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Physiological Role of D-3-Hydroxyacyl-CoA Dehydratase/D-3-Hydroxyacyl-CoA Dehydrogenase Bifunctional Protein[1]

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The second and third reactions of the peroxisomal β-oxidation spiral are thought to be catalyzed by enoyl-CoA hydratase/L-3-hydroxyacyl-CoA dehydrogenase bifunctional protein (L-bifunctional protein). Recently, we found the presence of D-3-hydroxyacyl-CoA dehydratase/D-3-hydroxyacyl-CoA dehydrogenase bifunctional protein (D-bifunctional protein) in mammalian peroxisomes. Therefore, we studied the physiological role of the D-bifunctional protein. The contents of the L- and D-bifunctional proteins were about 0.01 and 0.5 μg/mg protein, respectively, in cultured human skin fibroblasts. The activity of conversion of hexadecenoyl-CoA to 3-ketopalmitoyl-CoA by the D-bifunctional protein was estimated to be about 0.5 milliunit/mg of fibroblast protein. This value was about 100-fold that of the L-bifunctional protein in the fibroblasts. From comparison of the activities of the bifunctional proteins with the rate of palmitate oxidation and the activities of acyl-CoA oxidase and 3-ketoacyl-CoA thiolase, it is proposed that the D-bifunctional protein plays a major role in the peroxisomal oxidation of palmitate in the fibroblasts. The contents of both the L- and D-bifunctional proteins in liver were about 2.5 μg/mg protein. Therefore, it is suggested that the D-bifunctional protein also plays a significant role in human liver peroxisomal fatty acid oxidation. Actions of the bifunctional proteins on enoyl forms of other acyl-CoA derivatives were examined. The D-bifunctional protein but not the L-bifunctional protein reacted with 2-methylhexadecenoyl-CoA and 3α,7α,12α-trihydroxy-5β-cholest-24-enoyl-CoA. We propose that, among the reactions of the distinct group of carboxylates oxidized specifically in peroxisomes, oxidation of 2-methyl-branched fatty acids and side-chain shortening of cholesterol for bile acid formation are catalyzed by the D-bifunctional protein, but not the L-bifunctional protein.

Key words: bifunctional protein, D-3-hydroxyacyl-CoA dehydratase, D-3-hydroxyacyl-CoA dehydrogenase, β-oxidation, peroxisomal.

One of the main functions of peroxisomes in mammals is the β-oxidation of fatty acids and related compounds: very-long-chain fatty acids, dicarboxylic acids, pristanic acid, eicosanoids, and di- and trihydroxycoprostanoic acids, whose carboxylates are activated to their CoA esters, and the carbon chains are shortened inside peroxisomes in four consecutive steps (*1, 2*).

Dehydrogenation of acyl-CoA esters to their corresponding *trans*-2-enoyl-CoAs is catalyzed by various acyl-CoA oxidases. The first acyl-CoA oxidase (*3*) acts on various saturated fatty acyl-CoA esters, but not on pristanoyl-CoA (2,6,10,14-tetramethylpentadecanoyl-CoA), a branched-chain fatty acyl-CoA ester. The second oxidase, pristanoyl-CoA oxidase, catalyzes 2-methyl-branched-chain acyl-CoA and straight-chain acyl-CoA, and the third oxidase reacts with trihydroxycoprostanoyl-CoA (*4*).

The second and third reactions, hydration and dehydrogenation of different enoyl-CoA esters to 3-ketoacyl-CoAs are assumed to be catalyzed by a single enzyme, enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase bifunctional protein (L-bifunctional protein) (*5*), which was later confirmed to be a trifunctional protein additionally having the isomerase activity (*6*).

Recently, we have purified D-3-hydroxyacyl-CoA dehydratase/D-3-hydroxyacyl-CoA dehydrogenase bifunctional protein (D-bifunctional protein), which converts enoyl-CoAs to 3-ketoacyl-CoAs *via* D-3-hydroxyacyl-CoAs in peroxisomes, and suggested that the enzyme(s) named D-3-hydroxyacyl-CoA dehydratase (*7-9*) is the D-bifunctional protein (*10, 11*).

We plan to study the physiological and pathological roles

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Abbreviations: D-bifunctional protein, D-3-hydroxyacyl-CoA dehydratase/D-3-hydroxyacyl-CoA dehydrogenase bifunctional protein; L-bifunctional protein, enoyl-CoA hydratase/L-3-hydroxyacyl-CoA dehydrogenase bifunctional protein; trifunctional protein, enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase/3-ketoacyl-CoA thiolase trifunctional protein; 24E-THC-CoA, 3α,7α,12α-trihydroxy-5β-cholest-24-enoyl-CoA.

of the D-bifunctional protein. We suspect that the peroxisomal β-oxidation in the fibroblasts proceeds from enoyl-CoA through D-3-hydroxyacyl-CoA to 3-ketoacyl-CoA by the D-bifunctional protein, because the content of the L-bifunctional protein is very low in cultured human skin fibroblasts compared to that of the D-bifunctional protein (*12*). The other consideration is to examine whether enoyl-CoA esters produced from dicarboxylic acids, 2-methyl-branched-chain fatty acids, and trihydroxycoprostanoic acid are catalyzed by the L-bifunctional protein as previously assumed.

In this study, we estimated the contents and the enzyme activities of the peroxisomal enzymes catalyzing conversion of enoyl-CoA to 3-ketoacyl-CoA in cultured human skin fibroblasts. The data indicate that the D-bifunctional protein plays a major role in the peroxisomal β-oxidation in the fibroblasts. An experiment focusing on bile acid formation was also carried out. The data suggest that conversion of $3\alpha,7\alpha,12\alpha$-trihydroxy-5β-cholest-24-enoyl-CoA (24E-THC-CoA) to the keto form, presumably $3\alpha,7\alpha,12\alpha$-trihydroxy-24-keto-5β-cholestanoyl-CoA, is catalyzed by the D-bifunctional protein but not the L-bifunctional protein.

MATERIALS AND METHODS

Materials—The D- and L-bifunctional proteins (*10*), crotonase (*10*), and enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase/3-ketoacyl-CoA thiolase trifunctional protein (trifunctional protein) (*12*) were purified from human liver. Antibodies against these human enzymes were prepared, except that the anti-crotonase antibody was raised against the rat enzyme (*13*). Since human acyl-CoA oxidase and peroxisomal 3-ketoacyl-CoA thiolase were not purified, rat enzymes and their antibodies were used (*3, 14*).

Straight-chain fatty acyl-CoA derivatives were prepared as described previously (*15*). 2-Methylpalmitoyl-CoA was synthesized by the mixed anhydride method and purified (*15*). Hexadecanedionoyl-mono-CoA and CoA derivative of *N*-(α-methylbenzyl)azelaamic acid were donated by T. Suga, Tokyo College of Pharmacy. These CoA esters were converted to the enoyl forms by incubating with *Candida* sp. acyl-CoA oxidase (Toyobo, Osaka). 24E-THC-CoA was synthesized (*16, 17*).

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 (*10, 11*).

Conversion of enoyl-CoAs to 3-ketoacyl-CoAs by the purified bifunctional proteins was assayed by either of the following methods A and B. The reaction mixture of method A contained 20 mM potassium phosphate, pH 7.0, 100 mM KCl, 20 μM enoyl-CoA, 100 μM NAD^+, 50 μM CoA, and 100 μM dithiothreitol in the presence or absence of 0.2 mg/ml BSA in a total volume of 1 ml. On addition of 5 μg or more of either the L-bifunctional protein or the D-bifunctional protein, absorbance at 340 nm started to increase and reached at a plateau. Then, the reaction was started by addition of 5 μg or more of rat liver peroxisomal 3-ketoacyl-CoA thiolase. The mixture of method B contained 100 mM Tris, 100 mM KCl, 20 μM enoyl-CoA, 200 μM NAD^+. Increase in absorbance at 340 nm was also followed after addition of the enzyme.

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

Procedures for Protein Analysis—Protein concentration was determined by a modification (*18*) of a procedure of Lowry *et al.* (*19*). SDS-PAGE was carried out as described by Laemmli (*20*). Blotting of the electrophoresed polypeptides to a nitrocellulose sheet was done electrophoretically (*21*), and color development was done with the use of the second antibody conjugated with alkaline phosphatase.

The contents of the enzyme proteins were determined by immunoblot analysis. Various amounts of the purified enzymes and the extracts, which were prepared as described below, were subjected to SDS-PAGE and immunoblot analysis. The signal bands were quantified using a densitometer (Cliniscan 2®, Helena, Beaumont, TX).

Cell Culture and Preparation of the Extract—Human skin fibroblasts and HepG2 cells, which were derived from a human hepatoblastoma, were cultured in a medium containing Dulbecco's modified Eagle's medium, 10% (v/v) fetal calf serum, 0.1 mM nonessential amino acids, 1× antibiotic-antimycotic solution (GIBCO/BRL Oriental, Tokyo) and 4.5 mg/ml D-glucose. The cells were collected after trypsinization.

The cell pellet was suspended in 50 mM potassium phosphate, pH 7.5/0.5% Tween 20/0.2 M NaCl/2 mM mercaptoethanol/1 mM EDTA, and sonified. After standing on ice for 30 min, the suspension was centrifuged at 10,000×g for 10 min, and the supernatant was saved for use.

Human livers were obtained at autopsy and stored at −80°C. One of the liver samples was used in this study, because the D-bifunctional protein contents and the D-3-hydroxyoctanoyl-CoA dehydratase activities of four samples were similar. The frozen liver was homogenized with four volumes of 50 mM potassium phosphate, pH 7.5/1% Tween 20/0.2 M NaCl/2 mM mercaptoethanol/1 mM EDTA, and the mixture was centrifuged at 10,000×g for 30 min.

RESULTS AND DISCUSSION

Contents of L- and D-Bifunctional Proteins—Contents of the L- and D-bifunctional proteins in the cultured human skin fibroblasts were estimated by immunoblot analysis (Fig. 1).

The signal band for the D-bifunctional protein from the fibroblasts of three persons migrated at the same position as the purified enzyme (Fig. 1A). This band was specifically abolished when the antibody was previously mixed with the D-bifunctional protein.

A faint signal band was observed at nearly the same migration position as the purified L-bifunctional protein, together with several intense signal bands in the same area (Fig. 1B, lane 3). The slight difference in the position of the band for the fibroblasts from that of the purified enzyme was thought to be due to the use of a larger amount of the extract. To confirm the migration positions, a mixture of the purified enzyme and the fibroblast extract was examined. The suspected band for the fibroblasts and the band for the enzyme were migrated at the same position (Fig. 1B, lane 2). This band for the fibroblasts was speci-

fically abolished by the antibody which had been previously mixed with the L-bifunctional protein (data not shown), thus confirming it to be the signal band for the L-bifunctional protein.

Contents of catalase, acyl-CoA oxidase, the L-bifunctional protein, the D-bifunctional protein, and peroxisomal 3-ketoacyl-CoA thiolase in the fibroblasts, liver, and HepG2 cells estimated by immunoblot analysis are listed in Table I.

The ratios of the contents of the L- and D-bifunctional proteins in the cultured cells and liver seemed to differ remarkably, although the contents of these bifunctional proteins in various tissues were not studied. It is possible that the contents of these proteins varied with cell culture conditions.

Activities of the Two Bifunctional Proteins—The D-bifunctional protein catalyzes the reversible conversion of D-3-hydroxyacyl-CoA to enoyl-CoA. Carbon-chain-length specificity of the D-bifunctional protein was examined in direction of the enoyl-CoA hydratase reaction, because we had only D-3-hydroxyoctanoyl-CoA but not other D-3-hydroxyacyl-CoAs with various carbon-chain lengths. The enoyl-CoA hydratase reaction is catalyzed by crotonase, enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase/3-ketoacyl-CoA thiolase trifunctional protein (trifunctional protein), the L-bifunctional protein, and the D-bifunctional protein. The enzyme activities of crotonase and the D-bifunctional protein were distinguished by titration with the specific antibodies, but the native forms of the other proteins were not titrated by the corresponding antibodies.

As shown in Fig. 2A, treatment of the fibroblast extract with anti-crotonase antibody led to a marked decrease in the crotonyl-CoA hydratase activity, indicating virtually complete titration of crotonase. Treatment of the extract with anti-D-bifunctional protein antibody resulted in a decrease in the activities with medium-chain substrates. The carbon-chain-length specificity of the titrated activities with anti-D-bifunctional protein antibody (Fig. 2B) was similar to that of the purified D-bifunctional protein (Fig. 7 of Ref. *10*).

The octenoyl-CoA hydratase activity titrated by anti-D-bifunctional protein antibody was 240 milliunits/mg protein. The D-3-hydroxyoctanoyl-CoA dehydratase activity was 160 milliunits/mg protein, and all of this activity disappeared after treatment with the antibody. The content of the D-bifunctional protein was estimated to be 0.5 μg/mg protein (Table I). The calculated specific enzyme activities for the hydratase and dehydratase reactions were 480 and 320 units/mg of the enzyme protein, respectively. The specific activities of the purified D-bifunctional protein for the octenoyl-CoA hydratase and D-3-hydroxyoctanoyl-CoA dehydratase reactions were 360 and 380 units/mg, respectively (*10*). The estimated values were not significantly different from those of the purified enzyme.

The substrate specificity of the remaining activities after treatment with both anti-crotonase and anti-D-bifunctional protein antibodies was similar to that of the trifunctional protein (*12*) but quite different from that of the L-bifunctional protein (*10*). The remaining octenoyl-CoA hydratase activity of 160 milliunits/mg corresponded to the calculated activity of the trifunctional protein content of about 2

TABLE I. **Contents of peroxisomal proteins in human fibroblasts, liver, and HepG2 determined by immunoblot analysis.** The values are expressed as μg per mg of protein.

Enzyme	Fibroblast	Liver	HepG2
Catalase	0.15	4.0	0.15
Acyl-CoA oxidase	0.02	0.3	0.6
L-Bifunctional protein	0.01	2.5	0.1
D-Bifunctional protein	0.5	2.5	0.6
3-Ketoacyl-CoA thiolase	0.1	0.9	0.4

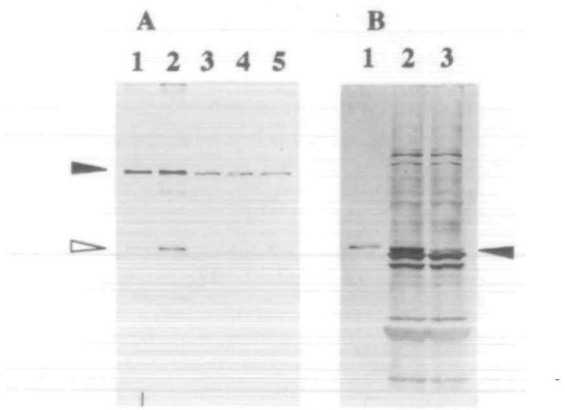

Fig. 1. **Immunoblot analysis of the D- and L-bifunctional proteins in the cultured human skin fibroblasts.** A, 10% gel; lane 1, D-bifunctional protein (10 ng); lane 2, D-bifunctional protein (20 ng); lanes 3, 4, and 5, fibroblasts from three persons (15 μg each). Solid and open arrowheads indicate the positions of 77- and 46-kDa polypeptides, respectively. B shows a part of the pattern obtained with a longer 8% gel. Lane 1, L-bifunctional protein (3 ng); lane 2, L-bifunctional protein (3 ng) plus fibroblasts (30 μg); lane 3, fibroblasts (30 μg). Arrowhead indicates the position of the band for the L-bifunctional protein.

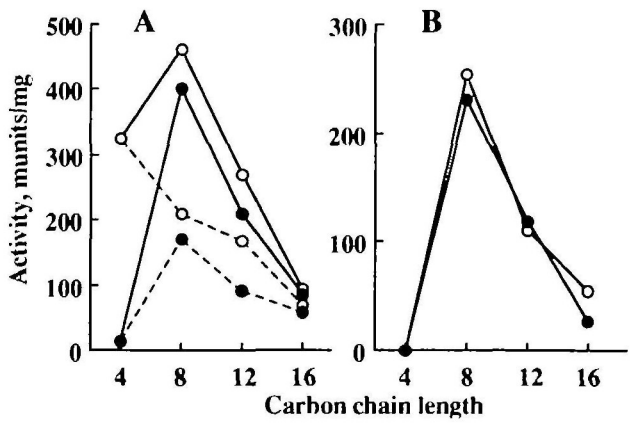

Fig. 2. **Carbon-chain-length specificities of enoyl-CoA hydratase activities in fibroblasts.** A: The activities of the fibroblast extract before and after immunoprecipitation with the antibodies against crotonase and the D-bifunctional protein. Open circles and solid line, fibroblast extract; open circles and dotted line, anti-D-bifunctional protein antibody; closed circles and solid line, anti-crotonase antibody; closed circles and dotted line, anti-crotonase and anti-D-bifunctional protein antibodies. B: The activities in the fibroblasts titrated by anti-D-bifunctional protein antibody. Open circles, differences between the activities before and after the treatment with anti-D-bifunctional protein antibody; closed circles, differences between the activity after treatment with anti-D-bifunctional protein antibody plus anti-crotonase antibody and that after treatment with anti-crotonase antibody.

μg/mg. The octenoyl-CoA hydratase activity of the L-bifunctional protein was about 3-fold that with crotonyl-CoA (*10*). The octenoyl-CoA hydratase activity of the L-bifunctional protein was estimated to be 40 milliunits/mg at maximum, assuming that all of the remaining crotonyl-CoA hydratase activity of 13 milliunits/mg was due to the L-bifunctional protein. The content of the L-bifunctional protein was 0.01 μg/mg (Table I). The activity is assumed to be about 4 milliunits/mg, based on the specific activity of 370 units/mg of the purified enzyme (*10*). Therefore, the L-bifunctional protein is thought to make no significant contribution to hydration of enoyl-CoA in the fibroblasts.

Figure 3A summarizes the results of titration of the liver extract. The octenoyl-CoA hydratase activity titrated by anti-D-bifunctional protein antibody was about 1.6 units/mg. The D-3-hydroxyoctanoyl-CoA dehydratase activity was also 0.74 unit/mg, and all of this activity was titrated by the antibody. The content of the D-bifunctional protein in liver was 2.5 μg/mg (Table I). The calculated specific enzyme activities were about 640 and 300 units/mg of the enzyme, respectively, for the hydratase and dehydratase activities of the D-bifunctional protein. The remaining liver crotonyl-CoA hydratase activity after titration with anti-crotonase antibody was 0.5 unit/mg. The specific activity was calculated to confirm the relation of the activity and the content of the D-bifunctional protein. The activity with octenoyl-CoA was calculated to be about 600 units/mg using the L-bifunctional protein content of 2.5 μg/mg (Table I). The results suggest that the contents of the L- and D-bifunctional proteins are nearly the same in human liver.

Figure 3B summarizes the results of titration of the HepG2 extract. The octenoyl-CoA hydratase activity of the extract of HepG2 cells titrated by anti-D-bifunctional protein antibody was 340 milliunits/mg. The D-3-hydroxyoctanoyl-CoA activity was 240 milliunits/mg, which was titrated by the antibody. The content of the D-bifunctional protein in HepG2 cells was 0.6 μg/mg. The calculated specific enzyme activities were about 570 and 400 units/mg, respectively, for the hydratase and dehydratase activities of the D-bifunctional protein. The remaining crotonyl-CoA hydratase activity after titration with anti-crotonase antibody was 34 milliunits/mg. The content of the L-bifunctional protein was 0.1 μg/mg (Table I). Using these values, the calculated specific activity with octenoyl-CoA was about 1,000 units/mg enzyme protein, which was higher than that of the purified L-bifunctional protein, perhaps due to incomplete titration of crotonase. The results suggest that the contents of the L-bifunctional protein in HepG2 cells was lower than the content of the D-bifunctional protein in spite of the incompatibility of the activity with the content.

It is noteworthy that determination of the D-3-hydroxyacyl-CoA dehydratase activity in the extract having a high enoyl-CoA hydratase activity is difficult, because the product of the dehydratase reaction is further converted to L-3-hydroxyacyl-CoA.

Conversion of Enoyl-CoAs to 3-Ketoacyl-CoAs by the Bifunctional Proteins—The activities of conversion of enoyl-CoAs to 3-ketoacyl-CoAs by the bifunctional proteins were determined by method A. The bifunctional proteins were preincubated with enoyl-CoA and NAD^+. After equilibrium of the hydratase and dehydrogenase reactions was attained, the reaction was started by addition of peroxisomal 3-ketoacyl-CoA thiolase in the presence of CoA (Fig. 4). Appropriate amounts of the enzymes differed when different carbon-chain-length substrates were used. But the maximal rate of increase in absorbance at 340 nm was attained in the presence of 2-5 μg of the peroxisomal 3-ketoacyl-CoA thiolase when 10 μg of either bifunctional protein was used.

Figure 5 shows the activities of the bifunctional proteins with various carbon-chain-length substrates. The L-bifunctional protein exhibited the highest activity with octenoyl-CoA, and the D-bifunctional protein exhibited maximal activity with decenoyl-CoA and dodecenoyl-CoA. Both enzymes were inactivated during preincubation with longer carbon-chain-length substrates such as hexadecenoyl-CoA, but the enzymes were protected from inactivation by addition of BSA at 0.2 mg/ml. The carbon-chain-length specificities of these two enzymes were different from those of the corresponding hydratase activities. Both the L- and D-bifunctional proteins exhibited similar specific enzyme activities with medium-chain-length substrates, but the activity of the D-bifunctional protein was much higher than

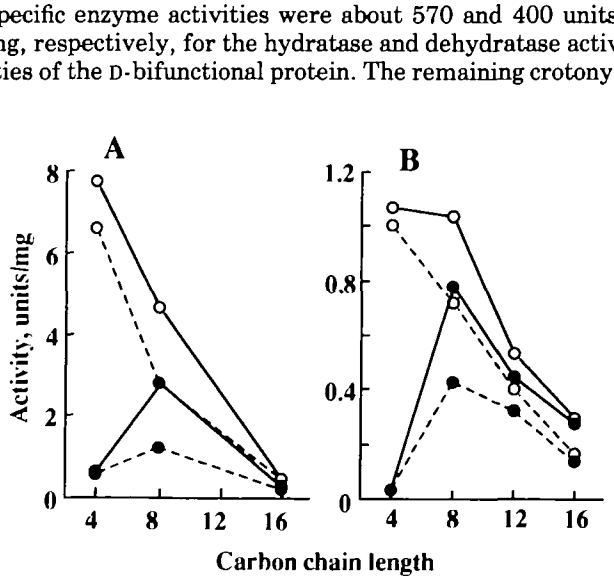

Fig. 3. **Enoyl-CoA hydratase activities in liver and HepG2 cells.** A, liver; B, HepG2 cells. Open circles and solid line, the extract; open circles and dotted line, anti-D-bifunctional protein antibody; closed circles and solid line, anti-crotonase antibody; closed circles and dotted line, anti-crotonase and anti-D-bifunctional protein antibodies.

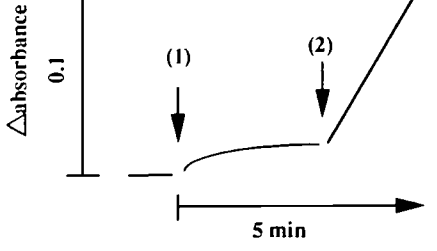

Fig. 4. **Determination of conversion rate of enoyl-CoA to 3-ketoacyl-CoA by method A.** The D-bifunctional protein (2 μg) was added to the reaction medium containing octenoyl-CoA, and the increase in absorbance at 340 nm was followed (arrow 1). After reaching equilibrium, 0.1 μg of 3-ketoacyl-CoA thiolase was added (arrow 2).

that of the L-bifunctional protein with longer carbon-chain-length substrates.

The activity was assayed with various concentrations of hexadecenoyl-CoA (Fig. 6). Both enzyme activities were higher at lower concentration of the substrate in the absence of BSA in the reaction mixture. When BSA was included at 0.2 mg/ml, the activity with low concentrations of the substrate was lower and reached maximal level at about 10 μM. The result indicates that the D-bifunctional protein exhibits a higher activity than the L-bifunctional protein.

Dependency of the activities on the substrate concentration should be considered. This was difficult to determine, however, because substrate inhibition was marked, especially for the L-bifunctional protein, and the activity was reduced owing to lowering of the substrate level by its binding to BSA added. However, apparent dependencies of the L- and D-bifunctional proteins on the substrate concentrations were similar in the presence of BSA.

Role of D-Bifunctional Protein in the Fibroblast β-Oxidation—The activity of acyl-CoA oxidase in the cultured human fibroblasts was determined to be 0.058 milliunit/mg of the fibroblast protein in our previous study (*22*). The content of acyl-CoA oxidase was 0.02 μg/mg of the fibroblast protein (Table I). The calculated specific enzyme activity of acyl-CoA oxidase was about 3 units/mg of the enzyme protein. This value was similar to those of the previously estimated values for human (*22*) and rat enzyme (*3*).

The calculated specific hydratase activity of the D-bifunctional protein in the fibroblasts was similar to that of the purified enzyme as described above. The 3-hydroxyacyl-CoA dehydrogenase activities of the D- and L-bifunctional proteins in the crude extracts could not be determined, because their activities were much lower than those of the epimerization reaction catalyzed by the hydratases and the D-bifunctional protein. Therefore, rates of conversion of hexadecenoyl-CoA to 3-ketopalmitoyl-CoA by the purified D- and L-bifunctional proteins were determined. The activities of the D- and L-bifunctional proteins were about 1 and 0.5 unit/mg of the enzyme, respectively (Fig. 6). Using these values and the contents of these enzymes, the rates of conversion by the D- and L-bifunctional proteins were calculated to be 0.5 and 0.005 milliunit/mg of the fibroblast protein, respectively.

We could not determine the peroxisomal thiolase activity, because only mitochondrial 3-ketoacyl-CoA thiolase was titrated by the antibody among the thiolases acting on 3-ketopalmitoyl-CoA. However, the peroxisomal 3-ketopalmitoyl-CoA thiolase activity was calculated to be about 3 milliunits/mg, using the contents of the peroxisomal thiolase shown in Table I and the specific enzyme activity of the human enzyme (unpublished data), which was similar to that of the rat enzyme (*23*).

The activities in the forward reaction in the fibroblasts assayed with palmitoyl-CoA and its intermediate derivatives in optimal *in vitro* conditions were as follows: acyl-CoA oxidase = 0.06, the D-bifunctional protein = 0.5, the L-bifunctional protein = 0.005, peroxisomal 3-ketoacyl-CoA thiolase = 3 milliunits/mg protein. The palmitate oxidation by peroxisomes in the cultured fibroblasts was 5 pmol/min per mg protein (*24*).

We propose, therefore, that the D-bifunctional protein plays a major role in the second and third reactions of the peroxisomal fatty acid oxidation in cultured human skin fibroblasts, and epimerization of D-3-hydroxyacyl-CoA, a product of polyunsaturated fatty acid oxidation, to the L-isomer by this enzyme is not important in the fibroblasts. The peroxisomal fatty acid oxidation system is established to play a major role in oxidation of very-long-chain fatty acids. We could not determine the individual enzyme activities because the substrates were not available. But we suspect that the D-bifunctional protein, but not the L-bifunctional protein, also plays a major role in oxidation of very-long-chain fatty acids in the fibroblasts. Evidence for

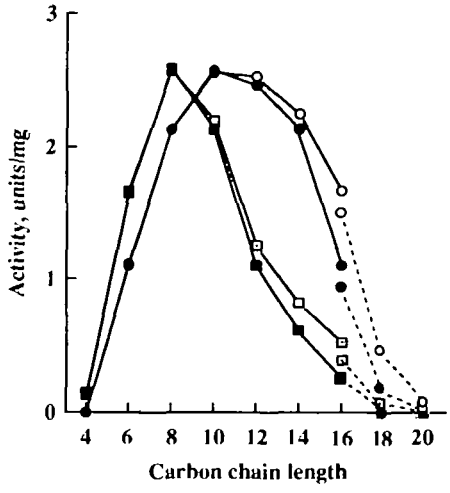

Fig. 5. **Conversion of enoyl-CoAs to 3-ketoacyl-CoAs by the bifunctional proteins.** Activities of the L- and D-bifunctional proteins with enoyl-CoAs having various carbon chain lengths from C_4 to C_{16} were determined by method A. These data are indicated by solid lines. Since enoyl-CoAs with 18-22 carbons were not available, palmitoyl-CoA and longer carbon chain acyl-CoAs were previously converted to enoyl-CoAs by incubation with one unit of *Candida* sp. acyl-CoA oxidase in the incubation mixture. These data are shown by dotted lines. ○ and ●, D-bifunctional protein; □ and ■, L-bifunctional protein; ○ and □, presence of BSA; ● and ■, absence of BSA.

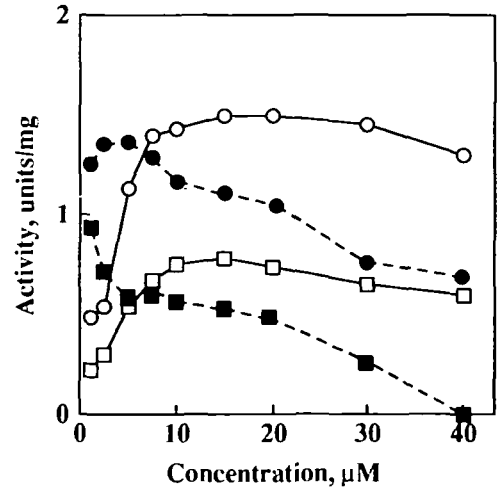

Fig. 6. **Relation of the activity and substrate concentration.** The activities with hexadecenoyl-CoA were assayed by method A. ○ and ●, D-bifunctional protein; □ and ■, L-bifunctional protein; ○ and □, presence of BSA; ● and ■, absence of BSA.

this idea is described below for the D-bifunctional protein deficiency.

The contents of the D- and L-bifunctional proteins in human liver were nearly the same (Table I). Therefore, the D-bifunctional protein is supposed to play a significant role in fatty acid oxidation even in human liver. In HepG2 cells, the content of the D-bifunctional protein was higher than that of the L-bifunctional protein. Therefore, the D-bifunctional protein is also more important in this type of cells.

Until recently, the second and third reactions of β-oxidation in the peroxisomal and mitochondrial systems were assumed to proceed *via* L-3-hydroxyacyl-CoA. The second and third reactions of β-oxidation in peroxisomes of *Saccharomyces cerevisiae* were demonstrated to proceed *via* D-3-hydroxyacyl-CoA (*25, 26*). It is interesting that two chirally different systems are present in mammalian cells, and in some cells, such as fibroblasts, the pathway *via* the D-isomer seems is dominant in peroxisomes.

D-Bifunctional Protein Catalyzes a Distinct Group of Fatty Acid Derivatives—Several acyl-CoA compounds other than very-long-chain and long-chain fatty acids are known to be oxidized predominantly in peroxisomes (*1*). The hydration and dehydrogenation of some of the enoyl-CoA derivatives were examined with the use of the D- and L-bifunctional protein (Table II).

Long-chain dicarboxylic acids (*27, 28*) and a xenobiotic acyl-compound, *N*-(α-methylbenzyl)azelaamic acid (*29*), are stated to be exclusively oxidized in rat liver peroxisomes. The rates of NADH formation with hexadecenedioyl-CoA and the enoyl form of a xenobiotic acyl-CoA derivative by the D- and L-bifunctional proteins were comparable to the oxidation rate of hexadecenoyl-CoA.

The β-oxidation of 2-methyl-branched fatty acids also takes place in peroxisomes (*30*). Deficiency of peroxisomal acyl-CoA oxidase resulted in accumulation of very-long-chain fatty acids, but not of pristanic acid (2,6,10,14-tetramethylpentadecanoic acid, an oxidative product of phytol) (*31*). On addition of acyl-CoA oxidase, the presence of two additional oxidases, which act on the CoA esters of 2-methyl-branched chain fatty acid and trihydroxycoprostanoic acid, was confirmed in peroxisomes (*32-34*). The second and third reactions of oxidation of these compounds have been generally assumed to be catalyzed by the L-bifunctional protein. Indeed, disturbance of the oxidation of pristanic acid and bile acid precursor has been described in patients with the L-bifunctional protein deficiency (*35-37*).

The oxidation rate of 2-methylhexadecenoyl-CoA by the D-bifunctional protein was about 1/10 of that of hexadecenoyl-CoA. But the oxidation of this CoA ester by the L-bifunctional protein was not detected (Table II).

Another characteristic feature of the peroxisomal β-oxidation is bile acid formation from di- and trihydroxy-5β-cholestanic acid to chenodeoxycholic acid and cholic acid, respectively. Two intermediates, 3α,7α,12α-trihydroxy-5β-cholest-24-enoic acid and 3α,7α,12α,24-tetrahydroxy-5β-cholestanoic acid, have been identified (*38*).

Conversion of 24*E*-THC-CoA to its keto form has been thought to be catalyzed by the L-bifunctional protein. Accumulation of 3α,7α,12α-trihydroxy-5β-cholestanoic acid in patients with isolated L-bifunctional protein deficiency has been thought to be evidence for this assumption. Recently, this has been called into question by results indicating that the L-bifunctional protein was not sufficient for the formation of the 24-keto product from 24*E*-THC-enoyl-CoA using the purified enzyme preparation (*39*).

The 24*E*-THC-CoA-dependent NADH formation was measured with the use of the D- and L-bifunctional protein. As shown in Table II, the D-bifunctional protein exhibited a high activity, but the L-bifunctional protein showed no activity.

The rate of NADH production in the presence of 24*E*-THC-CoA was not markedly pH-dependent in the range of pH 7-10, but the equilibrium was influenced by pH (Fig. 7). An apparent equilibrium constant of 1.2×10^{10} was obtained from the results for the dehydratase and dehydrogenase reactions.

The production of a keto form from 24*E*-THC-CoA was confirmed by effects of hydrazine and hydroxylamine on the enzyme reaction, because conventional spectrophotometric determination of the enolate form of the keto compound in

TABLE II. **Hydratase/dehydrogenase activities of human D- and L-bifunctional proteins with various substrates.** The NADH formation was assayed by method B with a fixed substrate concentration at 20 μM. The activity was expressed as units/mg enzyme.

Substrate/BSA	D-Bifunctional protein	L-Bifunctional protein
Hexadecenoyl-CoA		
+BSA	8.2	7.7
−BSA	4.3	6.4
Hexadecenedioyl-CoA		
+BSA	5.6	12
−BSA	4.6	7.3
E-C9-CoA*		
+BSA	5.6	13
−BSA	4.7	12
2-Methylhexadecenoyl-CoA		
+BSA	0.56	<0.007
−BSA	0.49	<0.003
24*E*-THC-CoA		
+BSA	2.5	<0.0001
−BSA	2.4	<0.001

*E-C9-CoA is an acyl-CoA oxidase product of CoA ester of *N*-(α-methylbenzyl)azelaamic acid.

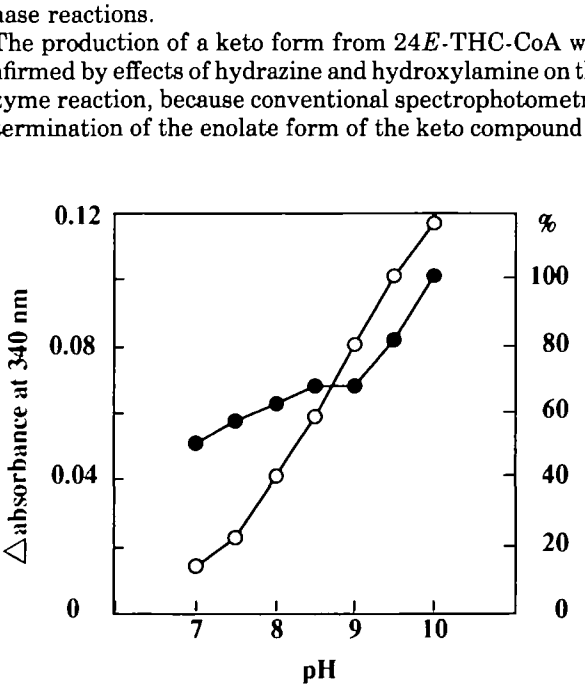

Fig. 7. **Effect of pH.** NADH production was determined by method B using 20 μM of 24*E*-THC-CoA, except that the pH of the reaction mixture was varied by addition of HCl. Open circles indicate the absorbance at 340 nm at equilibrium, and closed circles indicate the initial velocity expressed as %.

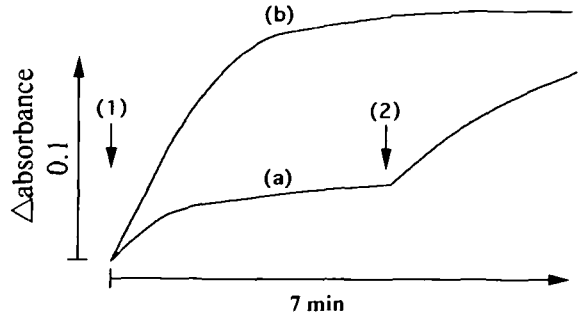

Fig. 8. **Effect of hydrazine on the reaction.** Method B was used, except the pH of the reaction mixture was adjusted to 8.0. The concentration of 24*E*-THC-CoA was 20 μM. (a) The D-bifunctional protein (1.5 μg) was added (arrow 1). When the equilibrium was attained, hydrazine was added to give a final concentration of 0.2 M (arrow 2). (b) The reaction was started in the reaction mixture containing 0.3 M hydrazine.

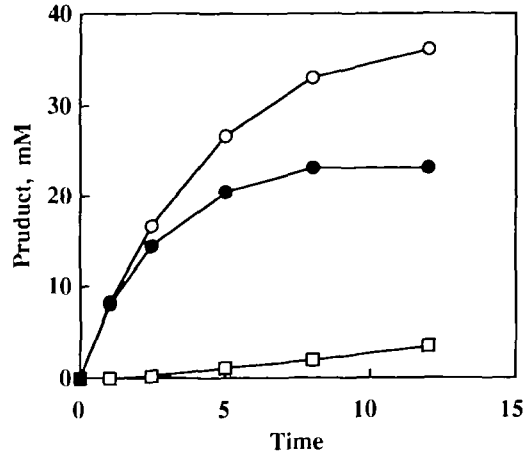

Fig. 9. **Accumulation of hydroxylamine-labile compound.** The reaction mixture of method B contained 40 μM 24*E*-THC-CoA and 400 μM NAD^+. The reaction was started by addition of the D-bifunctional protein. The NADH formation was followed. At appropriate time, 0.2 ml of the reaction mixture was taken and 20 μl of 2 M HCl was added to stop the reaction. The sample was neutralized by addition of 1 ml of 0.2 M Tris-HCl, pH 8.0, then freshly neutralized hydroxylamine was added to give a final concentration of 25 mM. Hydroxylamine-labile CoA was determined by following the increase in absorbance at 412 nm in the presence of 5,5′-dithiobis-(2-nitrobenzoate). ○, NADH; ●, hydroxylamine-labile CoA; □, free CoA.

the presence of Mg^{2+} was difficult (*40*).

As shown in Fig. 8, a previously attained equilibrium of the hydratase and dehydrogenase reactions at a lower pH was shifted by addition of hydrazine, which has been used as a trapping agent of ketones and aldehydes. When the enzyme reaction was started in the reaction mixture containing hydrazine, the reaction proceeded similarly to that observed in the reaction mixture with a high pH. It is known that 3-ketoacyl-CoA esters are extremely susceptible to attack by hydroxylamine, resulting in release of free CoA (*40*). The reaction was followed by measuring an increase in absorbance at 340 nm, and portions of the reaction mixture were taken and the hydroxylamine-labile CoA was determined. Accumulation of the hydroxylamine-labile CoA compound was confirmed (Fig. 9). A stoichiometrical relation between production of the hydroxylamine-labile CoA compound and an increase in NADH was not observed in the latter half of the reaction. The reason is not known, but it may be due to lability of the keto compound.

Enhancement of the reaction with 2-methylhexadecenoyl-CoA by hydrazine and accumulation of the hydroxylamine-labile compound were also confirmed (data not shown).

D-Bifunctional Protein Deficiency—We re-investigated of the fibroblasts from patients with peroxisomal disorders and found that two cell lines had no cross-reactive material to the antibody against the D-bifunctional protein. The disease of these two patients was previously diagnosed as the L-bifunctional protein deficiency. The D-3-hydroxyacyl-CoA dehydratase activity was not detected in the patients' fibroblasts. It was previously observed that peroxisomal oxidation of both palmitate and lignocerate in these cells was markedly reduced. The results indicate that the D-bifunctional protein plays a major role in the peroxisomal oxidation of long-chain and very-long-chain fatty acids. Furthermore, it is supposed that the D-bifunctional protein, but not the L-bifunctional protein, is responsible for the oxidation of 2-methyl-branched fatty acids and the side-chain oxidation of cholesterol for bile acid formation, since these metabolisms have been reported to be disturbed in patients whose disease was previously diagnosed as the L-bifunctional protein deficiency before elucidation of the presence of the D-bifunctional protein (*35-37*). The results of study on these two patients will be published elsewhere.

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