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

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When D-3-hydroxyacyl-CoA dehydratase/D-3-hydroxyacyl-CoA dehydrogenase bifunctional protein was purified from human liver, two preparations were obtained. One contained a 77-kDa polypeptide as the main component and minor smaller polypeptides including a 46-kDa polypeptide, and this preparation showed both the dehydratase and dehydrogenase activities. The other preparation was a homodimer of the 46-kDa polypeptide and showed only the dehydratase activity. Further analysis indicated that the native enzyme is a homodimer of 77-kDa polypeptide, which was proteolytically modified during purification. The cDNA for the human 77-kDa polypeptide was cloned. The amino acid sequences of the peptides derived from the components of the enzyme preparations were located in the deduced amino acid sequence of the cDNA. The preparation containing the 77-kDa polypeptide was treated with a protease, and two monofunctional fragments were separated. The dehydrogenase and dehydratase fragments were located on the amino- and carboxyl-terminal sides, respectively, of the deduced amino acid sequence of the cDNA. The protein expressed by the cDNA with the entire coding region exhibited both the dehydratase and dehydrogenase activities, and that expressed by a truncated version covering the carboxyl-terminal side exhibited only the dehydratase activity. The cloned cDNA was identical to the human 17β-hydroxysteroid dehydrogenase IV cDNA.

Key words: bifunctional protein, enzyme structure, human enzyme, D-3-hydroxyacyl-CoA dehydratase, D-3-hydroxyacyl-CoA dehydrogenase.

Two multifunctional enzymes catalyze the enoyl-CoA hydratase and L-3-hydroxyacyl-CoA dehydrogenase reactions in higher animals. The peroxisomal enoyl-CoA hydratase and L-3-hydroxyacyl-CoA dehydrogenase bifunctional protein (L-bifunctional protein), a monomeric protein, was first described as a multifunctional protein involved in the peroxisomal fatty acid β-oxidation (*1*), but later this enzyme was confirmed to be a trifunctional protein having an isomerase activity together with the hydratase and dehydrogenase activities (*2*). Enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase/3-ketoacyl-CoA thiolase trifunctional protein (trifunctional protein) is located in mitochondria (*3*). This enzyme is a multienzyme complex, consisting of four molecules of the α-subunit having domains of enoyl-CoA hydratase and L-3-hydroxyacyl-CoA dehydrogenase and four molecules of the β-subunit having a 3-ketoacyl-CoA thiolase domain.

Recently, we purified peroxisomal D-3-hydroxyacyl-CoA dehydratase/D-3-hydroxyacyl-CoA dehydrogenase bifunctional protein (D-bifunctional protein) (*4, 5*). Two preparations exhibiting the dehydratase activity were obtained. Preparation II contained a 77-kDa polypeptide as the main component and minor smaller polypeptides including a 46-kDa polypeptide, and this preparation showed the dehydratase and dehydrogenase activities with D-3-hydroxyacyl-CoA. Preparation I was a homodimer of the 46-kDa polypeptide and showed only the dehydratase activity. It was supposed that the native enzyme was a homodimer of the 77-kDa polypeptide, and that the two purified preparations contained proteolytically modified enzymes. It has recently been confirmed that peroxisomal multifunctional protein of *Saccharomyces cerevisiae* catalyzes both the D-3-hydroxyacyl-CoA dehydratase and D-3-hydroxyacyl-CoA dehydrogenase reactions (*6*).

The structural analyses of the peroxisomal L-bifunctional protein (*7*) and the α-subunit of the mitochondrial trifunctional protein (*8, 9*) indicate that in both cases the hydratase domain is located on the amino-terminal side and the dehydrogenase domain on the carboxyl-terminal side. In the *S. cerevisiae* enzyme, however, the locations are reversed: the hydratase domain is located on the carboxyl-terminal side and the dehydrogenase domain on the amino-terminal side (*6*).

In this study, the human D-bifunctional protein, preparation II, was subjected to limited proteolysis, and the two monofunctional fragments were separated: one preparation showed the dehydratase activity, and the other showed the dehydrogenase activity. The cDNA for the D-bifunctional protein was cloned, and its deduced amino acid sequence was compared with those of the peptide fragments from the

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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.

components of the purified enzyme preparations and the enzymatically active fragments produced by limited proteolysis. The D-bifunctional protein, the active fragments separated after limited proteolysis, and the cDNA-directed expressed enzymes showed similar catalytic activities. The results indicate that the native enzyme of the D-bifunctional protein is a homodimer of the 77-kDa polypeptide, and that the hydratase domain is located on the carboxyl-terminal side of this polypeptide and the dehydrogenase domain on the amino-terminal side.

MATERIALS AND METHODS

Human Enzyme Preparations and the Antibodies—Human liver enzyme preparations I and II and their antibodies were prepared as described previously (*4*). One antibody was raised against the 77-kDa polypeptide isolated from preparation II, and the other was raised against preparation I, a homodimer of the 46-kDa polypeptide.

Enzyme Assay—The activities of enoyl-CoA hydratase and D-3-hydroxyacyl-CoA dehydratase were assayed by following the change in absorbance at 280 nm as described previously (*4, 5*).

The 3-hydroxyacyl-CoA dehydrogenase activity in the forward reaction was assayed by an increase in absorbance 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) (*10*).

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.

Separation of Two Enzyme Activities by Protease K Digestion—Preparation II (0.25 mg) was incubated with proteinase K (5 μg) at 30°C for 30 min in a total volume of 1 ml of 100 mM potassium phosphate, pH 7.5/0.2% (v/v) Tween 20/5% (v/v) ethylene glycol/2 mM mercaptoethanol/1 mM EDTA/0.2 mM NADH. The digestion was stopped by addition of phenylmethylsulfonyl fluoride. All buffers used for subsequent procedures contained 2 mM mercaptoethanol and 1 mM EDTA. The digested mixture was desalted by passage through a Sephadex G-25 column with 20 mM potassium phosphate, pH 7.5/0.2% Tween 20/10% ethylene glycol (buffer A), then applied to a Reactive Green 19 column. The two enzyme activities were separated by elution with a NaCl concentration gradient from 0 to 2 M in buffer A. The dehydratase activity was eluted first, and the dehydrogenase activity was eluted in the fractions at a high NaCl concentration.

Procedures for Protein Analysis—Protein concentration was determined by a modification (*11*) of the procedure of Lowry *et al.* (*12*). SDS-PAGE was carried out as described by Laemmli (*13*). Blotting of the electrophoresed polypeptides onto a nitrocellulose sheet was done electrophoretically (*14*), and color development was done with the use of the second antibody conjugated with alkaline phosphatase. For amino acid sequence analysis, the samples were subjected to SDS-PAGE, then transferred to a polyvinylidene difluoride membrane. The membrane containing polypeptide was cut out and subjected to protein sequencing in an Applied Biosystems 477A protein sequencer. When internal amino acid sequence was determined, the samples were partially digested in the stacking gel of SDS-PAGE with the use of proteinase K, protease Glu-C or Lys-C according to the method of Cleveland *et al.* (*15*).

Cloning of the cDNA—Human hepatic λgt11 cDNA library constructed with oligo(dT) primers (*9*) was screened immunologically using the two antibodies raised against the 77- and 46-kDa polypeptides (*16*). Several positive clones were obtained after three rounds of screening using 3×10^5 plaques. These clones did not extend to the 5′ upstream side containing the initial codon. Then, 2×10^5 plaques of random primed human hepatic λgt11 cDNA library (*9*) were screened with a radio-labeled PCR fragment of the amino-terminal side of the longest immunologically positive clone as a probe. A clone containing the 5′-noncoding region overlapped with the immunopositive clones was obtained. The cDNA encoding the entire amino acid sequence was constructed with these two clones. Truncated cDNA fragments were obtained by PCR amplification using appropriate primers. The cDNA clones were sequenced by the dideoxy chain termination method (*17*), and bases were determined using an Applied Biosystems 373A DNA sequencer.

Expression of Proteins with the cDNAs in Insect Cells—The baculovirus transfer vector pBlueBacIII, the wild-type baculovirus *Autograph california* multiple nuclear polyhedrosis virus (AcMNPV), and a host culture of *Spodoptera frugiperda* (Sf9) cells were obtained from Invitrogen (San Diego, CA). Construction of pBlueBacIII with the cDNAs containing the entire or truncated coding region and the preparation of the recombinant viruses were achieved according to the manufacturer's protocol. Four days after infection of Sf9 cells with the recombinant viruses, the cells were collected by centrifugation. The cell pellet was lysed, and the expressed proteins were purified by a similar method to that used for purification of the D-bifunctional protein (*4*).

RESULTS AND DISCUSSION

Separation of the Two Activity Domains—When preparation II was treated with proteinase K, the dehydratase activity remained constant, while the amount of the 77- and 46-kDa polypeptides decreased, and that of 36- and 32-kDa and smaller polypeptides increased. The dehydrogenase activity and the content of the 36-kDa polypeptide decreased as the amount of proteinase K or the digestion period was increased in the absence of NADH. In the presence of NADH, decrease in both the dehydrogenase activity and the 36-kDa polypeptide was markedly prevented. Complete digestion of the 77- and 46-kDa polypeptides with a high recovery of the dehydrogenase activity was difficult, although the dehydratase activity was stable even after extensive digestion. Therefore, we fixed the conditions of digestion by measuring the dehydrogenase activity and detection of the remaining 77- and 46-kDa polypeptides by SDS-PAGE.

Figure 1 shows separation of the two enzyme activities by Reactive Green 19 column chromatography after digestion of preparation II. Most of the dehydratase activity was eluted first (fractions 4-13). The main component of this fraction was a 32-kDa polypeptide. The dehydrogenase activity was eluted later (fractions 14-20). In this fraction, the main polypeptide was of 36 kDa, but smaller minor polypeptides were also found.

These two fractions were each concentrated and subjected to Sephadex G-100 column chromatography. When a small amount of the 46-kDa polypeptide was found in the dehydratase fraction, this polypeptide was partially separated from the main polypeptide with a molecular mass of 32 kDa. When the dehydrogenase fraction was chromatographed, the 77-kDa polypeptide, if any, was removed from the main component of 36 kDa, although small amounts of smaller polypeptides were still found. For purification of these enzymatically active fragments, the fractions containing the 77- and 46-kDa polypeptides were discarded at the two column chromatographic procedures with Reactive Green 19 and Sephadex G-100.

Separation of the digested fragments was also achieved by phosphocellulose column chromatography. The 32-kDa polypeptide of the dehydratase fraction of Reactive Green 19 column chromatography was not adsorbed onto the column, which had been equilibrated with buffer A, and the 46-kDa polypeptide was adsorbed and eluted at around 60 mM potassium phosphate in buffer A. The 36-kDa polypeptide of the dehydrogenase fraction was adsorbed and eluted at around 150 mM potassium phosphate.

The two monofunctional preparations were purified by Reactive Green 19, Sephadex G-100, and phosphocellulose column chromatography, and were designated the dehydratase and dehydrogenase fragments, respectively.

Figure 2A shows the polypeptide compositions of these preparations. The major polypeptide of the dehydratase fragment was of 32 kDa. The main polypeptide of the dehydrogenase fragment was of 36 kDa, although two smaller polypeptides were also observed.

To investigate the steric relation of the two fragments, their immunochemical reactivities were examined (Fig. 2, B and C). Both the 36- and 32-kDa polypeptides were detected by anti-77-kDa polypeptide antibody, but only the 32-kDa polypeptide was detected by anti-46-kDa polypeptide antibody. The results indicate that these two fragments are derived from distinctly separate regions in the 77-kDa polypeptide.

Molecular masses of the two active fragments were estimated by Sephadex G-100 column chromatography. Both the dehydratase and dehydrogenase preparations were estimated to be of 65 kDa (Fig. 3). The data suggest that both active fragments are homodimers.

Similar catalytically active fragments were also obtained by use of proteases such as trypsin and proteinase Glu-C. Production of these fragments, the similarity of the catalytic properties of the active fragments to those of the purified liver enzyme preparations, and the dimeric forms of the fragments indicate that the two domains are relatively independent and connected by a part that is exposed to

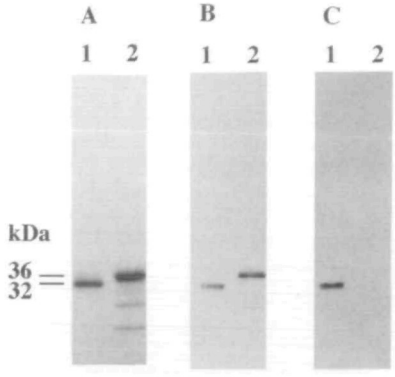

Fig. 2. **SDS-PAGE of the active enzyme fragments.** The samples were analyzed on 10% gel. A, protein staining; B, immunoblot analysis using anti-77-kDa polypeptide antibody; C, immunoblot analysis using anti-46-kDa polypeptide antibody. Lane 1, dehydratase fragment; lane 2, dehydrogenase fragment.

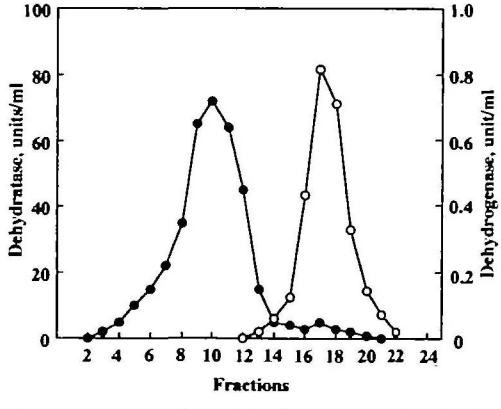

Fig. 1. **Separation of the dehydratase and dehydrogenase fragments.** The digestion mixture was desalted by passing through a Sephadex G-25 column with buffer A, then the enzyme was applied to a Reactive Green 19 column (0.4 ml) which had been equilibrated with buffer A. The column was developed with a NaCl concentration gradient. Fractions 1-4, adsorption of the digest; fractions 5 and 6, washing with buffer A; fractions 7-14, NaCl gradient from 0.05 to 0.4 M; fractions 14-24, NaCl gradient from 0.4 to 2.0 M. Volume of fractions 1-4 was 1 ml, and that for others was 0.4 ml. ●, dehydratase activity with D-3-hydroxyoctanoyl-CoA; ○, dehydrogenase activity with D-3-hydroxyoctanoyl-CoA.

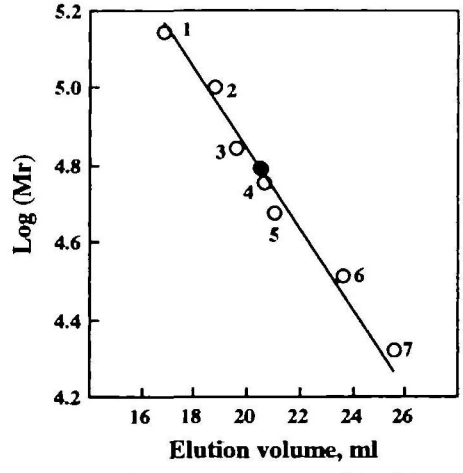

Fig. 3. **Estimation of molecular masses of the dehydratase and dehydrogenase fragments by molecular sieve column chromatography.** The samples were applied to a Sephadex G-100 column (1 × 48 cm) and developed with buffer A containing 0.2 M NaCl. The standard proteins were: 1, rabbit muscle lactate dehydrogenase (140 kDa); 2, pig heart citrate synthase (100 kDa); 3, pig heart malate dehydrogenase (70 kDa); 4, pigeon breast muscle carnitine acetyltransferase (55 kDa); 5, horseradish peroxidase (44 kDa); 6, bovine erythrocyte superoxide dismutase (32.6 kDa); 7, rabbit muscle myokinase (21 kDa). Elution positions of both the dehydratase and dehydrogenase activities were nearly the same, as indicated by ●.

attack by proteases.

Structure of the cDNA and Partial Amino Acid Sequences of the Enzyme—Only one type of cDNA was obtained by immunoscreening using two antibodies. Total length of the cloned cDNA was 2,620 bp, consisting of 70 bp in the 5′-noncoding region, an open reading frame of 2,208 bp encoding 736 amino acids, and 342 bp in the 3′-noncoding region. The structure of the coding region of the cDNA exactly matched that of the human 17β-hydroxysteroid dehydrogenase IV cDNA (*18*). The deduced amino acid sequence is shown in Fig. 4.

The amino-terminal amino acid of the 77-kDa polypeptide in preparation II was blocked, but the main amino-terminal amino acid of the 46-kDa polypeptide in preparation II and that of preparation I was His-312. Ser-314 and Ala-316 were also found as minor residues. Amino acid sequences of the peptides derived from the 77-kDa polypeptide were located in the whole region, but those from 46-kDa polypeptide in preparation I were found only in the carboxyl-terminal side (Fig. 4).

The amino-terminal amino acid sequence of the dehydratase fragment was not homogeneous, but the main terminal amino acids were identified as Ser-318 and Ala-320. The molecular mass of the polypeptide of this preparation was of 32 kDa. Therefore, this polypeptide is concluded to be derived from the region stretching from these amino acid residues to the vicinity of residue number 620.

The amino-terminal amino acid of the dehydrogenase fragment was blocked. The molecular mass of 36 kDa of this polypeptide suggests that is consists of about 310 amino acids on the amino-terminal side of the 77-kDa polypeptide.

Deblocking of the amino-terminal amino acid of the 36- and 77-kDa polypeptides by treatment with *N*-acylaminoacyl-peptide hydrolase (Takara) failed. The *in vitro* transcribed-translated product with the cDNA having the entire coding region migrated to the same position as the 77-kDa polypeptide on SDS-PAGE (data not shown). Therefore, the terminal amino acid is supposed to be the initial methionine or a residue near this methionine. It is noteworthy that *N*-acylaminoacyl-peptide hydrolase is inactive with the X-Pro sequence.

Expression of 77-kDa Polypeptide and Its Truncated Polypeptide—Proteins were expressed in Sf9 cells by the full-length cDNA and a truncated cDNA encoding a region from His-312 to Leu-736. The D-3-hydroxyacyl-CoA dehydratase activity increased more than 50-fold after transfection with either of the recombinant viruses. The amounts of the expressed protein amounted to about 5% of the total protein in the cells.

The cell pellet was extracted sequentially as described for purification of the human enzyme. The truncated version was recovered in the first extract and the full-size version was solubilized in the second extract containing a detergent. These two expressed enzymes were purified by similar procedures to those used for the human enzyme preparations (*4*). The extracts were subjected to column chromatography on phosphocellulose and Reactive Green 19. During purification, these two expressed proteins behaved very similarly to the human liver enzymes.

The molecular mass of the enzyme protein expressed by the full-size cDNA was estimated by molecular sieve column chromatography on Sephadex G-150 and found to be indistinguishable from that of the human preparation II. The polypeptide of this expressed enzyme migrated to the same position as the 77-kDa polypeptide of preparation II on SDS-PAGE. The signal intensity of the expressed enzyme by immunoblot analysis was nearly the same as that of the 77-kDa polypeptide in preparation II with the antibodies against the 77-kDa and 46-kDa polypeptides.

Molecular masses of the native form and the subunit of the protein expressed by the truncated cDNA were very similar to those of preparation I. The truncated version was also detected at similar intensity to that of preparation I by immunoblot analysis with either of the antibodies.

Another truncated protein having the amino-terminal side, Met-1 to Asn-281, was expressed in the insect cells. Expression of this protein was confirmed by SDS-PAGE and immunoblot analysis. The expressed protein was recognized by the antibody against 77-kDa polypeptide, but not by the antibody against preparation I. However, neither the dehydratase nor the dehydrogenase activity was con-

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  1 MGSPLRFDGRVVLVTGAGAGLGRAYALAFAERGALVVVNDLGGDFKGVGKGSLAADKVVE
 61 EIRRRGGKAVANYDSVEEGEKVVKTALDAFGRIDVVVNNAGILRDRSFARISDEDWDIIH
121 RVHLRGSFQVTRAAWEHMKKQKYGRIIMTSSASGIYGNFGQANYSAAKLGLLGLANSLAI
181 EGRKSNIHCNTIAPNAGSRMTQTVMPEDLVEALKPEYVAPLVLWLCHESCEENGGLFEVG
241 AGWIGKLRWERTLGAIVRQKNHPMTPEAVKANWKKICDFENASKPQSIQESTGSIIEVLS
301 KIDSEGGVSANHTSRATSTATSGFAGAIGQKLPPFSYAYTELEAIMYALGVGASIKDPKD
361 LKFIYEGSSDFSCLPTFGVIIGQKSMMGGGLAEIPGLSINFAKVLHGEQYLELYKPLPRA
421 GKLKCEAVVADVLDKGSGVVIIMDVYSYSEKELICHNQFSLFLVGSGGFGGKRTSDKVKV
481 AVAIPNRPPDAVLTDTTSLNQAALYRLSGDWNPLHIDPNFASLAGFDKPILHGLCTFGFS
541 ARRVLQQFADNDVSRFKAIKARFAKPVYPGQTLQTEMWKEGNRIHFQTKVQETGDIVISN
601 AYVDLAPTSGTSAKTPSEGGKLQSTFVFEEIGRRLKDIGPEVVKKVNAVFEWHITKGGNI
661 GAKWTIDLKSGSGKVYQGPAKGAADTTIILSDEDFMEVVLGKLDPQKAFFSGRLKARGNI
721 MLSQKLQMILKDYAKL
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Fig. 4. **Deduced amino acid sequence of the cDNA.** Limited proteolysis of the enzyme preparations and amino acid sequencing were carried out as described under "MATERIALS AND METHODS." Amino acid sequences of peptide fragments from the 77-kDa polypeptide of preparation II and those from the 46-kDa polypeptide of preparation I confirmed are marked by thin and bold underlines, respectively. The amino-terminal amino acid of preparation II was blocked, but that of preparation I was His-312 as marked by a closed triangle. The amino-terminal amino acid of the dehydrogenase fragment was blocked, but the two amino acids, Ser-318 and Ala-320, marked by open triangles, were found as the terminal amino acids of the dehydratase fragment preparation.

firmed. The expressed protein was a monomer of about 30 kDa by analysis of SDS-PAGE and molecular sieve column chromatography (data not shown). The defined region may be shorter than is necessary for expression of the functional dimeric truncated protein.

Catalytic Properties of the Enzymatically Active Protease-Digested Fragments and the Expressed Proteins—Catalytic activities of the proteinase K-digested fragments were examined. The maximal activities of the dehydratase fragment with octenoyl-CoA and D-3-hydroxyoctanoyl-CoA were 1,200 and 1,700 units/mg, respectively (Table I). These activities were comparable to those of preparations I and II, in terms of activity per mole of the enzyme. The K_m values were also similar to those of preparations I and II. This preparation was monofunctional, like the human liver enzyme preparation I, and the dehydrogenase activity was not detected (less than 0.04 unit/mg).

Apparent maximal velocities and K_m values of the dehydrogenase fragment were similar to those of preparation II when compared on the basis of molecular mass (Table II). This fragment preparation after Reactive Green 19 column chromatography still showed a relatively high dehydratase activity (about 20 units/mg), although most of this activity was separated. The remaining dehydratase activity was partially separated by phosphocellulose column chromatography. The latter half of the dehydrogenase activity peak fraction was pooled as the final preparation of the dehydrogenase fragment. This preparation exhibited a very low dehydratase activity (about 0.5 unit/mg). The ratio of the dehydratase activity to the dehydrogenase activity was approximately 50 for preparation II. For the dehydrogenase fragment, this ratio varied slightly among the final preparations, but it was about 1/50 when the activities were assayed by the standard methods. Therefore, the dehydrogenase fragment was concluded to be monofunctional, although extensive removal of the dehydratase activity was not carried out.

Table III summarizes the enzyme activities of the expressed proteins. The protein having the entire region exhibited the reversible conversion of enoyl-CoA to D-3-hydroxyacyl-CoA. The specific activities of the hydratase and dehydratase with the substrates having eight carbons were very similar to the values previously reported for preparation II (*4*). This protein also exhibited the D-3-hydroxyoctanoyl-CoA dehydrogenase activity, and its specific enzyme activity was comparable to that of preparation II. A low L-3-hydroxyoctanoyl-CoA dehydrogenase activity was found in this preparation. This activity was hardly decreased after treatment with either of the antibodies against the human D-bifunctional protein under conditions under which most of the activities of hydratase, dehydratase, and D-3-hydroxyacyl-CoA dehydrogenase were markedly decreased. The data suggest that the L-3-hydroxyacyl-CoA dehydrogenase activity in this preparation is not related to the expressed enzyme.

The truncated version covering the carboxyl-terminal side exhibited the hydratase and dehydratase activities, and the specific activities were comparable to those of purified preparation I. Significant dehydrogenase activity with both D- and L-3-hydroxyoctanoyl-CoA was confirmed in this preparation. When this preparation was treated with the antibodies, the dehydratase and hydratase activities were decreased, but the dehydrogenase activities with the D- and L-isomers were not affected. The data also suggest that the L-3-hydroxyacyl-CoA dehydrogenase activity in this preparation is not related to the expressed truncated enzyme. It is noteworthy that a relatively high L-3-hydroxyacyl-CoA dehydrogenase activity was found in Sf9 cells, and this activity was not completely separated from the expressed enzymes by the procedures used.

Conclusion—In previous studies (*4, 5*), we have purified two enzyme preparations having D-hydroxyacyl-CoA dehydratase activity. One preparation contained a 77-kDa polypeptide as the main component and several smaller polypeptides including a 46-kDa polypeptide, and this preparation exhibited both the dehydratase and the dehydrogenase activities with D-3-hydroxyoctanoyl-CoA. The other preparation was a homodimer of a 46-kDa polypeptide and exhibited only the dehydratase activity.

Only one type of cDNA encoding for the 77-kDa polypeptide was obtained by immunoscreening of cDNA using the antibodies against the 77- and 46-kDa polypeptides. The deduced amino acid sequence of the cDNA was compared with the partial amino acid sequences of the peptide fragments of the components of the human purified enzyme preparations. The enzymatically active and monofunctional dehydrogenase and dehydratase fragments were separated after limited proteolysis of preparation II. Location of these fragments was also conducted by comparison of the amino acid sequences and immunochemical reactivities.

The protein expressed by the full-size cDNA was very

TABLE I. **Kinetic parameters for the hydratase and dehydratase reactions.** The hydratase and the dehydratase activities were assayed with octenoyl-CoA and D-3-hydroxyoctanoyl-CoA, respectively. V_{max} is expressed as units/mg protein, and K_m is expressed as μM.

	Hydratase		Dehydratase	
	V_{max}	K_m	V_{max}	K_m
Preparation II	470	40	720	100
Preparation I	759	30	1,200	90
Dehydratase fragment	1,200	43	1,700	140

TABLE II. **Kinetic parameters for the dehydrogenase reactions.** The enzyme assay was carried out with various concentrations of one substrate and a fixed concentration of the other. The fixed concentrations were: D-3-hydroxyoctanoyl-CoA = 20 μM, NAD^+ = 0.2 mM, 3-ketooctanoyl-CoA = 20 μM, NADH = 0.1 mM.

	Dehydrogenase reaction				Reductase reaction			
	D-3-Hydroxyoctanoyl-CoA		NAD^+		3-Ketooctanoyl-CoA		NADH	
	V_{max}	K_m	V_{max}	K_m	V_{max}	K_m	V_{max}	K_m
Preparation II	12	10	7.7	13	8.8	2.7	9.3	5.4
Dehydrogenase fragment	30	10	17	12	22	2.7	26	3.8

TABLE III. **Enzyme activity of the expressed proteins.** The hydratase and dehydratase activities were assayed with octenoyl-CoA and D-3-hydroxyoctanoyl-CoA, respectively. The dehydrogenase activity was assayed with the isomers of 3-hydroxyoctanoyl-CoA under the standard assay conditions. The values are expressed as units/mg protein.

	Hydratase	Dehydratase	Dehydrogenase	
			D-isomer	L-isomer
Full-size version	350	390	17	0.12
Truncated version	750	810	0.58	2.1

similar to the 77-kDa polypeptide of preparation II in respect to molecular, immunochemical, and catalytic properties. The protein expressed by the truncated cDNA encoding from His-312 to the carboxyl-terminal Leu-736 was very similar to the purified liver enzyme preparation I. The protein expressed by the cDNA encoding from Met-1 to Asn-281was inactive, but it was similar to the dehydrogenase fragment in immunochemical reactivity.

The data obtained in this study indicate that the D-bifunctional protein is a homodimer of the 77-kDa polypeptide, and the dehydrogenase domain is on the amino-terminal side, and the dehydratase domain on the carboxyl-terminal side. The stereospecificity of the substrates and the location of the domains of the D-bifunctional protein are quite different from previously confirmed mammalian multifunctional proteins involved in β-oxidation. Domains of the enoyl-CoA hydratase and L-3-hydroxyacyl-CoA dehydrogenase of the L-bifunctional protein and the α-subunit of the trifunctional protein are located on the amino-terminal and carboxyl terminal side, respectively (*2, 7-9*).

It is interesting that the structure and function of the D-bifunctional protein are very similar to those of *S. cerevisiae* D-3-hydroxyacyl-CoA dehydratase/D-3-hydroxyacyl-CoA dehydrogenase bifunctional protein (*6*).

It is noteworthy that the D-bifunctional protein is the same as 17β-hydroxysteroid dehydrogenase IV in respect to its primary structure (*18*). The purified 17β-hydroxysteroid dehydrogenase was shown to exhibit the enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase activities (*19*). The reported activities were very low, but this may be due to the use of crotonyl-CoA and acetoacetyl-CoA, which are poor substrates for the D-bifunctional protein.

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