Peroxisomal Bifunctional Protein from Rat Liver is a Trifunctional Enzyme Possessing 2-Enoyl-CoA Hydratase, 3-Hydroxyacyl-CoA Dehydrogenase, and Δ^3,Δ^2-Enoyl-CoA Isomerase Activities*

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The bifunctional protein having 2-enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase activities is one of the best characterized proteins in mammalian peroxisomes. The purified protein was monomeric, with an estimated molecular weight of 78,000. In immunoblotting, it was recognized by the antibody to peroxisomal bifunctional protein from rat liver. Comparison of the amino acid sequences of cyanogen bromide cleaved peptides and the known sequence of the peroxisomal bifunctional protein from rat liver identified them as the same molecule.

In control experiments, the peroxisomal bifunctional protein purified according to published methods also catalyzed Δ^3,Δ^2-enoyl-CoA isomerization. This means that the bifunctional protein of rat liver is in fact a bifunctional enzyme possessing Δ^3,Δ^2-enoyl-CoA isomerase, 2-enoyl-CoA hydratase (EC 4.2.1.17), and 3-hydroxyacyl-CoA dehydrogenase (EC 1.1.1.35) activities in the same polypeptide.

Multifunctional proteins metabolizing fatty acyl-CoA esters are typical of extramitochondrial β-oxidation systems (1). In contrast to mitochondrial β-oxidation pathways, which are characteristic of animals, extramitochondrial β-oxidation pathways are widely distributed among living organisms (2). In eukaryotes these systems are located in microbodies such as peroxisomes or glyoxisomes, whereas in prokaryotes they have a cytosolic location.

The bifunctional protein having 2-enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase activities is one of the best known mammalian peroxisomal proteins and has been purified from many mammalian species (3, 4) including man (5). The amino acid sequence of the rat enzyme is known (6), and the relevant gene in the rat has been cloned (7). The bifunctional protein from rat liver can act as a trifunctional hydratase-dehydrogenase-isomerase enzyme.

**MATERIALS AND METHODS**

**RESULTS**

Purification of the Peroxisomal Protein Catalyzing Isomerization—When studying Δ^3,Δ^2-enoyl-CoA isomerases in rat liver, we found recently that mitochondrial and peroxisomal activities can be separated using general dye ligand chromatography on Matrex gel red A and succeeded in demonstrating that the isomerase activity originating from peroxisomes elutes at 0.4-0.9 M KCl at pH 7.0, whereas the mitochondrial used when studying the biogenesis of peroxisomes, protein targeting to these organelles (8-10), and also the pathogenesis of human inherited peroxisomal disorders (11-13). Even though mitochondrial and peroxisomal β-oxidation are similar in terms of their chemical reactions, their physiological roles in the mammalian metabolism appear to be quite different. Peroxisomal β-oxidation is considered to be physiologically relevant to a large spectrum of fatty acids or fatty acid derivatives which are poor substrates for mitochondrial β-oxidation (for a review, see Ref. 14). Experiments with polyunsaturated fatty acids and isolated peroxisomes from rat liver have provided evidence that peroxisomes contain enzymatic activity for the Δ^3,Δ^2-isomerization of acyl-CoA (15), the later investigations into the distribution of the enzyme in liver subcellular organelles have demonstrated location of isomerase activity in both peroxisomes and mitochondria (16). The peroxisomal isoenzyme has never been characterized, however.

The aim of this work was to purify and characterize peroxisomal Δ^3,Δ^2-enoyl-CoA isomerase. In addition to its isomerization activity, the purified peroxisomal enzyme was found to catalyze hydration of trans-2-enoyl-CoA and dehydrogenation of 1,3-hydroxyacyl-CoA, indicating that it is a multifunctional protein. The physical, immunological, and kinetic properties of the protein purified here and identified it as the same polypeptide which is known as the peroxisomal bifunctional protein. Thus, the data indicate that the bifunctional protein from rat liver can act as a trifunctional hydratase-dehydrogenase-isomerase enzyme.

1 Portions of this paper (including "Materials and Methods," Table I, Figs. 1-3, and additional Refs. S1-S6) are presented in miniprint at the end. The abbreviations used are: ACoA, agarose-hexane-coen-zyme A; BA, benzamide hydrochloride; DTT, dithiothreitol; MES, 2(N-morpholino)ethanesulfonic acid; EGTA, [ethylenebis(oxy)-ethylenenitro]tetraacetic acid; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TFA, trifluoroacetic acid. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.
isomerase activity elutes at a lower salt concentration. In the same study we observed that the treatment of rats with clofibrate caused a remarkable increase in the activity of both isoenzymes. Taking advantage of the above-mentioned finding, liver extracts from clofibrate-treated rats were applied to Matrex gel red A, and the isomerase peak eluting at a high salt concentration was taken for further purification. When ammonium sulfate precipitation and chromatographies on carboxymethylcellulose (CM30) and hydroxylapatite were carried out, sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the isomerase preparation showed only one band with the molecular weight of 78,000. This and the appearance of one symmetrical peak in reverse phase column chromatography using an acetonitrile gradient indicated that the protein had been purified to apparent homogeneity. The specific activity of the purified enzyme preparation was 5.5 \( \mu \text{mol} \times \text{mg protein}^{-1} \times \text{min}^{-1} \) when 60 \( \mu \text{M} \) trans-3-hexenoyl-CoA was used as the substrate. Gel filtration on S-200 HR gave a molecular weight of 83,000 for the native protein, revealing that the purified protein was monomeric.

Kinetic Characterization of the Enzyme and Identification of 2-Enoyl-CoA Hydratase and 3-Hydroxyacyl-CoA Dehydrogenase Activities to the Same Polypeptide as \( \Delta^3, \Delta^2 \)-Enoyl-CoA Isomerase Activity—Enzyme activity during purification was measured with trans-3-hexenoyl-CoA. The ratio of the reaction velocity of trans-3-hexenoyl-CoA to that of trans-3-decenoyl-CoA was 2.7. If cis-3-hexenoyl-CoA was taken as a substrate and similar incubations were carried out (with crotonase, 1,3-hydroxyacyl-CoA dehydrogenase and NAD\(^+\) present), NADH was generated. This demonstrated that the protein functioned as a cis,trans-3,trans-2-enoyl-CoA isomerase.

In further experiments, the purified protein was incubated with trans-3-decenoyl-CoA and NAD\(^+\). Surprisingly, these factors were enough to generate NADH, a process which requires 2-enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase activities in addition to \( \Delta^3, \Delta^2 \)-enoyl-CoA isomerase. If pyruvate and lactate dehydrogenase were added to remove the NADH formed and the same reaction was followed at 303 nm in the presence of Mg\(^{2+}\), an increase in absorbance was observed. The fact that Mg\(^{2+}\) forms a complex with 3-ketoacyl compounds absorbed in the near-UV region indicates that trans-3-decenoyl-CoA was metabolized to a 3-keto derivative during the incubation. In control experiments, crotonase, 3-hydroxyacyl-CoA dehydrogenase, and the mitochondrial isomerase purified from rat heart were alone insufficient to catalyze this conversion, but all of them together were sufficient (Fig. 1, Miniprint).

The above data provide evidence that the purified protein is a multifunctional enzyme. This being the case, and assuming that the results were not caused by possible trace contaminants in the preparation, the enzyme activities and polypeptide should be chromatographically inseparable. To test this further, an aliquot from the hydroxylapatite eluate was dialyzed and applied to an agarose-hexane-CoA affinity column to which all the activities were bound. Elution of the bifunctional activity (2-enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase) in a KCl gradient was parallel to the isomerase activity (data not shown). An aliquot from the pooled activity fraction of the agarose-hexane-CoA column was again applied to a cation exchanger Mono S column of a fast protein liquid chromatography apparatus, and a linear sodium chloride gradient was developed. One sharp symmetrical protein peak was observed, and the \( \Delta^3, \Delta^2 \)-enoyl-CoA isomerase, 2-enoyl-CoA hydratase, and 3-hydroxyacyl-CoA dehydrogenase activities were all eluted together with this protein (Fig. 2, Miniprint). We have also tested a 5'-AMP-Sepharose affinity column, which has been proposed will bind NAD\(^+\)-linked enzymes, and found that all three activities were bound to this material and showed parallel elution with a KCl gradient.

Identification of the Novel Trifunctional Protein as the Earlier Bifunctional Protein—Because the molecular weight of the enzyme purified here was the same as that of the peroxisomal bifunctional protein and it possessed both known catalytic activities of the bifunctional protein, the question arose as to whether they were identical molecules. To test this possibility further, we purified peroxisomal bifunctional protein from livers of clofibrate-treated rats following the procedure described by Osumi and Hashimoto (3). After purification the enzyme preparation showed only one band on sodium dodecyl sulfate-polyacrylamide gel electrophoresis, indicating that the protein was purified to apparent homogeneity. When the catalytic activities of this protein were measured with either trans-3-decenoyl-CoA or trans-2-decenoyl-CoA in the presence of purified enzymes as auxiliary catalysts, the enzyme possessed separately measurable 2-enoyl-CoA hydratase, 3-hydroxyacyl-CoA dehydrogenase, and \( \Delta^3, \Delta^2 \)-enoyl-CoA isomerase activities and also had bifunctional and trifunctional properties when measured with trans-2- and trans-3-decenoyl-CoA, respectively, as substrates (Table I, Miniprint). The purified isomerase, bifunctional protein, and liver homogenate from clofibrate-treated rats were immunoblotted with antibody to the bifunctional protein from rat liver, and a band of the same molecular size was detectable in all of them.

The published amino acid sequence for the bifunctional protein shows 12 methionines. This and the known property of cyanogen bromide of cleaving peptides at methionine at neutral or acidic pH (17) provide a means to test whether the peroxisomal bifunctional protein from rat liver and the isomerase catalyzing enzyme purified here were identical. The protein purified according to the present procedure was therefore cleaved with cyanogen bromide and the peptides separated on a reverse phase column (Fig. 3, Miniprint). The amino acid sequences of two of the peptides were partially determined by means of automatic protein sequencer, allowing comparison with the known sequence of the bifunctional protein. Two identical sequences were found in the bifunctional protein. In both cases methionine was the amino acid preceding the sequence observed, demonstrating the reliability of the method used. No amino acid sequence was obtained from the first peptide emerging in the acetonitrile gradient. One explanation for this may be that the peptide represents the amino terminal part of the bifunctional protein, which is known to be blocked (18).

**DISCUSSION**

The discovery of \( \Delta^3, \Delta^2 \)-enoyl-CoA isomerase activity in the peroxisomal bifunctional protein was based on the following findings: 1) the purified peroxisomal protein which catalyzes the isomerization of 3-enoyl-CoA esters was observed to catalyze the formation of 3-ketoacyl-CoA and the reduction of NAD\(^+\) in the presence of \( \Delta^3 \)-enoyl-CoA substrates. 2) Edman degradation analysis of peptides obtained by cyanogen bromide cleavage of the enzyme showed the amino acid sequence to be identical to the published sequence for the rat liver bifunctional protein over the regions determined. 3) The bifunctional protein purified according to the published method and the isomerization catalyzing protein purified here

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2 P. M. Palosaaari, J. M. Kilponen, R. T. Sormunen, J. E. Hassinen, and J. K. Hiltunen, manuscript in preparation.
showed similar catalytic properties with different acyl-CoA intermediates of \( \beta \)-oxidation. In contrast to the present work, there is a short note in the literature that the bifunctional protein from liver peroxisomes is devoid of the cis-3-trans-2-enoyl-CoA isomerase activity (19). The reason for this discrepancy remains unclear.

The rat liver peroxisomal bifunctional protein contains homologous regions with mitochondrial enoyl-CoA hydratase close to its amino terminus, whereas the carboxyl-terminal end shows homology with mitochondrial 3-hydroxyacyl-CoA dehydrogenase. This has been taken to indicate that the bifunctional protein contains two functional domains (6). It has been demonstrated by kinetic studies with the purified bifunctional protein that intermediates are channelled from the active site of the enoyl-CoA hydratase domain to the 3-hydroxyacyl-CoA dehydrogenase domain without release into the bulk phase (20). The present finding that additions of 2-enoyl-CoA hydratase (crotonase) and 3-hydroxyacyl-CoA dehydrogenase to the incubation as auxiliary catalytes do not increase the observed rate of isomerization of \( \Delta^2 \)-enoyl-CoA esters can be interpreted as indicating that channelling also occurred between the active centers catalyzing isomerization and hydration. Experiments with liver peroxisomes isolated from rats treated with clofibrate have demonstrated chain shortening of \([1^{14}C] \) arachidonic acid by three acetyl groups, indicating that peroxisomal \( \beta \)-oxidation in vitro can proceed beyond a double bond positioned at an odd-numbered carbon atom in fatty acids (15). Furthermore, infusion of trans-3-dodecenoyl CoA in isolated rat liver stimulated \( \text{H}_2\text{O}_2 \) production (16), which is a sequence requiring isomerization of the double bond. The present data demonstrated isomerase activity in the bifunctional protein, which has a well documented peroxisomal location (3, 4). Taken together, these findings are in line with the proposal that one of the physiological functions of peroxisomal \( \beta \)-oxidation is the degradation of polyunsaturated fatty acids (14, 15), a process which utilizes \( \Delta^1, \Delta^2 \)-enoyl-CoA as an auxiliary catalyze.

In filamentous fungi and yeasts, peroxisomal \( \Delta^1, \Delta^2 \)-enoyl-CoA isomerase is separate from the multifunctional protein (2), which catalyzes hydration, dehydrogenation, and epimerization reactions in \( \beta \)-oxidation (21), but in rat liver the multifunctional protein of peroxisomal \( \beta \)-oxidation catalyzes isomerization, hydration, and dehydrogenation of acyl-CoA metabolites, although it is not capable of epimerization of 3-hydroxyacyl-CoA esters (22). The hydratase-dehydrogenase-epimerase enzyme in \( \text{Candida tropicalis} \), which is also a monomeric protein with an estimated molecular weight of 99,535, shows only limited amino acid homology over a short distance with the peroxisomal bifunctional protein in the rat (23), which was identified here as an isomerase-hydratase-dehydrogenase enzyme. This and the difference in enzyme pattern between the multifunctional proteins involved in fungal and rat peroxisomal \( \beta \)-oxidations indicate distinct phylogenetic divergence of these proteins in different phyla.

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Additional references are found on p. 2449.
Bifunctional Protein as Trifunctional Enzyme

Materials and Methods

Chemicals and reagents. NADP was purchased from Nacalai Tesque, Inc.; NADPH, 2-mercaptoethanol, and pyridine were from Kanto Chemical Co., Inc.; and glycylglycine was from Wako Pure Chemical Industries, Ltd. Maculene, gel, and an amino acyltransferase from Escherichia coli were from Sigma Chemical Co., Ltd. The other materials were from Showa Denko K. K. (Tokyo, Japan).

Appl-CoA esters. Aplic-CoA esters were synthesized by the mixed anhydride method (1) and purified on cellulose thin-layer plates (2).

Transter assay. The assay mixture consisted of 10 mM KCl and 10 μg/ml bovine serum albumin in 40 mM Tris/HC1 pH 7.5 and 1 mM NADP. Three arbitrary magnesium concentrations were used: 0, 10, and 20 mM. One unit of the enzyme was defined as the amount that caused a change of 1.0 μmol of the substrate in the assay mixture containing 1 μl of the enzyme solution.

Immunoblotting. The proteins from the SDS-PAGE were blotted onto nitrocellulose paper using the technique of the anti-bifunctional protein serum as the primary antibody, goat anti-rabbit IgG (whole molecule) as the second antibody, and horseradish-peroxidase for staining.

Results.

The data were compared with those obtained from the mixed anhydride method (1) and purified on cellulose thin-layer plates (2). The relative molecular weight of the protein was ascertained by SDS-PAGE. The enzyme activity was measured by the method of Yarosh et al. (11).

Discussion.

The data were compared with those obtained from the mixed anhydride method (1) and purified on cellulose thin-layer plates (2). The relative molecular weight of the protein was ascertained by SDS-PAGE. The enzyme activity was measured by the method of Yarosh et al. (11).

Fig. 1. Generation of NADH and 3-deoxy-D-xylulose-CoA from 3-keto-D-xylulose-CoA by the enzyme purified in the present work. 100 μM 3-keto-D-xylulose was transformed to NADH and 3-deoxy-D-xylulose-CoA in the presence of 8 μg of purified enzyme in 0.1 M potassium phosphate buffer, pH 7.5, at 37°C. The incubation mixture contained 0.1 M potassium phosphate buffer, pH 7.5, 50 μM NADPH, and 3-keto-D-xylulose at a final concentration of 100 μM. The generation of NADH was monitored at 340 nm.

Fig. 2. Gel filtration profile of the purified enzyme. The purified enzyme was subjected to gel filtration on a column of Sepharose 4B (2.6 cm × 30 cm) equilibrated with 20 mM Tris/HCl buffer, pH 7.5, containing 50 mM NaCl. Elution was monitored at 280 nm.

Fig. 3. Sedimentation profile of the purified enzyme. The purified enzyme was applied to a column of Hitachi 05.2.3.2 and the sedimentation was followed at 4°C. The enzyme was applied to a column of Hitachi 05.2.3.2 and the sedimentation was followed at 4°C.
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