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# Purification and Properties of Human D-3-Hydroxyacyl-CoA Dehydratase: Medium-Chain Enoyl-CoA Hydratase Is D-3-Hydroxyacyl-CoA Dehydratase[1]

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**Human medium-chain enoyl-CoA hydratase was purified from liver, because we noticed the presence of a high medium-chain enoyl-CoA hydratase activity in human skin fibroblasts catalyzed by an enzyme different from the known enzymes catalyzing the enoyl-CoA hydratase reaction. Two enzyme preparations were obtained. One of them, preparation I, consisted of 46-kDa polypeptide, and its molecular mass was estimated to be 86 kDa. The other, preparation II, consisted of a major 77-kDa polypeptide and minor smaller polypeptides including 46-kDa polypeptide. The molecular mass of preparation II was 154 kDa. Both enzyme preparations catalyzed reversible dehydration of medium-chain D-3-hydroxyacyl-CoA to 2-*trans*-enoyl-CoA, but did not react with L-3-hydroxyacyl-CoA. Catalytic properties and immunochemical reactivities of these enzyme preparations were nearly the same. The cross-reactive material to the antibody was confirmed to be in peroxisomes by immunohistochemical study of cultured human skin fibroblasts.**

**Key words: enoyl-CoA hydratase, human enzyme, D-3-hydroxyacyl-CoA dehydratase, medium-chain, peroxisomal.**

Enoyl-CoA hydratase catalyzes the second step of the fatty acid $\beta$-oxidation spiral. There are several enzymes catalyzing the enoyl-CoA hydratase reaction in higher animals. Crotonase (*1*) and enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase/3-ketoacyl-CoA thiolase trifunctional protein (trifunctional protein) (*2*) are present in mitochondria, and enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase bifunctional protein (bifunctional protein) (*3-5*) is present in peroxisomes. The presence of long-chain enoyl-CoA hydratase (*6, 7*) was also described, but its molecular properties are not well characterized.

A novel enzyme catalyzing the reversible hydration of 2-*trans*-enoyl-CoA to D-3-hydroxyacyl-CoA, referred to as D-3-hydroxyacyl-CoA dehydratase, has recently been recognized and purified from rat liver (*8-11*).

During analysis of cultured human skin fibroblasts, we noticed a high activity of the medium-chain enoyl-CoA hydratase (*12*). The substrate specificity of this enzyme was quite different from those of crotonase, the trifunctional protein, and the bifunctional protein. This enzyme accounted for more than 50% of the octenoyl-CoA hydratase activity in the fibroblasts.

We planned to characterize this enzyme. In an early stage of this study, we purified this medium-chain enoyl-CoA hydratase from human liver by monitoring the activities with crotonyl-CoA, octenoyl-CoA, and hexadecenoyl-CoA to distinguish this enzyme from crotonase, the trifunctional protein, and the bifunctional protein. Two enzyme preparations were obtained, and it was confirmed that these enzymes catalyzed the D-3-hydroxyacyl-CoA dehydratase reaction. The two enzyme preparations were different in molecular structure but very similar in immunochemical and catalytic properties.

## MATERIALS AND METHODS

*Materials*—The following enzymes were purified from rat liver and their antibodies were raised by the cited procedures: crotonase (*4*), the bifunctional protein (*4*), and the trifunctional protein (*2*). Enoyl-CoAs, L-3-hydroxyacyl-CoAs, and D-3-hydroxyoctanoyl-CoA were prepared as described previously (*13*).

Phosphocellulose, type P11, and DEAE-cellulose were obtained from Whatman (Maidstone, England). Sephadex G-25, G-100, G-150, DEAE-Sephadex A-50, PBE 94, and Polybuffer 74 for chromatofocusing were from Pharmacia Biotech (Uppsala, Sweden). AF-Blue Toyopearl 650 was from Tosoh (Tokyo). Reactive Green 19 and protein A-Sepharose were from Sigma (St. Louis, MO). Hydroxyapatite was from Nacalai Tesque (Kyoto).

Proteins and enzymes were purchased from Boehringer (Mannheim, Germany), Bio-Rad Laboratories (Richmond,

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Abbreviations: 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.

CA), and Sigma. Coenzyme A was obtained from Kyowa Hakko (Tokyo).

All other chemicals were of analytical grade.

*Enzyme Assay*—The enoyl-CoA hydratase activity was assayed by a decrease in absorbance at 280 nm as described previously (*4*) in the presence of 0.1 mM 2-*trans*-enoyl-CoAs at 30°C. For assay of activity, the purified crotonase preparation was diluted with 100 mM potassium phosphate, pH 7.5, containing 1 mg/ml BSA, and the other enzyme preparations were diluted with 50 mM potassium phosphate, pH 7.5, containing 2 mM mercaptoethanol. The reverse reaction was assayed under the same conditions except for the presence of 0.1 mM L-3-hydroxyacyl-CoAs. The D-3-hydroxyacyl-CoA dehydratase activity was assayed with 0.1 mM D-3-hydroxyoctanoyl-CoA.

One enzyme unit was defined as the activity converting 1 $\mu$mol of the substrate per min under the assay conditions.

*Preparation of Antibody*—The purified preparation I was used directly to raise the antibody. The final preparation II was subjected to a preparative SDS-PAGE, and the isolated 77-kDa polypeptide was used to raise the antibody, since this preparation contained several minor polypeptides. The antibodies against these two preparations were raised in rabbits, and the IgG fractions were obtained by ammonium sulfate precipitation and purified by DEAE-cellulose column chromatography.

*Procedures of Protein Analysis*—Protein concentration was determined by a modification (*14*) of the procedure of Lowry *et al.* (*15*). SDS-PAGE was carried out as described by Laemmli (*16*). Immunoblot analysis was carried out according to the procedure of Towbin *et al.* (*17*).

*Immunohistochemical Examination*—Cultured human skin fibroblasts were fixed with 4% paraformaldehyde/0.1 M potassium phosphate, pH 7.4, for 1 h. After washing with phosphate-buffered saline, the cells were permeabilized with 0.1% Triton X-100/phosphate-buffered saline for 15 min. Blocking was performed with 4% fetal calf serum/0.1% Triton X-100/phosphate-buffered saline for 1 h, then cells were treated with anti-preparation-I rabbit antibody and anti-human catalase guinea pig antibody. After washing with phosphate-buffered saline, the cells were further treated with fluorescein isothiocyanate-labeled goat antibody to rabbit IgG and rhodamine-labeled goat antibody to guinea pig IgG. Finally, the cells were washed with phosphate-buffered saline and immersed in 0.1% paraphenylenediamine/0.1 M Tris-HCl, pH 8.5/90% glycerol for examination under a fluorescence microscope (Axiophot, Zeiss, Germany).

*Extraction of Enzymes for Purification*—Fifty grams of frozen human liver was homogenized with 200 ml of 10 mM $K_2HPO_4$/1 mM benzamidine-HCl/1 mM phenylmethylsulfonyl fluoride/1 mM EDTA/5 mM mercaptoethanol, and centrifuged at 100,000 × *g* for 1 h. The supernatant (the first extract) was used for purification of crotonase and the bifunctional protein.

The precipitate was homogenized with 200 ml of 50 mM potassium phosphate, pH 7.5/1 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, and then centrifuged at 100,000 × *g* for 1 h. The supernatant (the second extract) was used for purification of D-3-hydroxyacyl-CoA dehydratase and the trifunctional protein.

*Purification of D-3-Hydroxyacyl-CoA Dehydratase*—D-3-Hydroxyacyl-CoA dehydratase was purified from the second extract.

*Step 1. Separation of the dehydratase from the hydratases:* Solid ammonium sulfate (220 g/liter) was added to the second extract. The precipitate was collected by centrifugation and suspended in 100 ml of 50 mM potassium phosphate, pH 7.5, containing 1% (v/v) Tween 20, 5 mM mercaptoethanol, and 1 mM EDTA using a glass homogenizer, then centrifuged. All buffers used in the following procedures contained 5 mM mercaptoethanol and 1 mM EDTA. Polyethylene glycol #6000 was added to the supernatant (0.22 g/ml). The precipitate was collected by centrifugation and suspended in 50 ml of 100 mM potassium phosphate, pH 7.5, containing 1% Tween 20. The insoluble material was removed. The supernatant was diluted 5-fold with 1% Tween 20 and applied to a phosphocellulose column (2.4 × 11 cm) which had been equilibrated with 20 mM potassium phosphate, pH 7.5/0.5% Tween 20. The column was washed with 100 ml of 50 mM potassium phosphate, pH 7.5/0.5% Tween 20. The enzyme was eluted with a linear concentration gradient from 50 to 250 mM potassium phosphate, pH 7.5, containing 0.5% Tween 20 in a total volume of 400 ml. Fractions containing the D-3-hydroxyoctanoyl-CoA dehydratase activity were pooled. The bifunctional protein started to be eluted in the tail of the dehydratase activity peak. The tail fractions containing a higher enoyl-CoA hydratase activity compared with the dehydratase activity were not saved, because the bifunctional protein was not clearly separated from the dehydratase by the subsequent purification procedures. Polyethylene glycol #6000 (0.25 g/ml) was added to the pooled fraction. The precipitate was collected by centrifugation at 100,000 × *g* for 30 min and suspended in 20 ml of 20 mM potassium phosphate, pH 7.5, 10% (v/v) ethylene glycol, 0.2% Tween 20 (buffer A). The insoluble material was removed by centrifugation.

*Step 2. Separation of the two D-3-hydroxyacyl-CoA dehydratases:* This fraction was applied to a Reactive Green 19 column (1.5 × 9 cm) which had been equilibrated with buffer A. The column was washed with 30 ml 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 80 ml.

*Step 3. Purification of preparation I:* The earlier peak fraction was dialyzed against 5 mM potassium phosphate, pH 8.0/10% ethylene glycol/0.2% Tween 20 and applied to a DEAE-Sephadex A-50 column (1.5 × 6 cm) which had been equilibrated with the dialysis buffer. The column was washed with 20 ml of the same buffer. The passed-through fractions were applied to a AF-Blue Toyopearl column (1.2 × 1.7 cm) which had been equilibrated with buffer A. After the column was washed with 4 ml of buffer A, the enzyme was eluted with a linear concentration gradient of NaCl from 0 to 0.5 M in a total volume of 30 ml of buffer A. The enzyme fraction was usually homogeneous. When impure materials were found, they were removed by Sephadex G-150 column chromatography with buffer A.

*Step 4. Purification of preparation II:* The later fraction from the Reactive Green 19 column was dialyzed and subjected to DEAE-Sephadex A-50 and AF-Blue Toyopearl column chromatographies as described for purification of preparation I, except that the concentration of NaCl was

TABLE I. **Summary of purification of D-3-hydroxyacyl-CoA dehydratase.** The hydratase and dehydratase activities were assayed with octenoyl-CoA and D-3-hydroxyoctanoyl-CoA, respectively.

| Step | Activity (units) | | Protein (mg) | Specific activity (units/mg) | |
|---|---|---|---|---|---|
| | Hydratase | Dehydratase | | Hydratase | Dehydratase |
| 2nd extract | 13,200 | 6,220 | 1,420 | 9.3 | 4.4 |
| Ammonium sulfate | 13,100 | 6,000 | 824 | 15.9 | 7.3 |
| Polyethylene glycol | 7,500 | 4,200 | 390 | 19.2 | 10.8 |
| Phosphocellulose | 4,370 | 3,800 | 133 | 32.9 | 28.6 |
| Reactive Green 19 | | | | | |
| Preparation I | 721 | 718 | 7.3 | 98.8 | 98.4 |
| DEAE-Sephadex | 496 | 485 | 1.4 | 354 | 346 |
| AF-Blue Toyopearl | 426 | 452 | 0.75 | 568 | 603 |
| Preparation II | 2,140 | 1,950 | 20 | 107 | 97.5 |
| DEAE-Sephadex | 1,220 | 1,270 | 3.4 | 359 | 374 |
| AF-Blue Toyopearl | 1,010 | 1,060 | 2.8 | 361 | 379 |

TABLE II. **Summary of purification of crotonase.**

| Step | Activity[a] (units) | Protein (mg) | Specific activity (units/mg) |
|---|---|---|---|
| 1st extract | 33,600 | 4,370 | 7.7 |
| 1st phosphocellulose | 30,700 | 3,930 | 7.8 |
| Ammonium sulfate | 26,700 | 1,080 | 24.7 |
| Polyethylene glycol | 25,000 | 875 | 28.6 |
| 1st hydroxyapatite | 15,500 | 50 | 310 |
| 2nd phosphocellulose | 10,400 | 11.2 | 929 |
| 2nd hydroxyapatite | 7,560 | 7.2 | 1,050 |
| Chromatofocusing | 5,720 | —[b] | —[b] |
| 3rd hydroxyapatite | 5,700 | 3.4 | 1,680 |

[a]Activity was assayed with crotonyl-CoA. [b]Protein was not determined.

TABLE III. **Summary of purification of the bifunctional protein.**

| Step | Activity[a] (units) | Protein (mg) | Specific activity (units/mg) |
|---|---|---|---|
| Phosphocellulose | 2,700 | 32.2 | 84 |
| Ammonium sulfate | 2,130 | 9.1 | 234 |
| Sephadex G-100 | 1,440 | 4.7 | 306 |
| AF-Blue Toyopearl | 930 | 2.5 | 372 |

[a]The activity was determined with octenoyl-CoA.

increased from 0.1 to 1 M in the latter step.

The protocols of purification of these enzyme preparations are shown in Table I. Preparations I and II could both be kept without loss of activity for several months when dissolved in 50% (v/v) glycerol/50 mM potassium phosphate, pH 7.5/5 mM mercaptoethanol/1 mM EDTA and stored at −20°C.

*Separation of Crotonase and the Bifunctional Protein*—The first extract was applied to a phosphocellulose column (2.4×11 cm, equilibrated with 10 mM potassium phosphate, pH 6.5). The column was washed with 100 ml of 50 mM potassium phosphate, pH 7.5, then eluted with a linear concentration gradient system composed of 100 ml each of 50 and 350 mM potassium phosphate, pH 7.5. Most of the crotonase was not adsorbed, but the bifunctional protein was adsorbed and eluted at a relatively high concentration of the buffer.

*Purification of Crotonase*—Solid ammonium sulfate (370 g/liter) was added to the passed-through fraction, and the precipitate was centrifuged off. To the supernatant, ammonium sulfate (170 g/liter of the passed-through fraction) was further added. The precipitate was collected and dissolved in 100 ml of 50 mM potassium phosphate, pH 6.5. Polyethylene glycol #6000 (0.3 g/ml) was added to precipitate the enzyme. The precipitate was collected by centrifugation and suspended in 25 ml of 50 mM potassium phosphate, pH 6.5, using a glass homogenizer. The suspension was centrifuged. The supernatant was applied to a hydroxyapatite/cellulose column, which was made with 15 g of hydroxyapatite and 3 g of cellulose. The column volume was about 40 ml. The column was washed with 40 ml of 0.2 M potassium phosphate, pH 6.5, then the enzyme was eluted with a linear concentration gradient composed of 0.2 and 2.0 M potassium phosphate, pH 6.5, formed of 100 ml of each solution. Active fractions were pooled, and the enzyme was precipitated by addition of ammonium sulfate (500 g/liter). The precipitate was dissolved in a small volume of 0.2 M potassium phosphate, pH 6.5, and desalted by passing through a Sephadex G-25 column which had been equilibrated with 10 mM potassium phosphate, pH 6.5/10% ethylene glycol. All buffers used in this and subsequent steps contained 10% ethylene glycol to stabilize the enzyme. This enzyme fraction was passed through a 5-ml phosphocellulose column equilibrated with 10 mM potassium phosphate, pH 6.5. The passed-through fraction was applied to a hydroxyapatite/cellulose column (4 ml). The column was washed with 8 ml of 0.2 M potassium phosphate, pH 6.5, then the enzyme was eluted with a linear concentration gradient from 0.3 to 1.4 M potassium phosphate, pH 6.5, in a total volume of 30 ml. The active fractions were pooled and concentrated by centrifugation in an Ultracent tube (Tosoh, Tokyo). After desalting with a Sephadex G-25 column, chromatofocusing was carried out on a 2-ml PBE 94 column which had been equilibrated with imidazole-HCl, pH 7.5, using polybuffer 74-HCl, pH 5.0, as elution solution according to the manufacturer's protocol except for the addition of 10% ethylene glycol. The active fractions were applied onto a small hydroxyapatite/cellulose column. The column was washed with 0.1 M potassium phosphate, pH 6.5, to remove the polybuffer, then the enzyme was eluted with 1 M potassium phosphate, pH 6.5. The enzyme was dialyzed against 0.2 M potassium phosphate, pH 6.5/20% ethylene glycol. The enzyme was stored at −20°C for several months without loss of activity. The purification of crotonase is summarized in Table II.

*Purification of the Bifunctional Protein*—The activities of octenoyl-CoA hydratase and D-3-hydroxyoctanoyl-CoA dehydratase in the eluates from the phosphocellulose column were determined in order to discard the D-3-hydroxyacyl-CoA dehydratase eluted in the front part of the

hydratase activity peak, because the contaminant dehydratase was not efficiently removed by the following procedures. Solid ammonium sulfate (180 g/liter) was added to the pooled active fractions, the mixture was centrifuged, and further ammonium sulfate (120 g/liter of the original volume) was added to the supernatant. The precipitate was collected by centrifugation and dissolved in a small volume of 100 mM potassium phosphate, pH 7.5. All buffers used for purification of this enzyme contained 2 mM mercaptoethanol and 1 mM EDTA. This fraction was passed through a Sephadex G-100 column (1.4×70 cm) using the same buffer. The active fractions were pooled, diluted with an equal volume of water, then applied onto a 2-ml AF-Blue Toyopearl column which had been equilibrated with 50 mM potassium phosphate, pH 7.5. The enzyme was eluted with a linear concentration gradient of NaCl from 0 to 2 M in 50 mM potassium phosphate, pH 7.5, consisting of 7 ml of each solution. The active fractions were usually homogeneous on SDS-PAGE. When impurity was found, the pooled fraction was subjected to phosphocellulose column chromatography. The final enzyme preparation was dialyzed against 100 mM potassium phosphate, pH 7.5, containing 50% glycerol, and stored at −20°C for more than a year without loss of activity. The purification of the bifunctional protein is summarized in Table III.

*Purification of the Trifunctional Protein*—Purification of the trifunctional protein was carried out as described previously (*2, 12*).

## RESULTS

*Purification of the Enzymes*—Crotonase, the bifunctional protein, and the trifunctional protein were purified from human liver for comparison with D-3-hydroxyacyl-CoA dehydratase. A sequential extraction of the enzymes was carried out. The dehydratase activity in the first extract was about 1/4 of that in the second extract. Therefore, the dehydratase was purified from the second extract.

Crotonase was purified from the first extract, because most of the crotonase was extracted in this fraction. About a half of the bifunctional protein was extracted in the first extract, and the remainder in the second extract. The bifunctional protein was purified from the first extract. The trifunctional protein extracted in the second extract was purified independently, because solubilization after polyethylene glycol precipitation was a key step to obtain a pure preparation, as noted in the previous papers (*2, 12*).

The D-3-hydroxyacyl-CoA dehydratase activity was separated into two peaks by Reactive Green 19 column chromatography. These two fractions were separately subjected to further purification. We named the purified preparations preparation I and preparation II in the order of elution.

A typical result of purification of D-3-hydroxyacyl-CoA dehydratase is summarized in Table I. The activities of octenoyl-CoA hydratase and D-3-hydroxyoctanoyl-CoA dehydratase were determined during purification. The specific D-3-hydroxyoctanoyl-CoA dehydratase activities of preparations I and II were 603 and 379 units/mg, respectively. The recovery of the dehydratase activity was usually about 25% (Table I).

Upon SDS-PAGE, preparation I gave one polypeptide band with a molecular mass of 46 kDa (Fig. 1). Preparation II gave several polypeptide bands, of which the major polypeptide was of 77 kDa, and the minor ones were of 74, 64, 58, 46, and 36 kDa.

Human crotonase (Table II), the bifunctional protein (Table III), and the trifunctional protein were purified for comparison with D-3-hydroxyacyl-CoA dehydratase. Both the bifunctional protein and the trifunctional protein were purified by similar procedures to those used for purification of rat enzymes. For purification of human crotonase, heat-shock treatment,which was very efficient for purification of bovine and rat enzymes, was not adopted, because of the lability of the human enzyme. Mobilities of the subunits of human crotonase, the bifunctional protein, and the trifunctional protein on SDS-PAGE (Fig. 1) were very similar to those of the corresponding rat enzymes. Molecular masses of these native human enzymes were also nearly the same as those of the rat enzymes as determined by molecular sieve column chromatography (data not shown).

*Molecular Structures*—The molecular masses of prepa-

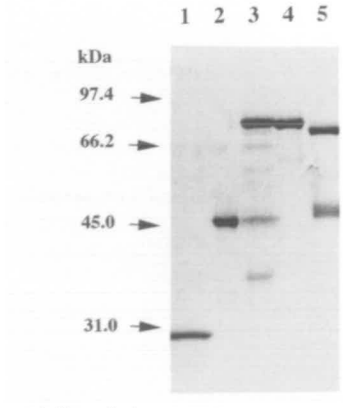


Fig. 1. **SDS-PAGE of the purified enzyme preparations.** The enzymes were analyzed on 10% gel. Lane 1, crotonase; lane 2, preparation I; lane 3, preparation II; lane 4, bifunctional protein; lane 5, trifunctional protein. Proteins used as molecular standards were rabbit muscle phosphorylase *b* (97.4 kDa), BSA (66.2 kDa), hen egg white ovalbumin (45.0 kDa), bovine carbonic anhydrase (31.0 kDa).

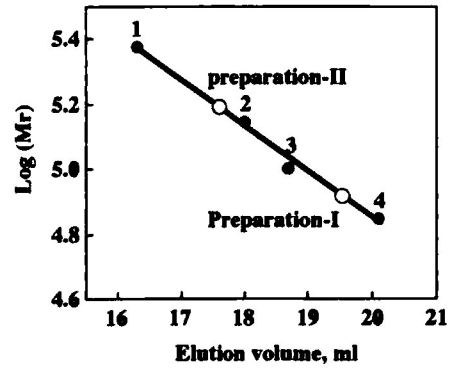


Fig. 2. **Estimation of molecular mass by molecular sieve column chromatography.** Samples were applied to a TSK-GEL G3000SW column (7.5 mm I.D.×60 cm; Tosoh, Tokyo) and developed with 20 mM potassium phosphate, pH 7.5/0.2 M NaCl/0.1% Tween 20/5 mM mercaptoethanol/1 mM EDTA. The standard proteins were: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

rations I and II were estimated to be 86 and 154 kDa, respectively, by gel filtration (Fig. 2).

Therefore, the preparation I enzyme was assumed to be a homodimer of 46-kDa polypeptide. Preparation II was subjected to various separation procedures, but separation of the constituent polypeptides was not attained and the distribution of these polypeptides corresponded closely to the dehydratase activity. Only the procedure described below was successful.

The result of AF-Blue Toyopearl column chromatography of preparation II in the presence of 4 M urea is shown in Fig. 3. The elution profiles of the 77- and 46-kDa polypeptides were different from those in the absence of urea. The 46-kDa polypeptide was eluted slightly earlier, and the later fractions contained the 77-kDa polypeptide with a small amount of the 46-kDa polypeptide. Other polypeptides in preparation II were not detected in the fractions exhibiting the dehydratase activity. Most of the enzyme activity applied was recovered, and the pattern of the enzyme activity matched neither that of the 46-kDa nor the 77-kDa polypeptide but that of the sum of these polypeptides. The specific activities of the main fractions ranged from 420 to 520 units/mg. These values seemed to be higher than that of preparation II, although a different protein assay method was used. The result indicates that

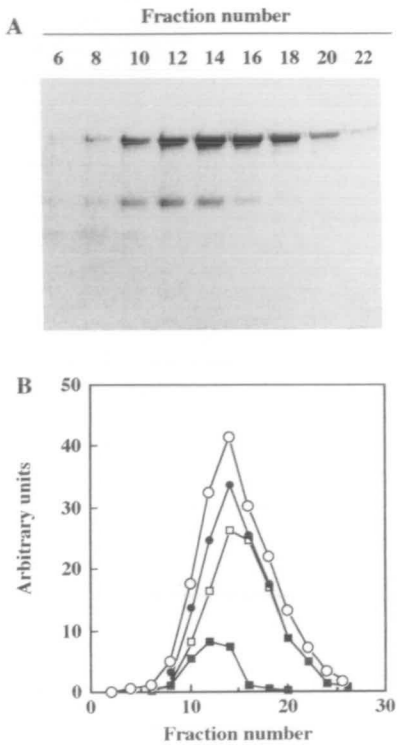


Fig. 3. **Partial separation of the polypeptides of preparation II.** Preparation II was chromatographed on an AF-Blue Toyopearl column as described under the purification procedure, except for the presence of 4 M urea. No loss of the enzyme activity was observed when the experiment was carried out at 5°C. Panel A shows the result of SDS-PAGE using a fixed amount of the fractions. Panel B shows elution profiles of the octenoyl-CoA hydratase activity (○) and those of 77- and 46-kDa polypeptides quantified by densitometric measurement of Panel A with the use of BSA as standard: 77-kDa polypeptide (□) and 46-kDa polypeptide (■), the sum of these polypeptides (●). The calculated specific activities of the fractions from 10 to 22 were 420-520 units/mg.

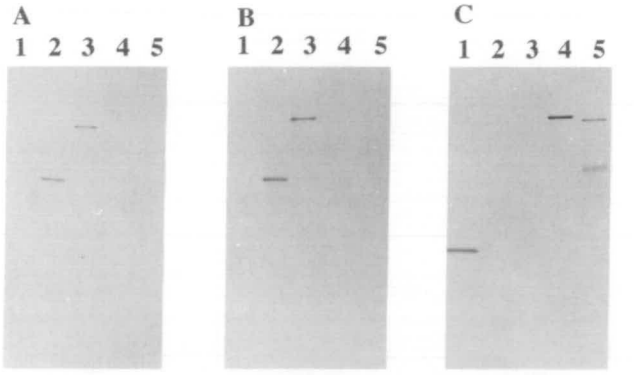


Fig. 4. **Immunoblot analysis of human enzymes catalyzing the enoyl-CoA hydratase reaction.** Panel A, anti-preparation-I antibody; panel B, anti-77-kDa polypeptide antibody; panel C, a mixture of the antibodies against crotonase, the bifunctional protein, and the trifunctional protein. Lane 1, crotonase; lane 2, preparation I; lane 3, preparation II; lane 4, bifunctional protein; lane 5, trifunctional protein. The amounts of these enzymes used were 20 ng.

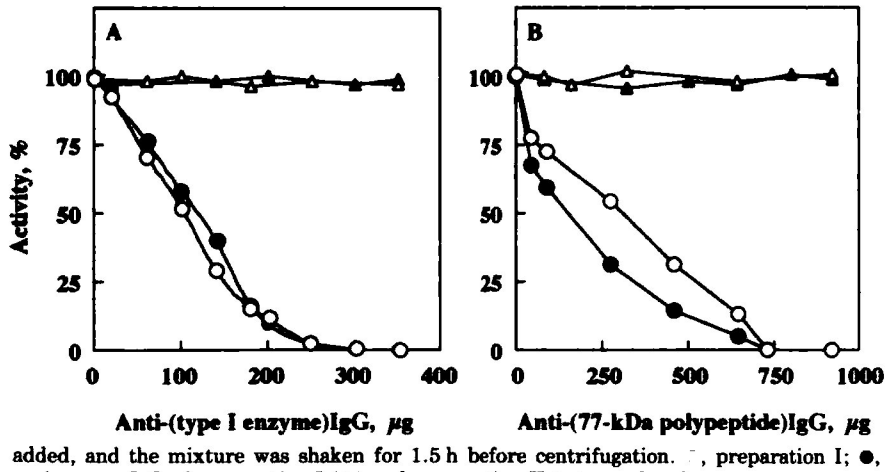


Fig. 5. **Titration of preparations I and II with the antibodies.** Preparation I (0.6 μg) and preparation II (1.0 μg) were mixed with antibodies at indicated amounts at room temperature in 50 μl of 150 mM NaCl, 10 mM potassium phosphate, pH 7/0.02% Tween 20/2 mM mercaptoethanol/1 mM EDTA. The following protease inhibitors were added to this mixture at 20 μg/ml: antipain, bestatin, chymostatin, E-64, leupeptin, pepstatin A, and phosphoramidon. The mixture was centrifuged at 10,000×g for 5 min, and the octenoyl-CoA hydratase activity in the supernatant was assayed. Panel A, titration with anti-(preparation I)-IgG. The preincubation time was 30 min. Panel B, titration with anti-(77-kDa polypeptide)IgG. Protein A-Sepharose was added, and the mixture was shaken for 1.5 h before centrifugation. ○, preparation I; ●, preparation II. The results of treatment with a preimmune IgG of preparation I (△) and preparation II (▲) are also shown.

both the 77- and 46-kDa polypeptides are responsible for the enzyme activity. We did not include this procedure in the purification of preparation II, because the removal of urea sometimes resulted in a decrease in the enzyme activity to about one half.

*Immunochemical Properties*—As shown in Fig. 4, A and B, the antibody against preparation I recognized the subunit of preparation I and the 46- and 77-kDa polypeptides of preparation II in immunoblot analysis. The antibody against the 77-kDa polypeptide of preparation II also recognized both preparation I and the 46- and 77-kDa polypeptides of preparation II.

Preparations I and II were not detected with the antibodies raised against crotonase, the bifunctional protein, and the trifunctional protein. Crotonase, the bifunctional protein, and the trifunctional protein were clearly detected only with the corresponding antibodies against rat enzymes (Fig. 4C).

Preparations I and II were titrated with the antibody against preparation I (Fig. 5A). Equivalence amounts of the antibody to titrate 0.6 $\mu$g of preparation I and 1.0 $\mu$g of preparation II were nearly the same. This indicates that anti-preparation-I antibody titrates the same molar amounts of the preparations I and II, because the ratio of molecular masses of these enzymes was 0.56 to 1.0. Preparations I and II were also titrated with the antibody against 77-kDa polypeptide in the presence of protein A-Sepharose (Fig. 5B). The equivalence amounts of the antibody to these two enzyme preparations were also similar.

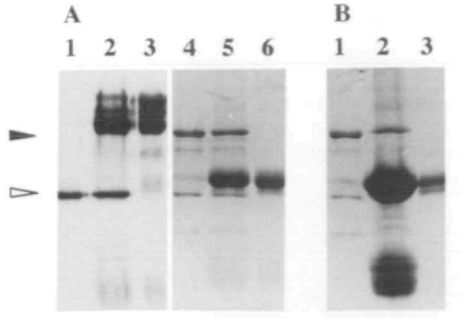


Fig. 6. **SDS-PAGE of immunoprecipitates.** Preparation I (1.5 $\mu$g) and preparation II (2 $\mu$g) were treated with antibody in a total volume of 100 $\mu$l of the same reaction mixture used for the experiment in Fig. 5. The immunoprecipitates were washed with phosphate-buffered saline and subjected to SDS-PAGE. Panel A, anti-preparation-I antibody was used. Lane 1, preparation I (1.5 $\mu$g); lane 2, immunoprecipitate of preparation I; lanes 3 and 6, antibody (5 $\mu$g); lane 4, preparation II (2 $\mu$g); lane 5, immunoprecipitate of preparation II. The immunoprecipitates were treated with sampling buffer with (lanes 4, 5, 6) or without (lanes 1, 2, 3) mercaptoethanol. Panel B, anti-77-kDa polypeptide antibody was used. Lane 1, preparation II (2 $\mu$g), lane 2, immunoprecipitate of preparation II; lane 3, antibody (3 $\mu$g). Closed and open arrowheads indicate the positions of 77 and 46 kDa, respectively.

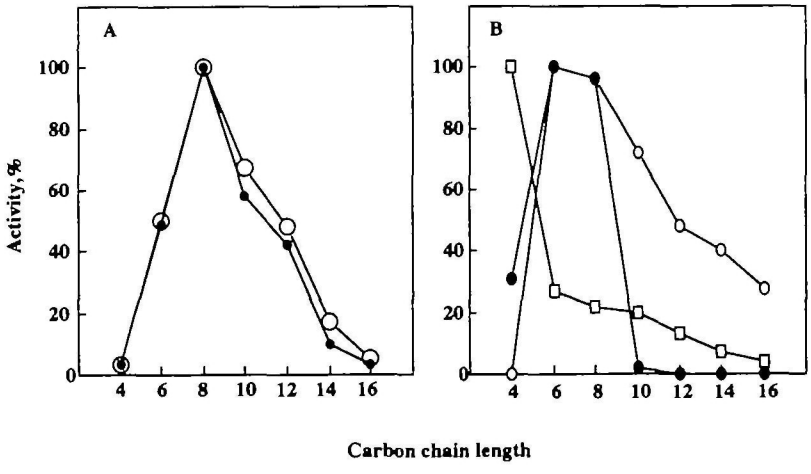


Fig. 7. **Carbon chain length specificities.** Panel A, preparation I (○) and preparation II enzyme (●); panel B, crotonase (□), bifunctional protein (●), and trifunctional protein (○). The activities were assayed at a fixed concentration of 0.1 mM enoyl-CoAs.

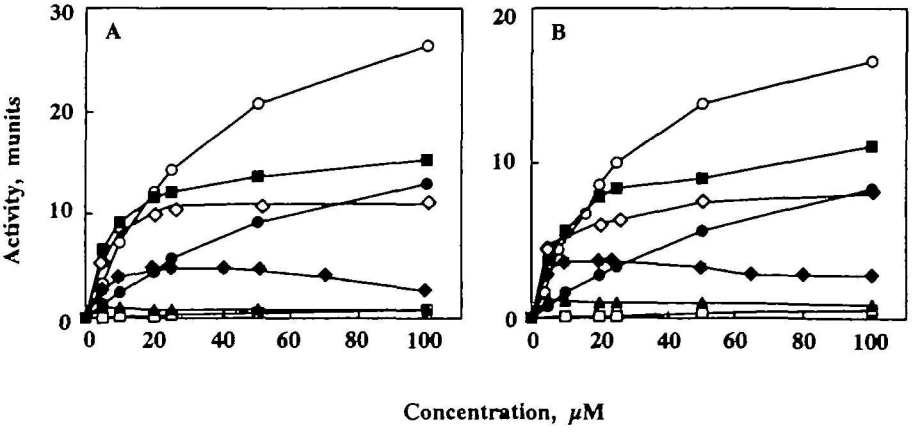


Fig. 8. **Velocity of the hydratase reaction as a function of substrate concentration.** Panel A, preparation I; panel B, preparation II. The amounts of preparations I and II were 0.05 $\mu$g, respectively. □, crotonyl-CoA; ●, hexenoyl-CoA; ○, octenoyl-CoA; ■, decenoyl-CoA; ◇, dodecenoyl-CoA; ◆, tetradecenoyl-CoA; ▲, hexadecenoyl-CoA.

TABLE IV. **Kinetic parameters of human enzymes catalyzing the enoyl-CoA hydratase reaction.**

| Substrate[a] | Crotonase | | BP[b] | | TP[b] | | Preparation I | | Preparation II | |
|---|---|---|---|---|---|---|---|---|---|---|
| | $V_{max}$ | $K_m$ | $V_{max}$ | $K_m$ | $V_{max}$ | $K_m$ | $V_{max}$ | $K_m$ | $V_{max}$ | $K_m$ |
| $C_4$ | 2,300 | 30 | 160 | 20 | – | – | – | – | – | – |
| $C_6$ | 530 | 15 | 530 | 28 | 95 | 110 | 745 | 100 | 348 | 100 |
| $C_8$ | 400 | 9 | 500 | 25 | 53 | 30 | 909 | 30 | 470 | 40 |
| $C_{10}$ | 360 | 7 | – | – | – | – | 364 | 12 | 197 | 9 |
| $C_{12}$ | 210 | 5 | – | – | – | – | 282 | 4 | 155 | 5 |
| $C_{14}$ | 149 | 5 | – | – | – | – | – | – | – | – |
| $C_{16}$ | – | – | – | – | – | – | – | – | – | – |

[a]Substrates are expressed as carbon chain length. [b]BP, the bifunctional protein; TP, the trifunctional protein. $V_{max}$ is expressed as units/mg protein, and $K_m$ is expressed as $\mu$M. –, values were not determined due to low activity or marked substrate inhibition.

TABLE V. **Relative velocities in the forward and reverse reactions.** The forward reaction was assayed with octenoyl-CoA, and the reverse reaction with L-(−)-3-hydroxyacyl-CoA (L-isomer) or D-(+)-3-hydroxyacyl-CoA (D-isomer). Activities of the reverse reaction were expressed relative to those of the forward reaction.

| Enzyme | Forward reaction | Reverse reaction | |
|---|---|---|---|
| | Octenoyl-CoA | L-isomer | D-isomer |
| Preparation I | 100 | <0.01 | 86 |
| Preparation II | 100 | <0.01 | 90 |
| Crotonase | 100 | 66 | <0.01 |
| Bifunctional protein | 100 | 53 | <0.01 |
| Trifunctional protein | 100 | 82 | <0.01 |

When preparations I and II were treated with either anti-preparation-I antibody or anti-77-kDa polypeptide antibody, recoveries of 46- and 77-kDa polypeptides from these enzyme preparations in the immunoprecipitates were not quantitative. It was noticed that the enzyme proteins, especially the 77-kDa polypeptide, disappeared during incubation with the antibody. Recoveries of 46- and 77-kDa polypeptides in the immunoprecipitates were improved by addition of protease inhibitors to the reaction mixture. Therefore, in the experiments of Figs. 5 and 6, protease inhibitors were used.

The immunoprecipitate of preparation I with anti-preparation-I antibody was treated with sampling buffer without mercaptoethanol, but that of the preparation-II enzyme was treated with the usual sampling buffer, because the migration positions of IgG interfered with the observation of the enzyme polypeptides (Fig. 6A). Nearly quantitative recoveries of the 46-kDa polypeptide of preparation I and the 77-kDa polypeptide and other polypeptides of preparation II were confirmed. When anti-77-kDa polypeptide antibody was used, the 77-kDa polypeptide and other polypeptides in preparationn II were confirmed in the immunoprecipitate (Fig. 6B), but quantitative recovery of the subunit of preparation I in the immunoprecipitate could not be confirmed, because a larger amount of IgG recovered by protein A-Sepharose gave polypeptide bands around the position of 46 kDa.

Human crotonase was immunoprecipitated by treatment with the antibody against rat crotonase, but the bifunctional protein and the trifunctional protein were not precipitated by the antibodies against the rat enzymes or by the antibodies against the human enzymes (data not shown). However, these antibodies immunoprecipitated the denatured human enzymes.

*Catalytic Properties*—Activities of preparations I and II and other human enzymes were determined with enoyl-CoAs. The carbon chain length specificities of these preparations were very similar (Fig. 7A), and quite different from those of crotonase, the bifunctional protein, and the trifunctional protein (Fig. 7B).

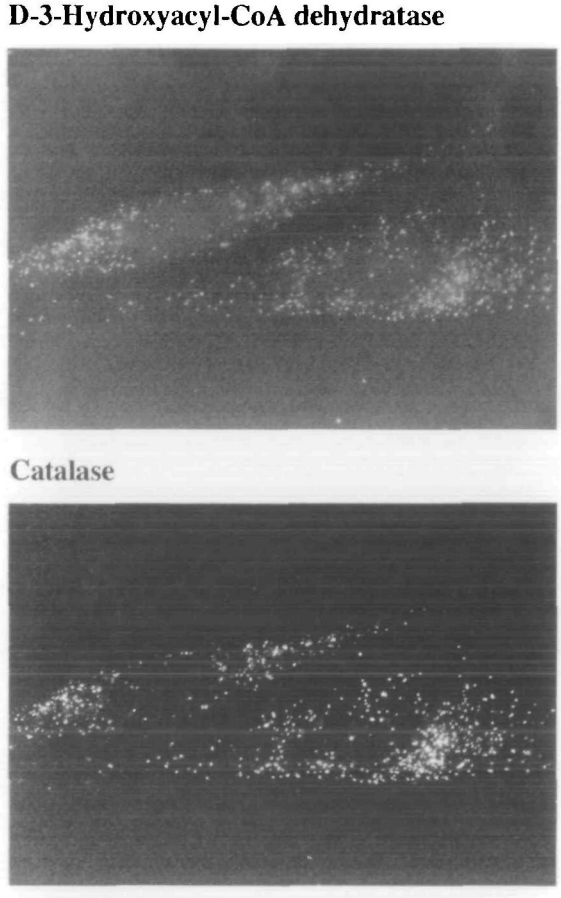


Fig. 9. **Localization of D-3-hydroxyacyl-CoA dehydratase in human skin fibroblasts.** Cultured human skin fibroblasts were doubly stained, and the same cells were photographed.

Both the enzyme preparations also showed nearly the same patterns of velocity-substrate concentration relationship (Fig. 8). Their activities with crotonyl-CoA were very low and linear with the substrate concentration up to 0.2 mM. The two enzymes showed normalized patterns for hexenoyl-CoA, octenoyl-CoA, decenoyl-CoA, and dodecenoyl-CoA. The kinetic parameters of the enzyme preparations were estimated only for these substrates (Table IV). $V_{max}$ and $K_m$ values of the two enzymes were very similar.

Kinetic parameters of crotonase were estimated for all of the substrates except hexadecenoyl-CoA, but those of the

bifunctional protein were obtained only for crotonyl-CoA, hexenoyl-CoA, and octenoyl-CoA, because of a marked substrate inhibition with longer carbon chain length substrates. The trifunctional protein exhibited a very low activity with crotonyl-CoA. Normalized patters were obtained with hexenoyl-CoA and octenoyl-CoA for this enzyme. The trifunctional protein was active with longer carbon chain substrates, but the parameters were not estimated, because double reciprocal plots gave nonlinear patterns.

All of the enzymes catalyzed the octenoyl-CoA hydratase reaction (Table V). However, the 3-hydroxyacyl-CoA dehydratase reactions of these enzymes were different. Crotonase, the bifunctional protein, and the trifunctional protein catalyzed reversible conversion of the L-3-hydroxyoctanoyl-CoA to octenoyl-CoA but did not react with the D-isomer. Inversely, preparations I and II both reacted with D-3-hydroxyoctanoyl-CoA but not with the L-isomer (Table V).

*Localization of the Dehydratase*—Subcellular fractionation could not be done for human liver. We confirmed the monospecificity of the antibody to the fibroblast extract: a signal corresponding to 77 kDa was clearly seen but a signal for 46 kDa was hardly detectable. The amount of the cross-reactive materials in the fibroblasts was determined to be 0.3 $\mu$g/mg protein using preparation II as standard. The octenoyl-CoA hydratase activity titer was 140 milliunits/mg. The D-3-hydroxyoctanoyl-CoA dehydratase titer was 120 milliunits/mg, and the activity disappeared on treatment with the antibody. The calculated specific activities of 470 and 400 units/mg enzyme for these reactions were comparable to the values for the purified preparation II. The carbon chain length susbstrate specificity of the titrated hydratase activity was similar to that of the purified enzyme (data not shown). Therefore, intracellular localization of the enzyme was carried out with cultured human skin fibroblasts by indirect immunofluorescence. The typical punctate fluorescence pattern shown in Fig. 9 clearly indicates that the cross-reactive material is localized in peroxisomes. The pattern of the fluorescence for D-3-hydroxyacyl-CoA dehydratase was superimposable on distribution of catalase, a peroxisomal marker, in the same cell.

## DISCUSSION

Human medium-chain enoyl-CoA hydratase was purified from liver, because we noticed that a high medium-chain enoyl-CoA hydratase activity was present in cultured human skin fibroblasts, and that its carbon chain length specificity was different from those of the known enzymes catalyzing the enoyl-CoA hydratase reaction. Two enzyme preparations were obtained. Preparation I was concluded to be a homodimer of a 46-kDa polypeptide. Preparation II had a molecular mass of 154 kDa and was composed of a main polypeptide of 77 kDa and several minor smaller polypeptides.

D-3-Hydroxyacyl-CoA dehydratase was purified from rat liver by three groups (*9-11*), but the molecular structures of the purified preparations were different. One of these preparations was structurally similar to our preparation I, and the others were structurally similar to our preparation II.

Preparations I and II were closely related. (i) Both preparations and their antibodies exhibited nearly the same cross-reactivity by immunoblot analysis, and titration of the enzymes. (ii) During purification, intensity of the immunoblot signal for the 77-kDa polypeptide was observed to be gradually decreased, and that for the 46-kDa polypeptide was inversely increased. (iii) The result shown in Fig. 3 indicates that both the 46- and 77-kDa polypeptides exhibit the dehydratase activity. (iv) Both preparations exhibited very similar catalytic properties in respect to reversible conversion of enoyl-CoA and D-3-hydroxyacyl-CoA, carbon chain length specificities, specific enzyme activities, and $K_m$ values.

By immunoblot analysis of liver extract, the main cross-reactive material was of 77 kDa, but a faint signal for the 46-kDa polypeptide was observed. The major signal was of 77 kDa in human skin fibroblasts, and the signal band corresponding to 46 kDa was very faint (data not shown). These results suggest that the enzyme consists of the 77-kDa polypeptide, a small amount of which is degraded inside the cells, and that preparations I and II, especially preparation I, are degradation products formed during purification. This speculation remains to be clarified.

$\beta$-Oxidation of polyunsaturated fatty acids requires auxiliary enzymes. Epimerization reaction of 3-hydroxyacyl-CoA is confirmed for peroxisomes but not for mitochondria (*18*). Epimerization involves two reactions. The first is dehydration of D-3-hydroxyacyl-CoA to 2-*trans*-enoyl-CoA catalyzed by an enzyme referred to as D-3-hydroxyacyl-CoA dehydratase. The second is hydration of 2-*trans*-enoyl-CoA to L-3-hydroxyacyl-CoA catalyzed by the bifunctional protein.

These two enzymes catalyze the reversible conversion of enoyl-CoAs to D-3-hydroxyacyl-CoAs but not to L-3-hydroxyacyl-CoAs. Therefore, the purified enzyme is not medium-chain enoyl-CoA hydratase, but D-3-hydroxyacyl-CoA dehydratase.

The enzyme, which was assumed to be medium-chain-specific enoyl-CoA hydratase, is peroxisomal D-3-hydroxyacyl-CoA dehydratase, not a mitochondrial enzyme. This dehydratase is involved in epimerization of D-3-hydroxyacyl-CoA, which is formed by $\beta$-oxidation of polyunsaturated fatty acids, to L-3-hydroxyacyl-CoA, which must proceed in combination with the bifunctional protein.

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