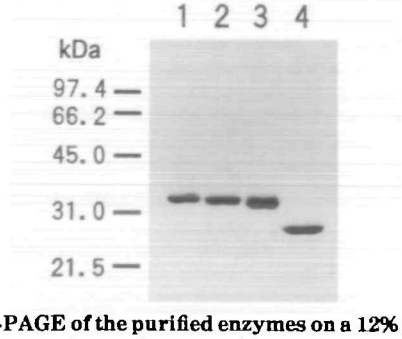


Fig. 1. **SDS-PAGE of the purified enzymes on a 12% gel.** Lane 1, heart type I enzyme, lane 2, liver type I enzyme, lane 3, liver type I enzyme purified after acid precipitation of the liver extract; lane 4, liver type II enzyme. Standard proteins, indicated with bars at left of the gel, were: phosphorylase *b*, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, from top to bottom.

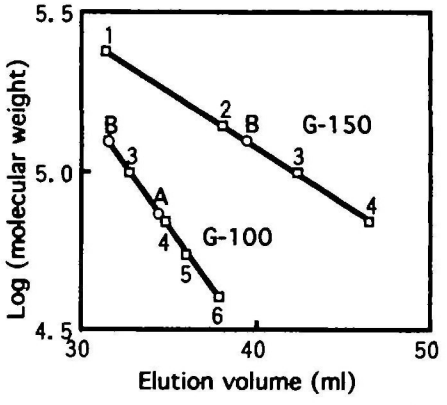


Fig. 2 **Estimation of molecular masses of type I and type II enzymes.** The columns of Sephadex G-150 and Sephadex G-100 (1.5×45 cm) were equilibrated and eluted with 50 mM potassium phosphate, pH 7.5/2 mM 2-mercaptoethanol/1 mM EDTA The standard enzymes (□) were: 1, rabbit muscle pyruvate kinase (237 kDa), 2, rabbit muscle lactate dehydrogenase (140 kDa); 3, pig heart citrate synthase (100 kDa), 4, pig heart malate dehydrogenase (70 kDa), 5, pigeon breast muscle carnitine acetyltransferase (55 kDa), 6, horseradish peroxidase (40 kDa) A, type I enzyme; B, type II enzyme

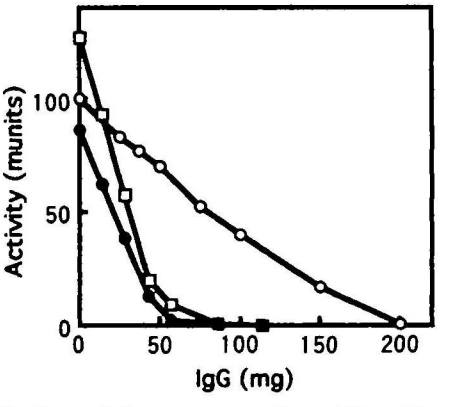


Fig 3 **Titration of the enzymes.** About 100 milliunits of the enzymes were incubated with various amounts of the antibodies for 30 min at room temperature in a total volume of 50 $\mu$l of 50 mM potassium phosphate, pH 7 5/2 mM 2-mercaptoethanol/1 mM EDTA/1 mg/ml bovine serum albumin. The mixtures were centrifuged at 10,000×*g* for 5 min, and the activities in the supernatants were determined by the standard assay procedure with acetoacetyl-CoA and NADH Liver (●) and heart (□) type I enzymes were titrated with the anti-rat liver enzyme IgG, and liver type II enzyme (○) was titrated with the anti-type II enzyme IgG The results of other combinations of enzyme and antibody were not indicated since no decrease in the activity was confirmed.

preparations, and also human liver enzyme which had been purified by similar procedures described for type I enzymes except that acid precipitation of the liver extract was included (Hashimoto, T., unpublished). Equivalence amounts of the antibody for these enzymes were similar to that for rat enzyme. These enzymes were not titrated by anti-type II enzyme IgG. Therefore, bovine type I enzyme is concluded to belong to classical HADH, but type II enzyme is a new enzyme in respect to its immunochemical properties.

*Subcellular Localization*—Subcellular fractionation of bovine liver was carried out. Crude mitochondrial fraction was obtained by centrifugation from postnuclear fraction without separation of heavy and light mitochondria. Soluble and microsomal fractions were obtained from the postmitochondrial fraction. The crude mitochondrial fraction was subjected to a discontinuous sucrose density gradient centrifugation. A pellet which passed through the 45% (w/w) sucrose solution layer was collected as the peroxisomal fraction, and the particles sedimented between the layers of 45% (w/w) and 35% (w/w) sucrose solutions were taken as the mitochondrial fraction. Most of the glutamate dehydrogenase activity was recovered in the mitochondrial fraction.

The subcellular distribution of HADH activity was well correlated with that of glutamate dehydrogenase. The ratio of the HADH activity with acetoacetyl-CoA to that with 3-ketooctanoyl-CoA was about 2 for the homogenate, postnuclear fraction, crude mitochondrial fraction, and the purified mitochondrial fraction. As indicated in Table I, the activity ratios of type I and type II enzymes were about 20 and 1.2, respectively. The constant ratio of 2 indicates that these type I and type II enzymes are localized in mitochondria at about the same amounts of the enzyme proteins, but not localized in peroxisomes. The peroxisomal fraction showed a low HADH activity with the activity ratio of 0.6. This activity may be due to a contamination of mitochondria and to peroxisomal enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase bifunctional protein, which exhibits a higher activity with C8 substrate.

As shown in Fig. 4, panel A, type I and type II enzymes were clearly distinguishable by immunoblot analysis. The results of immunoblot analysis of the subcellular fractions

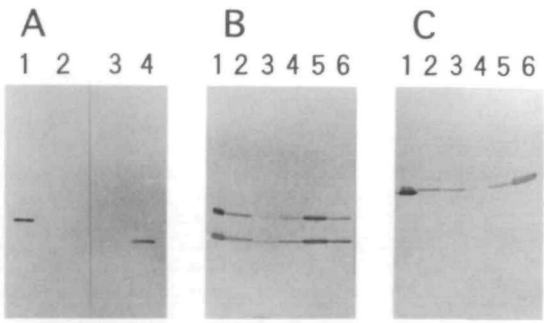


Fig 4 **Subcellular localization of type I and type II enzymes.** Panel A. 30 ng of type I enzyme (lanes 1 and 3) and type II enzyme (lanes 2 and 4) were used, and anti-(type I enzyme) IgG was used for lanes 1 and 2, and anti-(type II enzyme) IgG for lanes 3 and 4. Panel B lane 1, 30 ng each of type I and type II enzymes, lanes 2-6, 10 $\mu$g of protein of subcellular fractions: liver homogenate (lane 2), cytosolic fraction (lane 3), microsomal fraction (lane 4), mitochondrial fraction (lane 5), peroxisomal fraction (lane 6). Antibodies against type I and type II enzymes were used. Panel C· lane 1, 30 ng of rat peroxisomal 3-ketoacyl-CoA thiolase, lanes 2-6, bovine liver subcellular fractions as same as lanes 2-6 of panel B Antibody against rat peroxisomal 3-ketoacyl-CoA thiolase was used

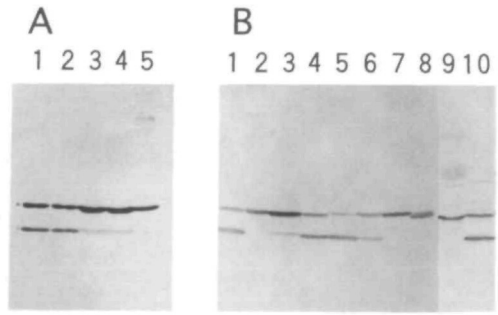


Fig. 5 **Tissue-specific and species-specific presence of type II enzyme.** The extracts of various tissues containing 10 milliunits by the standard assay for reverse reaction with acetoacetyl-CoA were used for immunoblot analysis Panel A. lane 1, 30 ng each of type I and type II enzymes; lane 2, bovine liver, lane 3, bovine kidney; lane 4, bovine heart; lane 5, bovine skeletal muscle Panel B: lane 1, 30 ng each of type I and type II enzymes, lane 2, human liver, lane 3, rat liver; lane 4, bovine liver, lane 5, sheep liver; lane 6, horse liver; lane 7, pig liver, lane 8, dog liver, lane 9, rabbit liver; lane 10, bovine liver Lanes 1-8 and lanes 9 and 10 of panel B are the results from separate experiments.

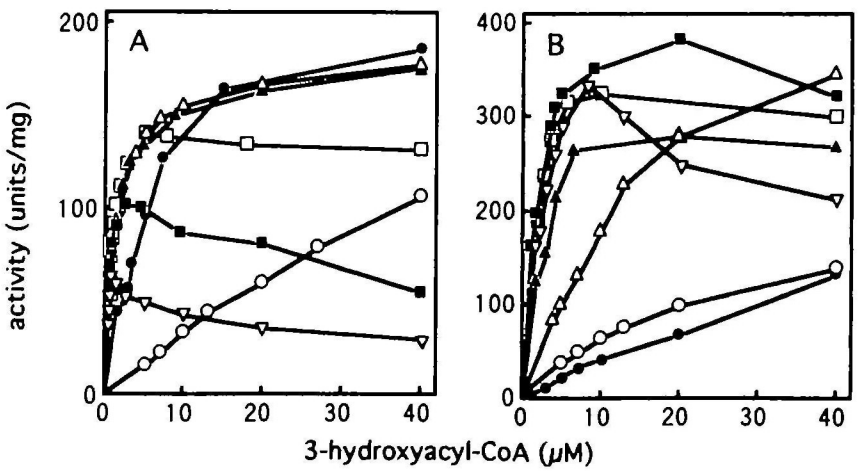


Fig. 6. **Velocities of forward reaction as a function of concentration of 3-hydroxyacyl-CoA at fixed concentration of 1 mM NAD$^+$.** Assays were carried out as described under "MATERIALS AND METHODS." A, type I enzyme, B, type II enzyme ○, 3-hydroxybutyryl-CoA; ●, 3-hydroxyhexanoyl-CoA; △, 3-hydroxyoctanoyl-CoA; ▲, 3-hydroxydecanoyl-CoA; □, 3-hydroxydodecanoyl-CoA; ■, 3-hydroxytetradecanoyl-CoA, ▽, 3-hydroxyhexadecanoyl-CoA.

indicated that both type I and type II enzymes were enriched in the mitochondrial fraction but not in other fractions, and the ratios of signal intensities for the two enzymes in each fraction were nealy the same (Fig. 4, panel B). Distribution of peroxisomes among the subcellular fractions was not judged by the calatase activity, because a large part of catalase is localized in cytosol in some animals such as bovine and guinea pig. But peroxisomal $\beta$-oxidation enzymes were localized in peroxisomes even in these animals (Hashimoto, T., unpublished observation). Therefore, immunoblot analysis was carried out with the use of the antibody against rat peroxisomal 3-ketoacyl-CoA thiolase (*14*). A strong signal was observed for the peroxisomal fraction, and the signal for mitochondrial fraction was faint (Fig. 4, panel C). The signal for the cytosolic fraction (panel C, lane 3) is supposed to be due to leakage of the enzyme during fractionation, because peroxisomes are fragile.

*Tissue and Species Specific Localization*—As described above, the contents of type I and type II enzymes could be roughly estimated by the ratio of the activity with acetoacetyl-CoA to that with 3-ketooctanoyl-CoA. The ratio of about 2 for bovine liver extract suggests that the amounts of type I and type II enzymes were nearly the same. The activity ratio of the bovine kidney extract and the heart extract was usually from 4 to 6 and from 6 to 8, respectively. The ratios of contents of type I and type II enzymes were estimated to be from 3:1 to 5:1 for kidney extract and from 5:1 to 7:1 for heart extract. The activity ratio of 15 for the skeletal muscle extract indicated lower content of type II enzymes. The contents of type I and type II enzymes were also estimated by titration of the enzymes with the antibodies. The values obtained by titration were similar to those estimated from the activity ratios (data not shown).

In immunoblot analysis, both type I and type II enzymes were detected in bovine liver, kidney, and heart, but not in skeletal muscle (Fig. 5, panel A).

Livers from various animals were examined (Fig. 5,

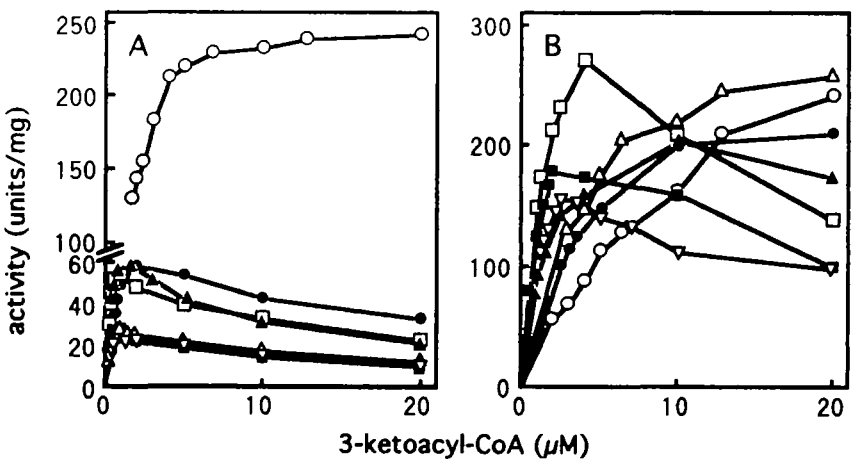


Fig. 7. **Velocities of reverse reaction as a function of concentration of 3-ketoacyl-CoA substrate at fixed concentration of 0.1 mM NADH.** Assays were carried out as described under "MATERIALS AND METHODS." A, type I enzyme; B, type II enzyme. ○, acetoacetyl-CoA; ●, 3-ketohexanoyl-CoA; △, 3-ketooctanoyl-CoA; ▲, 3-ketodecanoyl-CoA; □, 3-ketododecanoyl-CoA; ■, 3-ketotetradecanoyl-CoA; ▽, 3-ketohexadecanoyl-CoA.

TABLE III. **Kinetic constants of type I and type II enzymes.** Kinetic constants for 3-hydroxyacyl-CoAs and 3-ketoacyl-CoAs were calculated from the data for low substrate concentrations in Figs. 6 and 7, respectively. The constants for $NAD^+$ and $NADP^+$ were determined in the presence of 40 $\mu$M 3-hydroxybutyryl-CoA. Kinetic parameters for NADH and NADPH were determined in the presence of 20 $\mu$M acetoacetyl-CoA.

| Substrate | Type I enzyme | | Type II enzyme | |
|---|---|---|---|---|
| | Apparent $K_m$ ($\mu$M) | Apparent $V_{max}$ (units/mg) | Apparent $K_m$ ($\mu$M) | Apparent $V_{max}$ (units/mg) |
| Forward reaction | | | | |
| 3-Hydroxybutyryl-CoA | 75 | 400 | 28 | 290 |
| 3-Hydroxyhexanoyl-CoA | 15 | 380 | 56 | 360 |
| 3-Hydroxyoctanoyl-CoA | 1.9 | 220 | 24 | 550 |
| 3-Hydroxydecanoyl-CoA | 1.9 | 220 | 8.3 | 430 |
| 3-Hydroxydodecanoyl-CoA | 1.8 | 240 | 5.5 | 450 |
| 3-Hydroxytetradecanoyl-CoA | 1.5 | 230 | 2.1 | 400 |
| 3-Hydroxyhexadecanoyl-CoA | 1.5 | 200 | 1.5 | 380 |
| $NAD^+$ | 250 | 150 | 220 | 250 |
| $NADP^+$ | $2.0\times10^4$ | 11 | —[a] | —[a] |
| Reverse reaction | | | | |
| Acetoacetyl-CoA | 9.0 | 720 | 20 | 530 |
| 3-Ketohexanoyl-CoA | 5.7 | 320 | 5.9 | 320 |
| 3-Ketooctanoyl-CoA | 3.1 | 180 | 5.0 | 350 |
| 3-Ketodecanoyl-CoA | 2.3 | 240 | 2.3 | 320 |
| 3-Ketododecanoyl-CoA | 1.8 | 230 | 1.4 | 370 |
| 3-Ketotetradecanoyl-CoA | 1.3 | 120 | 1.4 | 300 |
| 3-Ketohexadecanoyl-CoA | 1.3 | 57 | 1.5 | 240 |
| NADH | 5.2 | 230 | 4.0 | 180 |
| NADPH | 380 | 180 | —[a] | —[a] |

[a]No activity was detected.

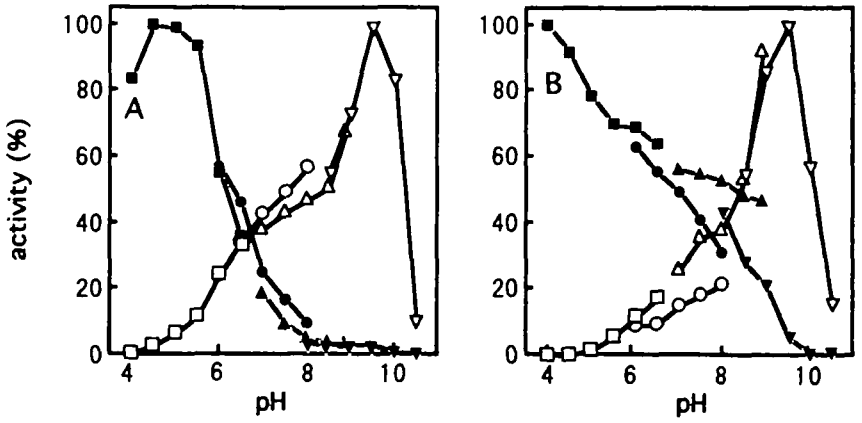


Fig. 8. **pH-activity profiles of type I and type II enzymes.** Velocity of the forward reaction was assayed under the standard assay conditions using 40 $\mu$M 3-hydroxybutyryl-CoA. Velocity of the reverse reaction was assayed under the standard assay conditions using 20 $\mu$M acetoacetyl-CoA. The buffers used were: 0.1 M potassium phosphate (○ and ●); 0.1 M Tris-Cl (△ and ▲); 0.1 M sodium acetate (□ and ■); 0.1 M $NH_4OH$-$NH_4Cl$ (▽); 40 mM borate-NaOH (▼). A, type I enzyme; B, type II enzyme. Open and solid marks represent the forward and reverse reactions.

panel B). Immunoblot signals for type I enzymes were clearly detected in all animals tested. For type II enzymes, intense signals were detected for bovine, sheep, and horse livers, and weak signals were found for rat and dog livers. But no signal was confirmed for human, rabbit, and pig livers.

*Catalytic Properties*—Velocities of the forward and reverse reactions of type I and type II enzymes at various concentrations of acyl-CoA substrates with carbon chain length from 4 to 16 are shown in Figs. 6 and 7. Kinetic parameters are summarized in Table III.

In the forward reaction, the dependency of type II enzyme on the 3-hydroxyacyl-CoA concentration was different from that of type I enzyme. Apparent $K_m$ values of type II enzyme for the substrates other than 3-hydroxypalmitoyl-CoA were higher. Both type I and type II enzymes were inhibited at higher concentration of the substrates, especially with longer carbon chain length substrates. But the substrate inhibition was more marked for type I enzyme. The specific activity of type II enzyme was much higher with longer carbon chain substrates than that of type I enzyme.

In the reverse reaction, the substrate concentration dependency and the substrate inhibition of type II enzyme were also different from those of type I enzyme.

Table III also shows the apparent $K_m$s for pyridine nucleotides. $K_m$ values of both enzymes for $NAD^+$ and NADH were similar. Very low activity was detected for type I enzyme when $NADP^+$ or NADPH was used. Similar low activities with these coenzymes were confirmed for purified rat HADH (*1*). These coenzymes were inactive for type II enzyme.

The pH-activity profiles of both enzymes are shown in Fig. 8. The optimal pH in the forward reaction was 9.5 for both enzymes. The value was comparable to beef liver enzyme (*15*), pig heart enzyme (*16*), and rat liver enzyme (*1*). The activity in the reverse reaction was highest at 4.5 for type I enzyme. This value was also similar to that for rat enzyme (*1*). Type II enzyme exhibited highest activity at pH 4, although the activity was not measured at pH below 4 in this experiment.

## DISCUSSION

Classical 3-hydroxyacyl-CoA dehydrogenase involved in mitochondrial fatty acid oxidation is often called monofunctional short-chain enzyme to distinguish it from mitochondrial enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase/3-ketoacyl-CoA thiolase trifunctional protein (*2*), which had been called long-chain 3-hydroxyacyl-CoA dehydrogenase before this enzyme complex was identified. These enzymes catalyze reversible conversion of L-(+)-3-hydroxyacyl-CoA to the corresponding 3-ketoacyl-CoA.

When we examined the HADH activity of human liver samples, we noticed several activity peaks by phosphocellulose column chromatography. To search for the presence of HADH isozymes, we used bovine liver. When the liver extract was subjected to column chromatographic procedures, and the HADH activity of the fractions was assayed with the different carbon chain length substrates, two different enzymes were found in bovine liver.

Type I enzyme of bovine liver consisted of two identical subunits with molecular mass of 35 kDa. The molecular structure, immunochemical reactivity, and carbon chain length specificity of this enzyme were very similar to the known HADH from various animals. Type II enzyme was different from type I enzyme in all of those respects.

Subcellular fractionation of bovine liver was carried out to locate these two enzymes. The enzyme assay with acetoacetyl-CoA and 3-ketooctanoyl-CoA and immunoblot analysis revealed that both type I and type II enzymes were located in mitochondria.

Sequential extraction of disrupted mitochondria was previously used to examine the association of very-long-chain acyl-CoA dehydrogenase and the trifunctional protein with the mitochondrial membrane (*2, 17*). In this study, a similar procedure was used. Frozen mitochondria from bovine liver were thawed and sequentially extracted with the following buffers: (i) 50 mM potassium phosphate, pH 7.5, (ii) 10 mM potassium phosphate, pH 7.5, (iii) 10 mM potassium phosphate, pH 7.5/0.1% Triton X-100, (iv) 10 mM potassium phosphate, pH 7.5/1% Triton X-100/0.2 M NaCl. The HADH activities were assayed with acetoacetyl-CoA, 3-ketooctanoyl-CoA, and 3-ketohexadecanoyl-CoA. The activities with acetoacetyl-CoA recovered in the first and second extracts were about 70 and 15% of the total activity, respectively. The ratios of the activities with three substrates were the same in these two extracts. Low enzyme activity was found in the third. A higher activity with 3-ketohexadecanoyl-CoA was observed in the last extract. This result suggests that solubilization of trifunctional protein was achieved in this extract. Immunoblot

analysis indicated that the signal intensities for type I and type II enzymes were highest in the first extract, although these signals were observed with similar intensity ratios in other extracts. The signal for trifunctional protein was mainly found in the last extract. These data suggest that both type I and type II enzymes are matrix enzymes.

Recently, it was reported that four new enzymes having the HADH activity were present in highly purified rat liver peroxisomes with minimal contamination of mitochondria (*18*). But none of them are similar in structure to type I and type II enzymes.

When D-3-hydroxyoctanoyl-CoA was used as substrate, no activity was detected for both type I and type II enzymes. It is considered that both type I and type II enzymes are located in the mitochondrial matrix and must be involved in the mitochondrial fatty acid $\beta$-oxidation.

The carbon chain length specificities of the purified rat and human enzymes in both the forward and reverse reactions were similar to those of bovine type I enzyme. The enzyme activity of type II enzyme was comparable to that of type I enzyme in bovine liver. Therefore, it is expected that carbon chain length specificity in the intramitochondrial compartment of bovine liver is different from those in rat and human mitochondria. Type II enzyme is abundant in liver and kidney. Type II enzyme is also rich in bovine, sheep, and horse livers, but it was hardly detected, if at all, in other animals so far tested. The different contents of type II enzyme among tissues and species may be related to the physiological and nutritional conditions of the animals concerned.

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