Purification, properties, and immunocytochemical localization of human liver peroxisomal enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase

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ABSTRACT A molecular understanding of genetic disease in which peroxisomal functions are impaired depends on analysis of the structure of normal and mutant enzymes of peroxisomes. We report experiments describing the isolation, characterization, and immunocytochemical localization of enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase bifunctional enzyme (PBE) of the peroxisomal fatty acid β-oxidation system from normal human liver and compared it with that of rat liver enzyme. The human enzyme, purified ~2300-fold by ion-exchange chromatography, is homogeneous as judged by NaDodSO4/PAGE. This PBE is localized exclusively in the matrix of peroxisomes in liver cells by the protein A/gold immunocytochemical method. The human PBE is similar to rat enzyme in size (Mr, ~79,000), isoelectric point (PI, 9.8), pH optima, molecular structure as observed by rotary shadowing, and peptide patterns on NaDodSO4/PAGE after proteolytic digestion with Staphylococcus aureus V8 protease.

The human and rat enzymes differed in their immunological properties by having partial identity with each other; this is reflected in their slightly dissimilar composition of the amino acids aspartic acid, threonine, glutamic acid, tyrosine, and glycine. COOH-terminal amino acids were similar for both the enzymes: Gly-Ser-Leu-Ile-COOH. These results suggest that the human and rat liver PBE may be different in their amino acid sequences at their antigenic sites.

The peroxisomal fatty acid β-oxidation pathway enzymes of rat liver have been well characterized. These enzymes are responsible for the successive oxidation and removal of two carbon atoms from the carboxyl-terminal end of the fatty acid molecules (1–3). The reaction sequence of fatty acid metabolism in rat liver peroxisomes can be summarized as follows: fatty acid is activated to acyl-CoA by ATP-dependent acyl-CoA synthetase, which is present in peroxisomes (4) and is sufficient to provide necessary acyl-CoA for the maximal β-oxidation in peroxisomes. The following reaction sequence then occurs in the peroxisomal matrix and is catalyzed by peroxisome-specific enzymes (2, 5, 6): the rate-limiting and initiating step in the peroxisomal β-oxidation is catalyzed by fatty acyl-CoA oxidase. The next two steps of this pathway are catalyzed by enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase bifunctional enzyme (PBE). The final step of the reaction is mediated by 3-ketoacyl-CoA thiolase, which breaks 3-ketoacyl-CoA into acyl-CoA. The saturated acyl-CoA having two carbon atoms less than the original molecule reenters the β-oxidation pathway (1, 2). In the rat liver, the peroxisomal β-oxidation pathway differs from that of mitochondrial β-oxidation (1, 2). The component enzymes of the β-oxidation system of these two organelles are not only different in their molecular, catalytic, and immunological properties, they also differ in their response to peroxisome proliferators (1, 2, 7–9). The peroxisomal β-oxidation enzymes are dramatically induced in the livers of rodents and some nonrodent species following treatment with certain hypolipidemic agents and the industrial phthalate ester plasticizer bis(2-ethylhexyl)phthalate (7–10).

The functional significance of peroxisomes in liver has been underscored by the discovery in the last decade of the hepatocarcinogenic property of several peroxisome proliferators (11) and the description of infants with Zellweger hepatorenal syndrome who manifest deficient oxidation and accumulation of very long-chain fatty acids, presumably due to the absence of intact peroxisomes in the liver and kidney (12–15). Available evidence suggests that Zellweger syndrome may be due to a failure of peroxisome membrane assembly or protein import either because of structural abnormalities of peroxisomal matrix proteins or lack of possible receptors on the membranes for specific recognition and packaging of peroxisomal enzymes (14). A molecular understanding of the inherited peroxisomal disorders depends on the characterization of the structure of normal and mutant enzymes of peroxisomes. As a first step, we now describe the purification and characterization of PBE of the peroxisomal fatty acid β-oxidation system from normal human liver. We have studied the molecular and immunological properties of this enzyme and compared it with that of rat peroxisomal enzyme.

MATERIALS AND METHODS

Purification of Peroxisomal PBE. Normal human liver obtained from immediate autopsy (Department of Pathology, University of Maryland, Baltimore) kept frozen at −80°C was thawed and homogenized in 50 mM P04 buffer (pH 7.0) containing 0.1% hexamethyolphosphoramic triamide (HMPTA), 2 mM EDTA, and 2 mM 2-mercaptoethanol. The purification steps were essentially the same as reported for rat enzyme (16, 17). Briefly, the supernatant 17,000 x g was passed on a phosphocellulose column (2.5 x 25 cm) equilibrated with the same buffer used for extraction of the enzyme. The protein from the column was eluted with a linear gradient (50–500 mM phosphate buffer in a total of 400 ml); the fractions with enoyl-CoA hydratase activity were pooled and fractionated with ammonium sulfate. The protein that precipitated between 20% and 40% ammonium sulfate was dissolved in the buffer; the ammonium sulfate was removed by chromatography on Sephadex G-25 column or by dialysis.

Abbreviations: PBE, peroxisomal enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase bifunctional enzyme; HMPTA, hexamethyolphosphoramic triamide.

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The enzyme solution was diluted with an equal volume of cold water containing 2 mM 2-mercaptoethanol, 0.1% HMPTA, and 5 mM EDTA and loaded on a CM-cellulose column with a 25-m1 bed volume, previously equilibrated with 25 mM potassium phosphate buffer (pH 7.0). It was eluted with a linear gradient of 50–300 mM phosphate or 0–0.3 M NaCl in 25 mM PO4 in a total volume of 300 ml. The protein with enoyl-CoA hydratase activity was pooled, concentrated by Amicon filtration, and dialyzed against 50% (vol/vol) glycerol in homogenization buffer. This protein was subsequently passed through a Sephadex G-200 column to remove denatured protein and to determine the molecular weight of the native protein. The protein from the CM-cellulose chromatography, which was 95% pure, was used for the biochemical, immunological, and protein analysis studies reported in this paper. The purification of rat peroxisomal enoyl-CoA hydratase was also performed from frozen rat liver as described (16, 17).

**Analytical Methods.** Enoyl-CoA hydratase was assayed as described by Steinman and Hill (18) in 0.3 M Tris-HCl buffer (pH 7.4) containing 5 mM EDTA, bovine serum albumin (0.05 mg/ml), and 200 μM crotonyl-CoA as substrate. The heat-labile peroxisomal enoyl-CoA hydratase activity was assayed after diluting the enzyme with 50 mM phosphate buffer (pH 7.0) and heating at 57°C for 5 min. The difference between total and heat-stable activities indicates the heat-labile activity. Protein content was determined by either measuring absorbance at 280 nm or by the method of Bradford (19). Electrophoresis was performed on NaDodSO4/polyacrylamide slab gels by the method of Laemmli (20). Isoelectric focusing was done on either slab gels (pH range, 3.5–10.0) or on tube gels (pH range, 9–11) as described (17). Amino acid analysis was performed following the method of Heinrikson and Meredith (21). Samples were hydrolyzed for 20, 44, and 68 hr at 112°C using 6 M HCl. Phenol (1 mg/ml) was included to minimize destruction of tyrosine and phenylalanine. Serine and threonine values were corrected for decompositional losses (21). Numbers were expressed based on the molecular weight of 78,250.

COOH-terminal amino acid composition of peroxisomal enzyme was determined after digestion with carboxypeptidase Y (22). The peptide mapping was done with Staphylococcus aureus V8 protease digestion as described by Cleveland et al. (23).

**Preparation of Monospecific Antibodies Against Purified PBE.** Monospecific polyvalent antibodies to purified human hepatic peroxisomal PBE were raised in New Zealand white male rabbits as described (17). Immunodiffusion was done at 22°C ± 2°C for 30–35 hr in 1% agarose in 0.05 mM PO4 buffer (pH 7.5) containing 1 mM NaN3 and 0.15 M NaCl (17). Immunoblotting with anti-human PBE was performed according to the method of Towbin et al. (24) and antigen–antibody complexes were visualized either by 125I-labeled protein A (New England Nuclear; 70–100 μCi/μg; 1 Ci = 37 GBq) autoradiography or by the peroxidase-antiperoxidase method.

**Electron Microscopy.** Immunocytochemical localization of PBE in human and rat liver was performed by the protein A gold method utilizing Lowicryl K4M-embedded tissues (25). The molecular structure of purified human and rat enzymes was ascertained by electron microscopy with the rotary shadowing technique (26).

**RESULTS**

**Purification.** A summary of the typical purification procedure of PBE from whole human liver extract by monitoring heat-labile peroxisomal enoyl-CoA-hydratase activity is given in Table 1. The specific activity at individual steps and the amount of enzyme recovered at the end of each step varied somewhat. However, the final product was usually of the same quality, with very low yields of ~1.0 mg per kg of liver. The enzyme specific activity was enriched during purification (Table 1) and the protein obtained by CM-cellulose chromatography was highly homogeneous as judged by NaDodSO4/PAGE (Fig. 1). However, after passage of this protein on a Sephadex G-200 column to determine the native molecular weight, the activity could not be monitored. Dialysis against 50% (vol/vol) glycerol, containing 0.1% HMPTA, 1 mM EDTA, or 1 mM 2-mercaptoethanol did not help regain the enzyme activity. Rat enzyme at this stage is highly active and fairly stable when kept frozen at −20°C in the presence of 50% (vol/vol) glycerol, 1 mM EDTA, and 1 mM 2-mercaptoethanol for as long as 1 year. Frequent freezing and thawing resulted in gradual loss of activity of the enzyme over a period of time, even for the rat enzyme.

**Purity of PBE.** Purity of the protein was determined by NaDodSO4/PAGE. Fig. 1 shows the electrophoretic pattern of proteins during purification; the purified human enzyme (lane 4) has a Mv of ≈78,250 ± 750 (n = 3). The isoelectric point (pI) of human enzyme was 9.8, which is similar to that of rat (16, 17).

The molecular structure of PBE from human liver and rat liver was determined by ascertaining the electron microscopic profiles of these proteins by rotary shadowing. The molecular structure of human PBE (Fig. 2A) appears similar to that of rat enzyme (Fig. 2B). Both proteins appear as globular proteins; this is consistent with the contention that many of the peroxisomal matrix proteins are globular in nature.

**Immunological Properties.** Antibodies to human PBE raised in rabbit were monospecific and gave a single precipitin line with pure protein as well as with crude liver extracts. The Ouchterlony double-diffusion patterns obtained by using antibodies against human PBE (Fig. 3A) show only a partial identity between highly purified rat and human

Table 1. Purification of peroxisomal enoyl-CoA hydratase/3-hydroxyacyl CoA dehydrogenase enzyme from normal human liver

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Vol, ml</th>
<th>Protein, mg/ml</th>
<th>Specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>17,000 × g supernatant</td>
<td>500</td>
<td>10</td>
<td>0.04</td>
</tr>
<tr>
<td>Phosphocellulose</td>
<td>75</td>
<td>5</td>
<td>82</td>
</tr>
<tr>
<td>Fraction from chromatography after concentration by Amicon CM-30 membrane</td>
<td>25</td>
<td>0.5</td>
<td>93</td>
</tr>
<tr>
<td>3-Hydroxyacyl-CoA dehydrogenase, units per mg of protein</td>
<td></td>
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</tr>
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</table>

Enzyme data on selected steps are presented here. The protein was measured as absorbance at 280 nm using an extinction coefficient of 1% protein = 5.8. Details of human liver peroxisomal enoyl-CoA hydratase purification are described in Materials and Methods. The heat-labile peroxisomal enoyl-CoA hydratase activity was determined using crotonoyl-CoA as substrate, and dehydrogenase activity was determined using acetoacetyl-CoA as substrate.
Fig. 1. NaDodSO4/PAGE of human liver protein samples obtained during purification after fractionation of phosphocellulose (lane 2), ammonium sulfate precipitation (lane 3), and CM-cellulose (lane 4). The protein obtained after the CM-cellulose chromatography step was 95–98% pure, as measured by densitometric scanning. Lanes: 1, molecular weight standards (×10^{-3}); 2, 50 μg of protein; 3, 25 μg of protein; 4, 5 μg of protein.

enzymes. This partial identity may reflect possible difference(s) in molecular structure. The immunodiffusion pattern in Fig. 3B was obtained using human liver extract (100,000 × g supernatant prepared from 10% homogenate) against antibodies against human PBE. A single precipitin line was observed, indicating the monospecific nature of the antibodies. Immunoblotting data presented in Fig. 4A also reveal a single polypeptide reacting with the anti-human PBE antibodies. Immunoblotting using anti-rat PBE antibodies revealed weak crossreactivity with human enzyme (Fig. 4B).

Immunotitration of human liver extracts with increasing amounts of anti-human peroxisomal PBE antibody resulted in complete precipitation of heat-labile peroxisomal enoyl-CoA hydratase activity from the supernatants (Fig. 5A). Similar results were obtained when rat liver extracts were immunotitrated with anti-rat PBE antibody (Fig. 5B). These antibodies partially precipitated the 3-hydroxyacyl-CoA dehydrogenase activity. Since the assay used in these studies cannot differentiate between peroxisomal and mitochondrial 3-hydroxyacyl-CoA dehydrogenase activities, it is not certain that the partial inhibition reflects the selective precipitation of peroxisomal dehydrogenase. However, since the antibodies recognize only one protein by immunoblotting and by immunoprecipitation and since both enzyme activities are enriched during purification, we conclude that human enoyl-CoA hydratase/dehydrogenase activities are exhibited by a bifunctional protein. It should be pointed out that the rat enoyl-CoA hydratase is a bifunctional enzyme that also has dehydrogenase activity (16).

Molecular Properties. Following proteolytic digestion of purified human PBE by S. aureus V8 protease, the peptide patterns observed were identical to those yielded by rat liver PBE (data not shown). The results of amino acid analyses expressed in mol of amino acids per mol of Mr 78,250 monomeric polypeptide are summarized in Table 2. Except for some differences in aspartic acid, threonine, glycine, glutamic acid, and tyrosine amino acid residues, the amounts of the other amino acids in the human liver PBE are similar to those in rat liver PBE. The analysis of the rates of release of COOH-terminal amino acids as a function of time from the human and rat enzymes showed a time-dependent release of amino acids with closely identical rates (data not shown). At least four amino acids were identified from the carboxyterminal end of these proteins. The COOH-terminal sequence was deduced as -Gly-Ser-Leu-Ile-COOH.

Immunocytochemical Localization of PBE in Human and Rat Liver. Using the antibody produced in rabbits against the purified human liver PBE, we studied the intracellular localization of this enzyme in Lowickryl-embedded normal human liver by the immunocytochemical protein A gold technique. The electron-dense particles are localized exclusively on the matrix.

Fig. 2. Rotary-shadowed replicas of human liver PBE (A) and rat liver PBE (B). Protein (~0.05 mg/ml) was sprayed on freshly prepared mica, dried in vacuum at room temperature, and then rotary-shadowed at an angle of 10^0 with platinum evaporated from an electron gun using a freeze-etching apparatus. The carbon film with the platinum replica was examined in an electron microscope. Proteins appear as small spheres. (×550,000.)

Fig. 3. (A) Immunodiffusion of human hepatic enoyl-CoA hydratase and rat hepatic enoyl-CoA hydratase against anti-human PBE antibodies. 1, Antiserum against human PBE; 2, 1 μg of rat enzyme (pure); 3, 1 μg of human enzyme (pure). (B) Immunodiffusion of human liver extract against anti-human liver PBE antiserum. 1, Antiserum; 2–6, various volumes of 10% extract (2, 5, 10, and 15 μl, respectively); 7, nonimmune serum.

Fig. 4. (A) Immunoblotting using anti-human hepatic PBE antibodies. Lanes 1–3, various concentrations of normal human hepatic extracts (50, 100, and 150 μg of protein); lane 4, purified human liver PBE (0.5 μg). (B) Immunoblotting with anti-rat hepatic PBE antibodies. Lane 1, pure human liver PBE (2 μg); lane 2, rat liver PBE (2 μg). Dilution of antiserum, 1:1000. The resulting immune complexes were detected by treatment with 125I-labeled protein A autoradiography. A Mr 35,000 subunit of enoyl-CoA hydratase in addition to major Mr 79,000 polypeptide is evident for rat enzyme (B) (lane 2).
of the human hepatic peroxisomes (Fig. 6A), clearly confirming the peroxisomal origin of the purified protein. We also used the anti-human PBE antibodies to ascertain whether they recognize the rat liver peroxisomal PBE (Fig. 6B). Once again, the labeling was highly specific for the peroxisome matrix. The number of gold particles appeared to be more on human liver peroxisomes than on the rat liver peroxisomes.

**DISCUSSION**

In this report, the purification of human peroxisomal PBE from liver to homogeneity is described and its properties are compared with those of rat enzyme. On the basis of molecular, biochemical, peptide mapping, and immunochromical data presented here, these two enzymes appear similar in some properties, but they seem to differ in their immunological identity and in catalytic stability.

The PBE purified from normal human liver is a single polypeptide of $M_t$ 78,250 ± 750 and with a pH value of ≈ 9.8. The purified human and rat enzymes displayed a globular molecular structure by rotary shadowing. The peptide patterns following *S. aureus* V8 protease digestion on NaDodSO₄/polyacrylamide gels were also similar for both proteins. However, our data indicated certain differences in these two enzymes. First, the human enzyme is not as stable as the rat enzyme. Second, immunologically the proteins showed only partial identity. Third, the amino acid composition of human and rat liver PBEs is slightly different. These immunological differences underscore the importance of purification of human peroxisomal enzymes for critically evaluating the molecular defects in human peroxosomal disorders. The use of antibodies prepared against rat peroxisomal enzymes for evaluating the abnormalities in human livers may be misleading.

Rat peroxisomal PBE is a bifunctional protein capable of catalyzing the second and third reactions of the β-oxidation pathway. It appears that normal human liver enzyme is also a bifunctional protein, since both the dehydrogenase activity and enoyl-CoA hydratase activity were copurified. The immunotitration data are also suggestive of the bifunctional nature of the human enoyl-CoA hydratase. However, further studies such as sequencing and peptide maps following trypsinolysis may be necessary to confirm the presence of the two enzyme domains in this human PBE.

Ouchterlony double-diffusion analysis revealed a partial identity between human and rat PBE proteins, suggesting that there is at least 60% or more sequence homology between these two proteins (27). This homology is reflected by the presence of the same COOH-terminal sequence for these two enzymes and slightly different amino acid composition. Similar differences in immunological properties do exist among cytochrome P-450 (28) and lysozyme (29) from
different species, which also share some common immunological determinants. The immunological differences of rat and rabbit cytochrome P-450 enzymes are shown to be associated with variations in amino acid composition (28).

Sustained peroxisome proliferation is associated with continued ingestion of several structurally unrelated chemicals with hypolipidemic properties (7, 9). In rodents, hepatocellular carcinomas develop after prolonged treatment with these chemicals (11). Studies in rodents have revealed that peroxisome proliferation is associated with a dramatic increase in all three enzymes of the peroxisomal \( \beta \)-oxidation pathway system. In addition, citrate acetyltransferase (30), carnitine octanoyltransferase (30, 31), acetyl-CoA-dihydroxyacetone-phosphate acetyltransferase (32), and catalase (11) are also induced. Using cDNA probes, it has been shown that hypolipidemic drugs induce a rapid and marked increase in the rates of transcription of mRNAs for the fatty acyl-CoA-oxidase and PBE in rat liver (10). Similar direct studies on the mechanism of induction of peroxisomal enzymes by xenobiotics in humans may be difficult to undertake. However, the immunocytochemical localization information on human liver biopsy presented here suggests that quantitative immunocytochemical analyses of peroxisomal enzyme changes in individuals treated with peroxisome proliferator hypolipidemic drugs may be feasible to establish whether the therapeutic doses of these drugs induce peroxisomal \( \beta \)-oxidation enzymes. Alternatively, to study the effect of peroxisome proliferators on normal human liver and to investigate the cellular, biochemical, and molecular events occurring in human peroxisomal disorders, it may be necessary to establish these cells in culture or maintain in athymic nude mice as xenotransplants. Recently, liver cells of rats transplanted into the anterior chamber of the eye or subcutaneous fat of a syngeneic host (33, 34) were found to respond to the peroxisome proliferator effects of a hypolipidemic drug. Systems such as these can be exploited to investigate the effects of various xenobiotics or to determine the defects in peroxisomal deficiency disorders with antibodies raised against peroxisomal enzymes. The availability of antibodies to human liver peroxisomal enzymes should assist in isolating and characterizing the cDNAs for these enzymes.

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