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Purification and Properties of Rat D-3-Hydroxyacyl-CoA Dehydratase: D-3-Hydroxyacyl-CoA Dehydratase/D-3-Hydroxyacyl-CoA Dehydrogenase Bifunctional Protein[1]

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We have previously purified two D-3-hydroxyacyl-CoA dehydratase preparations from human liver. One preparation contained a 77-kDa polypeptide and smaller polypeptides, and the other was a homodimer of a 46-kDa polypeptide. Three different purified rat peroxisomal D-3-hydroxyacyl-CoA dehydratase preparations have been reported. Therefore, rat enzyme was purified in this study to confirm the enzyme structure. Two preparations with similar molecular structures to the human enzyme preparations were obtained, and these were similar to each other in immunochemical and catalytic properties. It was suggested that the native enzyme was a homodimer of the 77-kDa polypeptide, and this enzyme was modified to a homodimer of the 46-kDa polypeptide, because conversion of the 77-kDa polypeptide to smaller polypeptides including the 46-kDa polypeptide was clearly observed during purification. Rat liver subcellular fractionation study indicates that this enzyme is located in peroxisomes. The enzyme preparation containing the 77-kDa polypeptide catalyzed the D-3-hydroxyacyl-CoA dehydrogenase reaction as well as the dehydratase reaction. Thus, it is proposed that this enzyme is D-3-hydroxyacyl-CoA dehydratase/D-3-hydroxyacyl-CoA dehydrogenase bifunctional protein.

Key words: bifunctional protein, D-3-hydroxyacyl-CoA dehydratase, D-3-hydroxyacyl-CoA dehydrogenase, peroxisomal, rat enzyme.

We have purified human D-3-hydroxyacyl-CoA dehydratase and obtained two enzyme preparations. One preparation contained a 77-kDa polypeptide and minor smaller polypeptides, and the other was a homodimer of a 46-kDa polypeptide. These preparations were concluded to be derived from a homodimer of the 77-kDa polypeptide (*1*). In this study, rat enzyme was purified, for the following reasons. (i) It is necessary to compare human enzyme with rat enzyme, because three different D-3-hydroxyacyl-CoA dehydratase preparations have been purified by different groups from rat liver: a homodimer of a 44-kDa subunit (*2*), a preparation containing 78-, 71-, and 47-kDa polypeptides with a molecular mass of 150 kDa for the native enzyme (*3*), and a homodimer of a 33.5-kDa subunit (*4*). (ii) Subcellular localization of the human enzyme was confirmed by immunohistochemical procedure with cultured human skin fibroblasts. A study on distribution of the enzyme activity and the cross-reactive material to the antibody among the subcellular fractions is also necessary. (iii) The use of fresh tissue is preferable to confirm the idea that the homodimer of the 46-kDa polypeptide is derived from a homodimer of the 77-kDa polypeptide.

In this report, we describe the purification and properties of rat liver D-3-hydroxyacyl-CoA dehydratase. The enzyme activity was concentrated by the procedures used for purification of the human enzyme, and two enzyme preparations similar to the human enzyme preparations were obtained. We noticed that the rat enzyme was much more susceptible to proteolytic degradation than the human enzyme during purification. The results obtained in this study suggest that the native rat enzyme is also a homodimer of the 77-kDa polypeptide, that it is D-3-hydroxyacyl-CoA dehydratase/D-3-hydroxyacyl-CoA dehydrogenase bifunctional protein located in peroxisomes, and that the homodimer of the 46-kDa polypeptide is a proteolytically modified enzyme having only the dehydratase activity.

MATERIALS AND METHODS

Materials—Materials were obtained as described in a previous report (*1*).

Enzyme Assay—The activities of enoyl-CoA hydratase and D-3-hydroxyacyl-CoA dehydratase were assayed by following change in absorbance at 280 nm as described previously (*1*): the reaction mixture was 0.3 M Tris-Cl, pH 7.5, and the substrate concentration was fixed at 0.1 mM under the standard assay conditions.

The 3-hydroxyacyl-CoA dehydrogenase activity in the forward reaction was assayed by the increase in absorbance

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Abbreviations: DEHP, di(2-ethylhexyl)phthalate; bifunctional protein, enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase bifunctional protein; trifunctional protein, enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase/3-ketoacyl-CoA thiolase trifunctional protein.

at 340 nm in the presence of 0.2 mM NAD^+ and 20 μM D-3-hydroxyoctanoyl-CoA or L-3-hydroxyoctanoyl-CoA in 0.1 M Tris/0.1 M KCl (pH 10.2) (*5*). The 3-hydroxyacyl-CoA dehydrogenase activity in the reverse reaction was assayed by 3-ketooctanoyl-CoA-dependent NADH oxidation (*6*).

The enzyme activities were assayed at 30°C, and one enzyme unit was defined as the amount of the enzyme converting 1 μmol of the substrate per min under the assay conditions.

Rats and Subcellular Fractionation of Liver—Male Wistar rats were fed a diet with or without 2% (w/w) di(2-ethylhexyl)phthalate (DEHP). Subcellular fractionation of rat liver was carried out by a standard method (*7*) without separation of heavy and light mitochondria. The crude mitochondrial fraction was subjected to sucrose density gradient centrifugation (*8*). The marker enzymes, catalase (*9*), glutamate dehydrogenase (*10*), and NADPH cytochrome *c* reductase (*11*), were assayed by the cited procedures.

Other Procedures—Protein concentration was determined by a modification (*12*) of the procedure of Lowry *et al.* (*13*). SDS-PAGE was carried out as described by Laemmli (*14*). Blotting of the electrophoresed polypeptides to a nitrocellulose sheet was done electrophoretically (*15*), and color development was done with the use of the second antibodies conjugated with horseradish peroxidase or alkaline phosphatase.

Enzyme Purification—Twenty-five grams of rat liver was homogenized with 100 ml of 50 mM potassium phosphate, pH 7.5, containing 1 mM benzamidine-HCl/1 mM phenylmethylsulfonyl fluoride/1% (w/v) sodium cholate, 0.5 M NaCl/1 mM EDTA/5 mM mercaptoethanol. The homogenate was kept on ice for 30 min, then centrifuged at 100,000 × *g* for 1 h.

Solid ammonium sulfate (100 g/liter) was added to the supernatant, and the mixture was centrifuged. To the supernatant, ammonium sulfate (100 g/liter of the original volume) was further added. The precipitate was collected by centrifugation and suspended in 25 ml of 100 mM potassium phosphate, pH 7.5, containing 2% (v/v) Tween 20, 5 mM mercaptoethanol, and 1 mM EDTA. All buffers used for the following procedures contained 5 mM mercaptoethanol and 1 mM EDTA. The suspension was dialyzed against 100 mM potassium phosphate, pH 7.5, containing 0.2% Tween 20. The dialysate was centrifuged at 100,000 × *g* for 30 min.

The supernatant was diluted 5-fold with 0.5% Tween 20 containing mercaptoethanol and EDTA, then applied to a phosphocellulose column (2.4 × 7 cm) which had been equilibrated with 20 mM potassium phosphate, pH 7.5/0.5% Tween 20. The column was washed with 60 ml of 25 mM potassium phosphate, pH 7.5/0.5% Tween 20. The enzyme was eluted with a linear gradient system from 25 to 150 mM potassium phosphate, pH 7.5, containing 0.5% Tween 20 in a total volume of 240 ml. The active fractions were pooled. Polyethylene glycol #6000 (0.25 g/ml) was added, and the mixture was centrifuged at 100,000 × *g* for 30 min.

The precipitate was dissolved in 3 ml of 100 mM potassium phosphate, pH 7.5, containing 0.2% Tween 20 and 5% (v/v) ethylene glycol. The fraction was desalted by passing through a Sephadex G-25 column using 5 mM potassium phosphate, pH 8.0, containing 0.2% Tween 20 and 5% ethylene glycol, then applied to a DEAE-Sephadex A-50 column (1.5 × 12 cm) which had been equilibrated with the same buffer. The column was washed with 2 column volumes of the same buffer.

The passed-through fractions, which contained the enzyme, were pooled and applied to a Reactive Green 19 column (1.4 × 3 cm) which had been equilibrated with 20 mM potassium phosphate, pH 7.5/0.2% Tween 20/10% ethylene glycol (buffer A). The column was washed with 2 column volumes of buffer A containing 0.1 M NaCl, then the enzyme was eluted with a NaCl concentration gradient system from 0.1 to 2 M in buffer A in a total volume of 40 ml. To remove small amounts of impurities, the enzyme fraction was dialyzed against buffer A, then rechromatographed on a Reactive Green 19 column.

The active fraction was collected and dialyzed against buffer A. The dialyzed enzyme was adsorbed on a small phosphocellulose column and eluted with a small volume of 200 mM potassium phosphate, pH 7.5, containing 0.02% Tween 20 and 10% ethylene glycol. The enzyme preparations were dialyzed against buffer A containing 50% (v/v) glycerol. The final preparations were kept for more than one year without loss of activity.

RESULTS

Purification of the Enzyme—The purification procedures were essentially the same as those used for purification of human enzyme (*1*). The purification is summarized in Table I.

Determination of the dehydratase activity of the liver extract was difficult, because the expected linear increase in octenoyl-CoA with time was masked by its conversion to L-3-hydroxyoctanoyl-CoA by high enoyl-CoA hydratase activities of crotonase (*16*), peroxisomal enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase bifunctional

TABLE I. **Summary of purification of the enzyme from rat liver.** Twenty-five grams of rat liver was used. The hydratase and dehydratase activities were assayed with octenoyl-CoA and D-3-hydroxyoctanoyl-CoA, respectively. The purification steps from phosphocellulose to Reactive Green 19 column chromatography were carried out within one day as noted under "MATERIALS AND METHODS."

Step	Activity (units)		Protein (mg)	Specific activity (units/mg)	
	Hydratase	Dehydratase		Hydratase	Dehydratase
Extract	43,700	7,840	5,040	8.7	1.6
Ammonium sulfate	10,600	3,150	608	17.4	5.2
Phosphocellulose	3,330	1,140	22.4	149	51
Polyethylene glycol	2,220	840	14.0	159	60
DEAE-Sephadex	666	601	4.03	165	149
Reactive Green 19	351	312	0.91	386	343
2nd Reactive Green 19	380	319	0.84	452	380

protein (bifunctional protein) (*16*), and mitochondrial enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase/3-ketoacyl-CoA thiolase trifunctional protein (trifunctional protein) (*17*).

Two enzyme activity peaks were separated by the Reactive Green 19 column chromatography. The former peak, eluted at the NaCl concentration of about 0.3 M, contained the enzyme consisting of 46-kDa polypeptide, and the latter peak, eluted at about 0.8 M NaCl, contained the enzyme consisting of 77-kDa and other smaller polypeptides. When the purification steps from phosphocellulose to Reactive Green 19 column chromatography were carried out within one day, 90% or more of the total enzyme activity was recovered in the latter peak. The preparation purified from this fraction is named preparation II. The purification protocol is listed in Table I, and the result of SDS-PAGE is shown in Fig. 1, lane 2. When purification was carried out after storage of the eluates from the phosphocellulose column at 4°C overnight, the activity in the former peak increased to 10–20% of the total activity. The enzyme preparation purified from this fraction consisted of the 46-kDa polypeptide (preparation I) (Fig. 1, lane 1). When purification was carried out after storage of the eluates from the phosphocellulose column at 4°C for 2 days, the final preparation from the latter peak fraction contained a smaller amount of the 77-kDa polypeptide with larger amounts of smaller polypeptides (preparation III) (Fig. 1, lane 3). When the enzyme was purified without Tween 20 from the step of phosphocellulose column chromatography, the yields of the enzyme activity and 77-kDa polypeptide in preparation II were lower. Phenylmethylsulfonyl fluoride and benzamidine were ineffective to improve the recoveries of the 77-kDa polypeptide and the activity.

The specific activity of D-3-hydroxyoctanoyl-CoA dehydratase of preparation II was 380 units/mg (Table I), and that of preparation I was 640 units/mg. These values were similar to those of the purified human enzyme preparations. The specific activity of preparation III was 390 units/mg. The specific activities of the octenoyl-CoA hydratase

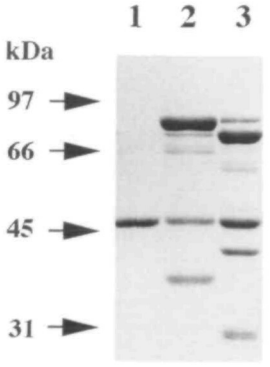

Fig. 1. **SDS-PAGE of the purified enzyme preparations.** The enzymes were analyzed on 10% gel. Lane 1, preparation I; lane 2, preparation II; lane 3, preparation III.

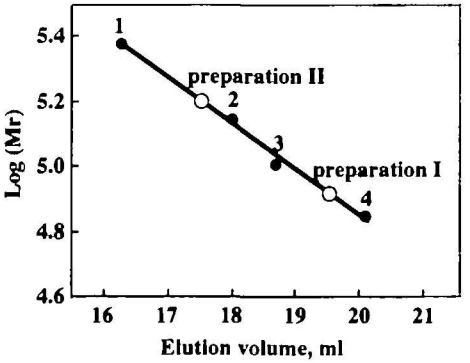

Fig. 2. **Estimation of molecular masses by molecular sieve column chromatography.** Samples were applied to a TSK-GEL G3000SW column (7.5 mm I.D. × 60 cm; Tosoh, Tokyo) and developed with 20 mM potassium phosphate, pH 7.5/0.2 M NaCl/0.1% Tween 20/5 mM mercaptoethanol/1 mM EDTA. The standard proteins 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).

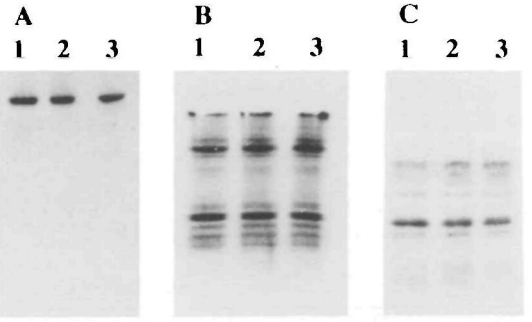

Fig. 3. **Peptide mapping of 46-kDa polypeptides in the different preparations.** Preparation I (sample 1), preparation II (sample 2), and preparation III (sample 3) were subjected to the first SDS-PAGE using 10% gel. Gels containing 46-kDa polypeptide were cut out and subjected to the second SDS-PAGE with a protease using 15% gel. Panel A, 2 μg of the samples without protease; panel B, 10 μg of the samples with 0.08 μg proteinase K; panel C, 6 μg of the samples with 0.04 μg endoproteinase Glu-C. Sample 1, lanes 1; sample 2, lanes 2; sample 3, lanes 3.

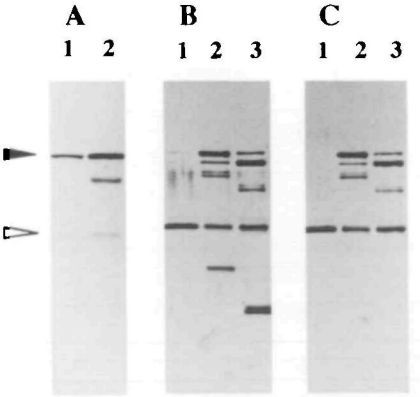

Fig. 4. **Immunoblot analysis of the liver extracts and the purified enzyme preparations.** Panel A, liver extract: lane 1, control rat; lane 2, DEHP-fed rat. The antibody used was anti-human preparation-I antibody. Panel B, enzyme preparations examined with anti-77-kDa polypeptide antibody: lane 1, preparation I; lane 2, preparation II; lane 3, preparation III. Panel C, purified enzyme preparations examined with anti-preparation-I antibody: lane 1, preparation I; lane 2, preparation II; lane 3, preparation III.

reaction for these enzyme preparations were similar to those of the dehydratase reaction.

Molecular Structures—SDS-PAGE revealed that preparation II contained several polypeptides. The major polypeptide was of 77 kDa, and 46- and 31-kDa polypeptides were clearly seen (Fig. 1). Preparation I consisted of one polypeptide with a molecular mass of 46 kDa. Preparation III contained a small amount of 77-kDa polypeptide, and the major component was of 71 kDa.

The molecular masses of preparations I and II estimated by gel filtration were 87 and 166 kDa, respectively (Fig. 2). SDS-PAGE showed that all of the polypeptides in preparation II were eluted together. The molecular mass of preparation III was estimated to be 150 kDa, and again all polypeptides were eluted together.

Relation of the Subunit of Preparation I and the 46-kDa Polypeptide in Preparation II—Peptide mapping by limited proteolysis was carried out according to the method of Cleveland *et al.* (*18*). The samples used were the subunit of preparation I and the 46-kDa polypeptides of preparations II and III. Figure 3A shows the SDS-PAGE pattern of these three samples. The patterns of peptide mapping of these three polypeptides treated with proteinase K were very similar (Fig. 3B), and those of the polypeptides treated with endoproteinase Glu-C were also very similar to each other (Fig. 3C).

Immunochemical Properties—Two antibody preparations raised against the human enzymes (*1*) were used: anti-77-kDa polypeptide antibody was raised against the 77-kDa polypeptide of human preparation II isolated by preparative SDS-PAGE, and anti-preparation-I antibody was raised against human preparation I.

Figure 4A shows the result of immunoblot analysis of the liver extract. The main signal was of 77 kDa, and a very faint signal was found at the 46-kDa position in the control. When DEHP-fed rat liver was tested, the signal intensity for 77-kDa polypeptide was much higher. Signals corresponding to 68 and 46 kDa were also seen. The D-3-hydroxyoctanoyl-CoA dehydratase activity in the liver extract was increased about 5-fold by administration of DEHP to rats.

Figure 4B shows the result of the enzyme preparations with the anti-77-kDa polypeptide antibody. The patterns of signals for preparations I and II were similar to those of stained polypeptides shown in Fig. 1. In preparation III all of the polypeptides except the 41-kDa polypeptide were detected. When anti-preparation-I antibody was used, the signals for the 46-kDa polypeptide and larger polypeptides were seen, but no signal for smaller polypeptides was detected, as shown in Fig. 4C. None of these signals were seen when the antibodies were previously mixed with the 77-kDa polypeptide isolated from preparation II by preparative SDS-PAGE.

Preparations I and II were titrated with anti-human preparation-I antibody (Fig. 5A). The D-3-hydroxyoctanoyl-CoA dehydratase activities of both preparations I and II were diminished to an undetectable level by increasing the amount of the antibody, but neither activity was affected by treatment with the IgG fraction from a preimmune serum. In this experiment, the amount of the enzyme was 125

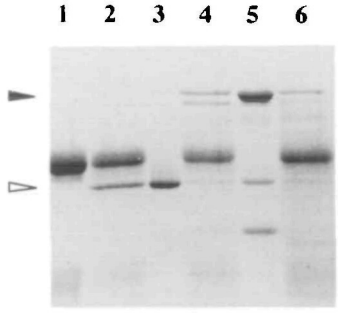

Fig. 6. **SDS-PAGE of immunoprecipitates.** Preparation I (1 μg), preparation II (2 μg), and ammonium sulfate fraction of liver extract (440 milliunits of the dehydratase) were treated with anti-preparation-I antibody in a total volume of 100 μl of 150 mM NaCl/10 mM potassium phosphate, pH 7.5/0.02% Tween 20/2 mM mercaptoethanol/1 mM EDTA. Protease inhibitors were added as described in legend to Fig. 5. The immunoprecipitates were washed with phosphate-buffered saline and subjected to SDS-PAGE. Lane 1, antibody (5 μg); lane 2, immunoprecipitate of preparation I; lane 3, preparation I (1 μg); lane 4, immunoprecipitate of preparation II; lane 5, preparation II (2 μg); lane 6, immunoprecipitate of the ammonium sulfate fraction. Closed arrowhead, 77 kDa; open arrowhead, 46 kDa.

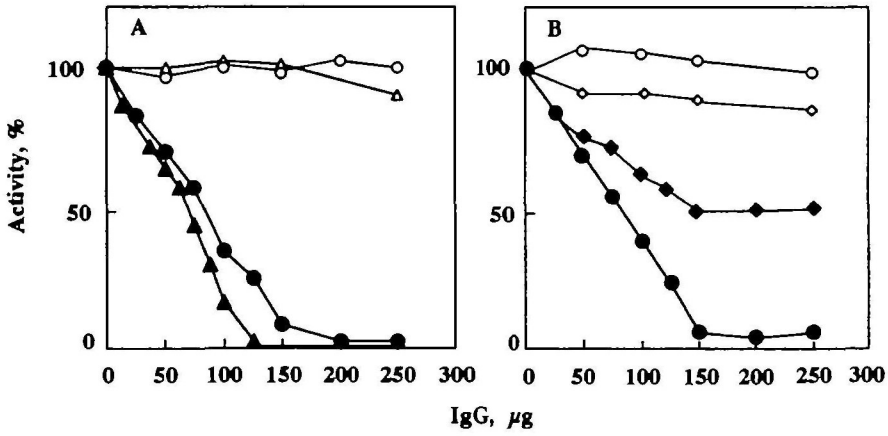

Fig. 5. **Titration of the enzyme with the antibody.** The enzyme preparations I and II (125 milliunits of D-3-hydroxyoctanoyl-CoA dehydratase) and the ammonium sulfate fraction (130 milliunits of the dehydratase) from the liver extract were incubated with various amounts of anti-preparation-I antibody for 30 min at room temperature in a total volume of 50 μl of 150 mM NaCl/10 mM potassium phosphate, pH 7.5/0.02% Tween 20/2 mM mercaptoethanol/1 mM EDTA. Antipain, bestatin, chymostatin, E-64, pepstatin A, and phosphoramidon were added to this mixture at 20 μg/ml each to protect the enzyme from degradation during incubation. The mixtures were centrifuged at 10,000×g for 5 min, and the activities in the supernatants were determined. Panel A, preparation II (●) and preparation I (▲) titrated with the antibody; preparation II (○) and preparation I (△) treated with a preimmune IgG. The dehydratase activity was expressed as % activity. Panel B, ammonium sulfate fraction treated with the antibody or with the preimmune IgG. The activities of D-3-hydroxyoctanoyl-CoA dehydratase (●, ○) and octenoyl-CoA hydratase (◆, ◇) are shown. Solid and open marks indicate the results with the antibody and the preimmune IgG, respectively.

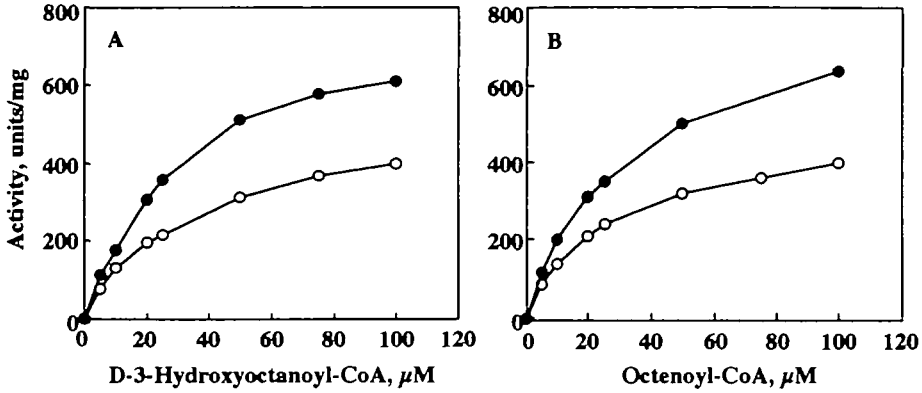

Fig. 7. **Velocities of the dehydratase and hydratase reactions as a function of substrate concentration.** Panel A, D-3-hydroxyoctanoyl-CoA dehydratase reaction. Panel B, octenoyl-CoA hydratase reaction. ○, preparation II; ●, preparation I.

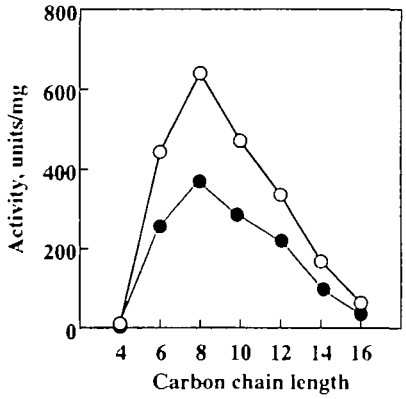

Fig. 8. **Carbon chain length specificities.** The enoyl-CoA hydratase activities were assayed at a fixed concentration of 0.1 mM. ●, preparation II; ○, preparation I.

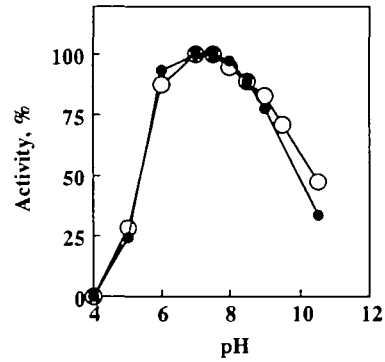

Fig. 9. **The pH-octenoyl-CoA hydratase activity profiles of the two types of the enzyme.** The reaction mixture contained 25 mM acetic acid, citric acid, phosphoric acid, and barbital sodium, and pH was adjusted with NaOH. ●, preparation II; ○, preparation I.

milliunits for both preparations I and II, and the equivalence amounts were 130 and 170 μg of the IgG, respectively. Assuming a ratio of the specific enzyme activities of these enzyme preparations of about 1.6, and a ratio of the molecular masses of the two enzyme preparations of 1.9, the equivalence amounts per mole of the enzyme were not much different for these two enzyme preparations.

Titration of the ammonium sulfate fraction of the crude extract was carried out because (i) most of the dehydratase activity was recovered in this fraction, and (ii) assay of the enzyme activity was more reliable than that of the extract due to removal of crotonase. The equivalence amount to titrate the D-3-hydroxyoctanoyl-CoA dehydratase of the ammonium sulfate fraction was similar to that of preparation II (Fig. 5B). The octenoyl-CoA hydratase activity was reduced to about one half. This may be due to titration of the dehydratase, because about half of the hydratase activity of this sample is thought to be due to the dehydratase.

When anti-77-kDa polypeptide antibody was used, addition of protein A-Sepharose was necessary. The results of titration of preparations I and II with this antibody were very similar to those shown in Fig. 5A (data not shown).

Recovery of the enzyme proteins in the immunoprecipitates was very low, especially that of the 77-kDa polypeptide at the beginning of this experiment, as observed for precipitation of the human enzymes. Therefore, protease inhibitors were added to the incubation mixture. Figure 6 shows that the subunit of preparation I was quantitatively recovered by anti-preparation-I antibody. The polypeptides in preparation II were also recovered by this antibody, but the recovery was lower, and degradation of the 77-kDa polypeptide was found. When anti-77-kDa polypeptide was used, a larger amount of IgG recovered by protein A-Sepharose interfered with the detection of 46-kDa polypeptides, although the 77-kDa polypeptide was clearly seen (data not shown).

Catalytic Properties—Preparations I and II catalyzed reversible conversion of octenoyl-CoA to D-3-hydroxyoctanoyl-CoA at similar velocities as shown in Table I, but both preparations were inactive with L-3-hydroxyoctanoyl-CoA. The product of octenoyl-CoA was examined by coupling 3-hydroxyacyl-CoA dehydrogenase, which specifically catalyzes the L-isomer. The conversion to 3-ketooctanoyl-CoA was followed by increase in absorbance at 303 nm in the presence of Mg^{2+} (*19*). The rates of production of 3-ketooctanoyl-CoA from octenoyl-CoA were nearly equivalent to the hydratase activity when crotonase, the bifunctional protein, or the trifunctional protein were used. But virtually no change in the absorbance with octenoyl-CoA was detectable when preparations I and II were used. The results suggest that these preparations catalyze reversible conversion of 2-*trans*-enoyl-CoAs to D-3-hydroxyacyl-CoAs, but not to the L-isomers.

Velocity-substrate concentration relationships of the

TABLE II. **Distribution of the marker enzymes in subcellular fractions of rat liver.** The marker enzymes were: hydratase, octenoyl-CoA hydratase; dehydratase, D-3-hydroxyoctanoyl-CoA dehydratase; GDH, glutamate dehydrogenase; reductase, NADPH cytochrome *c* reductase. The enzyme activities are expressed as units/mg protein, except for catalase activity, which is expressed as kunits/mg.

	Homogenate	Peroxisomes	Mitochondria	Microsomes	Cytosol
Hydratase	6.1	38	23	3.7	1.3
Dehydratase	1.5	22	1.3	1.8	0.21
Catalase	0.45	9.00	0.72	0.21	0.25
GDH	0.83	0.26	2.9	0.12	<0.01
Reductase	0.05	0.05	0.02	0.24	0.05

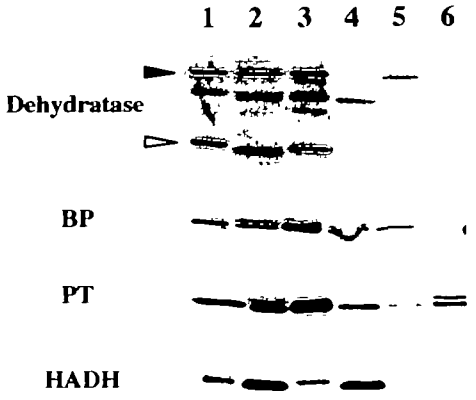

Fig. 10. **Subcellular distribution of the cross-reactive materials to the antibodies against the dehydratase and other proteins.** Lane 1, purified human enzyme (20 ng); lane 2, homogenate (30 μg protein); lane 3, peroxisomes (5 μg); lane 4, mitochondria (5 μg); lane 5, microsomes (5 μg); lane 6, cytosol (5 μg). Antibodies against the following proteins were used: human preparation I (dehydratase), the bifunctional protein (BP), peroxisomal 3-ketoacyl-CoA thiolase (PT), mitochondrial 3-hydroxyacyl-CoA dehydrogenase (HADH).

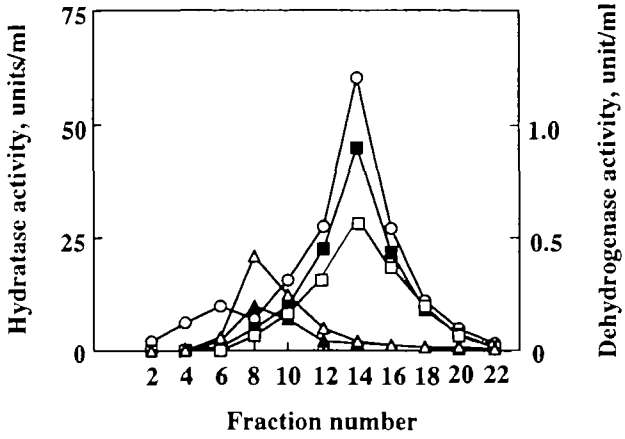

Fig. 11. **Separation of D-3-hydroxyoctanoyl-CoA dehydrogenase and L-3-hydroxyoctanoyl-CoA dehydrogenase.** A sample after DEAE-Sephadex column chromatography was applied to a 3-ml Reactive Green 19 column. After washing the column with 10 ml of 0.1 M NaCl in buffer A, the column was eluted with a linear concentration gradient system composed of 15 ml each of 0.1 and 1.5 M NaCl in buffer A. Fractions of 1 ml were collected. The activities with various substrates are shown: ○, octenoyl-CoA hydratase; □, D-3-hydroxyoctanoyl-CoA dehydrogenase; △, L-3-hydroxyoctanoyl-CoA dehydrogenase; ■, 3-ketooctanoyl-CoA reductase; ▲, acetoacetyl-CoA reductase.

octenoyl-CoA hydratase reaction of preparations I and II, and those of the D-3-hydroxyoctanoyl-CoA dehydratase reaction are summarized in Fig. 7. V_{max} and K_m values for the hydratase reaction of preparation I were 860 units/mg and 40 μM, respectively, and those of preparation II were 420 units/mg and 40 μM, respectively. These V_{max} and K_m values were comparable to those of the human enzymes. V_{max} and K_m values for the dehydratase reaction of preparation I were 870 units/mg and 30 μM, respectively, and those of preparation II were 500 units/mg and 30 μM, respectively. When the V_{max} values for preparations I and II were compared based on their molecular masses, the catalytic activities per mole of enzyme were found to be similar.

Activities of the preparations I and II were determined with enoyl-CoAs having various carbon-chain lengths. The carbon chain length specificities of these enzyme preparations were very similar to each other (Fig. 8), and the patterns resembled those of the human enzyme preparations. The patterns indicate that these enzyme preparations are active with medium-chain substrates.

Effect of pH—The effect of pH on preparations I and II is shown in Fig. 9. The patterns for the two enzyme preparations are similar to each other, but slightly different from those of the human enzyme preparations.

Localization of the Enzyme—Localization of the enzyme was studied by examination of the subcellular fractions of rat liver. Separation of the organelles was judged by distribution of the marker enzyme activities among the fractions (Table II), and distribution of the cross-reactive material to the antibody (Fig. 10).

The peroxisomal fraction was rich in the D-3-hydroxyoctanoyl-CoA dehydratase activity. Intensities of the signals for the dehydratase subunits, the 77- and 46-kDa polypeptides, were highest in the peroxisomal fraction, although the major signal was of the 77-kDa polypeptide. Both the activity of the dehydratase and its protein content visualized by immunoblot analysis were lower in other subcellular fractions. Peroxisomes are thought to be fragile and susceptible to leakage of some of the matrix proteins during subcellular fractionation. Therefore, we examined the distribution of the cross-reactive materials with the antibodies against the bifunctional protein (*16*) and 3-ketoacyl-CoA thiolase (*19*). The most intense signals for these proteins were confirmed in the peroxisomal fraction.

The glutamate dehydrogenase activity was assayed as the marker enzyme for mitochondria. This enzyme activity was concentrated in the mitochondrial fraction. The cross-reactive material to the antibody against 3-hydroxyacyl-CoA dehydrogenase (*5*) was also concentrated in the mitochondrial fraction, and its content was lower in the peroxisomal fraction. The data suggest that the dehydratase is located in peroxisomes.

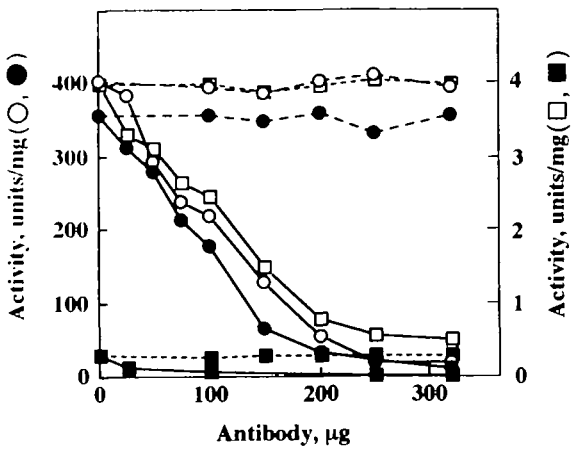

Fig. 12. **Parallel decrease in the activities of D-3-hydroxyacyl-CoA dehydratase and D-3-hydroxyacyl-CoA dehydrogenase by treatment with the antibody.** Fraction 14 of Fig. 11 was treated with the anti-preparation-I antibody as described in legend to Fig. 5. The activities of octenoyl-CoA hydratase (○), D-3-hydroxyoctanoyl-CoA dehydratase (●), D-3-hydroxyoctanoyl-CoA dehydrogenase (□), and L-3-hydroxyoctanoyl-CoA dehydrogenase (■) are shown. Solid and dotted lines indicate the results with the antibody and the preimmune IgG, respectively.

The contents of the dehydratase in various rat tissues were examined by immunoblot analysis. The major component was the 77-kDa polypeptide. The signal intensity was highest in liver. A faint signal was detected in kidney, but no signal was observed in heart, skeletal muscle, and brain. A similar distribution was observed for catalase and peroxisomal 3-ketoacyl-CoA thiolase (data not shown).

Search for D-3-Hydroxyacyl-CoA Dehydrogenase—Cook *et al.* (*3*) reported that $NADH^+$-dependent 3-ketododecanoyl-CoA reductase was co-purified with the dehydratase, whose preparation contained a 78-kDa polypeptide as its main component.

The activity of NAD^+-dependent 3-hydroxyoctanoyl-CoA dehydrogenation was examined with the L- and D-isomers in this study. These two activities were nearly the same in the liver extract, and decreased in a parallel during purification. The D-3-hydroxyoctanoyl-CoA dehydrogenase activity in the crude sample may be due to the presence of the hydratase(s) and L-3-hydroxyacyl-CoA dehydrogenase (*20*).

No dehydrogenase activity was detected in preparation I. The final preparation II exhibited a higher dehydrogenase activity with the D-isomer than that with the L-isomer, although these activities were very low compared to the dehydratase activity.

To search for D-3-hydroxyacyl-CoA dehydrogenase, the purification procedures were slightly modified. (i) The tail part of the activity peak on phosphocellulose column chromatography was not saved, in order to remove the bifunctional protein. (ii) A larger column of DEAE-Sephadex was used to maximize removal of the activities of enoyl-CoA hydratase and L-3-hydroxyacyl-CoA dehydrogenase. The octenoyl-CoA hydratase activity as measured by the L-3-hydroxyoctanoyl-CoA dehydratase activity in this preparation was less than 0.01% of the D-3-hydroxyoctanoyl-CoA dehydratase activity. (iii) The procedure for Reactive Green 19 column chromatography was slightly changed.

The results of this column chromatography are shown in Fig. 11. The L-3-hydroxyoctanoyl-CoA dehydrogenase activity was eluted between the two dehydratase activity peaks, and the D-3-hydroxyoctanoyl-CoA dehydrogenase activity was eluted with a similar pattern to that of the main dehydratase activity. However, the activity ratios of the dehydrogenase to the dehydratase were slightly higher in the later fractions. The elution pattern of the dehydrogenase seemed to be nearly the same as that of the 77-kDa polypeptide as examined by SDS-PAGE. The enzyme activities expressed as unit/ml in fraction 14 were as follows: octenoyl-CoA hydratase = 60, D-3-hydroxyoctanoyl-CoA dehydrogenase = 0.50, L-3-hydroxyoctanoyl-CoA dehydrogenase = 0.043, and 3-ketooctanoyl-CoA reductase = 0.83.

Fraction 14 was examined by titration with the antibody (Fig. 12). The D-3-hydroxyoctanoyl-CoA dehydrogenase activity was decreased in a similar manner to the D-3-hydroxyoctanoyl-CoA dehydratase and octenoyl-CoA hydratase activities by increasing the amount of the antibody. When fraction 8 was used, the dehydratase was completely titrated, but the L-3-hydroxyoctanoyl-CoA dehydrogenase activity was unchanged.

The carbon chain length specificity of fraction 14 was examined by use of 3-ketoacyl-CoAs. The pattern was very similar to that of the dehydratase (Fig. 8), and quite different from those of short-chain 3-hydroxyacyl-CoA dehydrogenase (*5*), the bifunctional protein (unpublished observation), and the trifunctional protein (*17*) purified from rat liver.

The human enzyme preparation corresponding to rat preparation II was subjected to Reactive Green 19 column chromatography to separate the D-3-hydroxyacyl-CoA dehydrogenase activity from that with the L-isomer, but the two activities were not clearly separated. However, these activities were separated by Sephadex G-150 column chromatography.

DISCUSSION

Rat D-3-hydroxyacyl-CoA dehydratase was purified and two preparations were obtained. Preparation I was supposed to be a homodimer of a 46-kDa polypeptide. Preparation II, which was purified as noted under "MATERIALS AND METHODS" to minimize modification, contained a major component with a molecular mass of 77 kDa. When the human enzyme was purified, it was difficult to study the relation of the 46- and 77-kDa polypeptides. But the rat enzyme was much more labile, and conversion of the 77-kDa polypeptide to the 46-kDa polypeptide was observed.

Preparations I and II were closely related, although the molecular structures were different. Their immunochemical properties as examined by immunoblot analysis and titration of the enzymes with the antibody were nearly the same.

The two enzyme preparations catalyzed reversible conversion of medium-chain 2-*trans*-enoyl-CoAs to D-3-hydroxyacyl-CoAs, and their carbon-chain length specificities were nearly the same. The specific enzyme activities were slightly different when expressed as units/mg protein, but the values were similar when expressed in terms of

molecular mass. The K_m values with octenoyl-CoA or D-3-hydroxyoctanoyl-CoA were also nearly the same. The effects of pH on the enzyme activities of preparations I and II were also similar.

The results indicated that preparation I and the minor, smaller polypeptides in preparation II were derived from the 77-kDa polypeptide, and that the native enzyme is a dimer of the 77-kDa polypeptide. (i) A decrease in the content of the 77-kDa polypeptide and accompanying increases in contents of the smaller polypeptides, including the 46-kDa polypeptide, were clearly observed when the rat enzyme was purified. (ii) Peptide mapping patterns of the 46-kDa polypeptides in preparations I and II were very similar. (iii) Preparation I and all of smaller polypeptides in preparation II were cross-reactive with the antibody raised against human 77-kDa polypeptide. The polypeptides of 46 kDa and larger in preparation II were recognized by the antibody raised against human preparation I. The polypeptides of less than 46 kDa seemed to be derived from a region outside of the 46-kDa region of the 77-kDa polypeptide, because these polypeptides were not detected by the preparation-I antibody. (iv) Equivalence amounts to titrate the two enzyme preparations were very similar using either antibody. (v) Catalytic properties of preparations I and II were very similar.

It is obvious from the titration of the enzyme in the ammonium sulfate fraction of the liver extract with the antibody that there is one type of D-3-hydroxyacyl-CoA dehydratase in rat liver, and subcellular fractionation study indicates that this enzyme is located in peroxisomes. Three different preparations have been purified from rat liver, and all of these enzymes were claimed to be peroxisomal (*2-4*). The difference in the molecular structures of these purified preparations probably depends on the purification procedures. We noticed that recovery of preparation II was low when the enzyme was purified without a detergent. One group purified the enzyme in the presence of a detergent, and obtained a preparation containing a 78-kDa polypeptide and several smaller polypeptides (*3*). The structure is similar to that of preparation II. But the other two groups obtained different preparations without using a detergent (*2, 4*). The structures of these preparations were similar to that of preparation I, although the molecular sizes of the subunits were different.

It has recently been reported that a peroxisomal D-3-hydroxyacyl-CoA dehydratase/D-3-hydroxyacyl-CoA dehydrogenase bifunctional protein of *Saccharomyces cerevisiae* catalyzes conversion of 2-*trans*-enoyl-CoA to 3-ketoacyl-CoA through D-3-hydroxyacyl-CoA, and that these two catalytic domains are located on the same polypeptide (*21*).

Epimerization to the L-isomers of D-3-hydroxyacyl-CoAs produced by β-oxidation of polyunsaturated fatty acids is thought to be involved in the peroxisomal fatty acid oxidation system but not in the mitochondrial one (*22*). The presence of D-3-hydroxyacyl-CoA dehydrogenase in higher animals has not been reported, and an apparent dehydrogenation activity of D-3-hydroxyacyl-CoA in peroxisomes has been explained by coupling of D-3-hydroxyacyl-CoA dehydratase and the bifunctional protein having the activities of enoyl-CoA hydratase and L-3-hydroxyacyl-CoA dehydrogenase (*20*). The D-3-hydroxyacyl-CoA dehydrogenase was detected in preparation II, which is practically free of the bifunctional protein, in this study (Fig. 11), although the dehydrogenase activity was very low compared with the dehydratase activity. No D-3-hydroxyacyl-CoA dehydrogenase activity was found in preparation I.

Peroxisomal β-oxidation cycle is catalyzed by acyl-CoA oxidase (*23*), the bifunctional protein (*16*), and 3-ketoacyl-CoA thiolase (*19*). The activities of these enzymes in the control rat liver were as follows in our experiments (units/g liver): acyl-CoA oxidase activity with palmitoyl-CoA = 0.3-0.6; octenoyl-CoA hydratase activity of the bifunctional protein = 50-100; 3-ketoacyl-CoA thiolase activity with 3-ketooctanoyl-CoA = 6-10. The L-3-hydroxyoctanoyl-CoA dehydrogenase activity of the bifunctional protein and D-3-hydroxyoctanoyl-CoA dehydrogenase of the dehydratase were 2-4 units/g liver. These two 3-hydroxyacyl-CoA dehydrogenase activities were much higher than the activity of acyl-CoA oxidase, which has been considered to be a rate-limiting enzyme. Therefore, we think that the dehydrogenase activity of the dehydratase plays a physiological role not only in epimerization of D-3-hydroxyacyl-CoAs derived from β-oxidation of polyunsaturated fatty acids, but also in the direct conversion of D-3-hydroxyacyl-CoAs to 3-ketoacyl-CoAs.

The 77-kDa polypeptide in preparation II is of comparable size to such multifunctional enzymes as the bifunctional protein, a monomer, and the α-subunit of the trifunctional protein, which carries the domains of enoyl-CoA hydratase and L-3-hydroxyacyl-CoA dehydrogenase (*24-27*). In all of these proteins, the hydratase domain and the dehydrogenase domain are located on the amino-terminal and carboxyl-terminal side, respectively. In a preliminary experiment, preparation II was treated with proteinase K and subjected to Reactive Green 19 column chromatography, and it was confirmed that the activities of the dehydratase and dehydrogenase were eluted in the different fractions. Further studies are required to demonstrate that the structure of D-3-hydroxyacyl-CoA dehydratase/D-3-hydroxyacyl-CoA dehydrogenase multifunctional protein purified in this study is similar to that of *S. cerevisiae* multifunctional protein, whose hydratase and dehydrogenase domains are located on the carboxyl- and amino-terminal side, respectively.

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