Molecular Cloning and Expression of cDNA Encoding 3α,7α,12α-Trihydroxy-5β-cholestanoyl-CoA Oxidase from Rabbit Liver*

Jan I. Pedersen†‡§, Gösta Eggertsen†§, Ulf Hellman*‡, Ulla Andersson*‡, and Ingemar Björkhem*‡

From the Division of Clinical Chemistry, Karolinska Institute, Huddinge University Hospital, 14186 Huddinge, Sweden, the Institute for Nutrition Research, University of Oslo, 0316 Oslo, Norway, and the Ludwig Institute for Cancer Research, Biomedicum, Uppsala University, 75124 Uppsala, Sweden

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The steroid side chain cleavage in bile acid formation is catalyzed by liver peroxisomal enzymes (Pedersen, J. I. and Gustafsson, J. (1980) FEBS Lett. 121, 345–348; Kase, F., Björkhem, I., and Pedersen, J. I. (1983) J. Lipid Res. 24, 1560–1567). We here describe the cloning and sequencing of a cDNA coding the first of these enzymes, a 3α,7α,12α-trihydroxy-5β-cholestanoyl-CoA oxidase (THCA-CoA oxidase) from rabbit liver peroxisomes. After tryptic digestion of purified protein in a polyacrylamide gel, five peptides were isolated and sequenced. Using two oligonucleotides deduced from the amino acid sequence data, two overlapping clones were isolated from a rabbit liver cDNA library, which together made up a unique cDNA sequence of 2139 base pairs. It contained an open reading frame of 2046 base pairs encoding a protein of 681 amino acids with a molecular mass of 76,209 daltons. All five peptides could be localized within the sequence. Transfection of COS cells with the coding part of the cDNA resulted in a significant expression of THCA-CoA oxidase activity. We were not able to demonstrate 3α,7α-dihydroxy-5β-cholestanoyl-CoA oxidase activity under the same conditions. The obtained sequence showed 73.6% similarity with a proposed rat THCA-CoA oxidase and 81% similarity with a recently reported human branched chain acyl-CoA oxidase, indicating that these three proteins represent the same enzyme. The similarity with rat palmitoyl-CoA oxidase was 41.8%. The C-terminal tripeptide of the protein was SNL, a previously undescribed variant of the main class of peroxisomal targeting signals. Northern blot analysis revealed that the gene is transcribed in liver and kidney, and the major mRNA fraction had a size of approximately 2.6 kilobase pairs.

Formation of bile acids from cholesterol takes place exclusively in the liver. The sequence of reactions involves a large number of enzymes localized to several compartments of the cell (1). The final steps start with activation to CoA esters of palmityl-CoA. The small amounts of protein available and because of the instability of the enzyme. Recently, Van Veldhoven et al. reported on the successful purification (13) and (during preparation of this manuscript) also on the cloning of this enzyme from rat liver (14) as well as of a similar enzyme from human liver (15). We have noted that a THCA-CoA oxidase from rabbit liver was more stable than its rat counterpart, and this source also provided more material. In the present work we report on the partial purification of THCA-CoA oxidase from rabbit liver, cloning and sequencing of a cDNA coding for the enzyme, and expression of enzyme activity in COS cells after transfection with this cDNA.

EXPERIMENTAL PROCEDURES

Materials—3α,7α,12α-Trihydroxy-5β-[7-3H]cholestan-27-oic acid as well as 3α,7α-dihydroxy-5β-cholestan-27-oic acid were prepared as described (16) using bile from Alligator mississippiensis as starting material. The natural form of THCA in bile of A. mississippiensis has been identified as the 25R-diestereoisomer (17), but the strong alkaline hydrolysis generally used during isolation causes isomerization at C-25 and the appearance of the 25S-isomer (18). The isolated material was separated by HPLC on a 5-μm C-18 Nucleosil column (0.5 × 25 cm) with 24% 30 mM trifluoroacetic acid (pH 2.9 with triethylamine) in methanol as eluting solvent. The 25R- and the 25S-isomers of THCA were almost completely separated in this system (19). The ratio between the 25R and the 25S isomers in the material primarily isolated was about 7:3. The combined fractions of tritium-labeled (25R-) and (25S)-THCA as well as unlabeled DHCA were converted into CoA esters using the same method as used for the synthesis of choloyl-CoA (20). Purified palmitoyl-CoA oxidase was a gift from Prof. T. Hashimoto (Shinshu University, Matsumoto, Japan). 2,7-Dichlorofluorescin diacetate was from Eastman Kodak Co. (Rochester, NY). The sources of special reagents and kits are given below. Other chemicals were commercial high purity material.

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‡ The first two authors contributed equally to this work.
§ To whom correspondence should be addressed: Institute for Nutrition Research, University of Oslo, P.O. Box 1046 Blindern, 0316 Oslo, Norway. Tel.: 47 22 85 13 58; Fax: 47 22 85 15 30; E-mail: j.i.pedersen@basalmed.uio.no.
¶ The abbreviations used are: THCA, 3α,7α,12α-trihydroxy-5β-cholestanolic acid; DHCA, 3α,7α-dihydroxy-5β-cholestanolic acid; Δ4-THCA, 3α,7α,12α-trihydroxy-5β-cholest-24(25)-enoic acid; 24-OH-THCA, 3α,7α,12α,24-tetrahydroxy-5β-cholestanolic acid; HPLC, high pressure liquid chromatography; bp, base pairs.

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Generation of Peptides by Digestion in Gel—Peptide in the peak fractions from the gel filtration above was concentrated by precipitation (24), and the dried sample was dissolved in 25 μl of sample buffer containing 5 μM dithiothreitol. After heating to 95 °C for 4 min, the reduced sample was alkylated by incubation at room temperature for 20 min with 16 μM iodoacetamide. The sample was subjected to polyacrylamide gel electrophoresis in SDS (25) on a 10% gel of 1-mm thickness using the Mini-Protean II Slab Cell (Bio-Rad). After Coomassie Blue staining, the band corresponding to a molecular mass of 72 kDa, using the ABI PRISMTM dye terminator cycle sequencing kit with AmpliTaq R polymerase FS (Perkin-Elmer). Enzyme Preparation—Liver peroxisomes were prepared from a male rabbit fasted for 24 h essentially as described for rat liver (21). The light mitochondrial fraction generated by centrifugation of the postmitochondrial supernatant at 16,200 rpm (24,000 × g) for 45 min in the Kontron T324 centrifuge (A8.24 rotor) was layered on a linear Nycodenz gradient ranging from 15% (w/v) in 0.25 M sucrose, 1 mM EDTA, and 1 mM Hepes at pH 7.4, to 45% in 1 mM EDTA, 1 mM Hepes and 1 mM Hepes at pH 7.4. The gradient tubes contained a prelayered 2-ml Maxidex cushion and were centrifuged at 30,000 rpm for 30 min at 4 °C in the Sorvall TV-850 vertical rotor. Fractions of 1.7 ml were collected and assayed for catalase activity (22, 23). The five fractions with the highest activity were combined, diluted with 0.25 M sucrose to a volume of 0.1 ml in an incubation medium that contained 0.1 M Tris-HCl, pH 8, 75 μM FAD, 1 mg/ml bovine serum albumin, 0.01% Triton X-100, and 42 μM (25R)- and (25S)-[3H]-THCA-CoA (60,000 cpn). The incubations were stopped with 2.5 μl of 6 M KOH and hydrolyzed at 60 °C for 30 min to remove bound CoA. After acidification with HCl and extraction with ethyl acetate, the samples were analyzed by reversed phase HPLC on a 5-μM YMC-Pack ODS-A column (250 × 4.6-mm inner diameter) (YMC Co., Ltd., Kyoto, Japan). The eluting solvent was 20% 30 trifluoracetic acid (pH 2.9 with triethylamine) in methanol. Radioactivity in the eluent was monitored by a Flo-One Beta radiocromatography detector (Radiocromatic Instruments & Chemical Co., Inc., Tampa, FL). The THCA-CoA racemase activity was assayed in the same incubation buffer as above by measuring the formation of (25R)-and (25S)-THCA-CoA from (25S)-[3H]-THCA-CoA after hydrolysis and extraction as above. Protein was determined by the method of Bradford (28) using bovine serum albumin as a standard. Protein in the eluent from the gel filtration column was estimated based on the UV absorbance in the eluent compared with that of a fixed amount of injected bovine serum albumin.

Analysis by Gas Chromatography-Mass Spectrometry—Extraction of incubations were dried under N2, and the material was methylated by treatment with methanol, 2,2-dimethoxypropane, concentrated HCl (1: 0.70.011, v/v) for 30 min at 55 °C. The mixture was dried under N2 and sialylated by treatment with 300 μl of pyridine, hexamethyldisilazane, trimethylchlorosilane (3:2:1, v/v) for 30 min at 60 °C. The solvent was removed by N2, and the material was dissolved in isooctane. Gas chromatography-mass spectrometry was performed on a Hewlett Packard 5890 series II Plus gas chromatograph equipped with an HP-ultra capillary column (25 m × 0.30 mm, 0.33-μm thickness), connected to an HP 5972 mass selective detector and an HP 7673A automatic sample injector. The oven temperature program was as follows: 180 °C for 1 min, 35 °C/min to 270 °C, and then 20 °C/min to 300 °C, where the temperature was kept for 19 min. Helium was used as carrier gas. The gas chromatograph was operated in the constant flow mode, with the flow rate set to 0.8 ml/min. The injector was operated in the splitless mode and kept at 270 °C, and the detector transfer line was kept at 280 °C. The mass spectrometer was operated in the selected ion monitoring mode, and two ions were detected simultaneously. The ions used for analysis were as follows: 3a,7α,12α-trihydroxy-5β-cholestanoyl-CoA oxidase (26). Even if it was possible after several purification steps to obtain a preparation that showed this band on SDS-polyacrylamide gel electrophoresis, the total amount of protein present was too small for further characterization (29). We therefore decided to reduce the number of purification steps and base our cloning strategy on in-gel digestion and peptide analysis of this protein band. The steps used are shown in Table I. Solubilization of the peroxisomes in hypotonic pyrophosphate buffer in the presence of detergent on ice was
found to be efficient. Heat treatment was avoided, since it was found that, unlike palmitoyl-CoA oxidase (26), THCA-CoA oxidase was very heat-labile. The acyl-CoA oxidase activity and the THCA-CoA activity were separated on a hydroxylapatite column. THCA-CoA oxidase eluted at 60 mM phosphate buffer, while acyl-CoA oxidase was eluted at 200 mM phosphate buffer. No acyl-CoA oxidase was detected in the 60 mM fraction eluate, while a small amount of THCA-CoA oxidase eluted in the 200 mM fraction. The 60 mM fraction eluate was concentrated by dialysis against polyethylene glycol and further fractionated by gel filtration (Fig. 1). Maximum THCA-CoA oxidase activity was detected in fraction 14 that eluted between 13.00 and 13.25 ml, which was immediately in front of the elution volume corresponding to bovine serum albumin (13.5 ml), suggesting a molecular mass of the oxidase of about 70 kDa. Fractions between 12.75 and 13.50 ml were used as a source of the oxidase. Activity measurement in the active fractions was complicated by the presence of a THCA-CoA racemase activity (interconversion of (25R)- and (25S)-THCA-CoA) (19) that started to elute immediately after the peak fraction of THCA-CoA oxidase activity.

Maximum THCA-CoA racemase was found to be in a fraction between 14.75 and 15.00 ml, corresponding to a molecular mass of about 40 kDa. This compares with the values given by Schmitz et al. of 45 kDa for a similar rat liver mitochondrial enzyme (30) and 47 kDa for a human liver peroxisomal enzyme (31). Fractions between 14.5 and 15.5 ml (fractions 20–23) were pooled and used as a source of racemase in incubations when required (see legend to Fig. 1). The racemase preparation was found to be contaminated with some THCA-CoA oxidase activity, corresponding to about 10% of that racemase preparation was found to be contaminated with some THCA-CoA oxidase activity, corresponding to about 10% of that.

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Very recently, the sequence for a branched chain acyl-CoA from CoA oxidase, the sequence of which is 17 amino acid residues shorter in the amino-terminal end than the other two enzymes. The most proximal of these methionine residues in the amino-terminal part of the deduced oxidase was allowed to transfect the same number of cells.

Expression Pattern of THCA-CoA Oxidase—To visualize the mRNA for THCA-CoA oxidase, a cDNA probe of 1135 bp (corresponding to positions 47–1172 in the cDNA sequence) was used. A strong signal was obtained with rabbit liver total RNA corresponding to a band at 2.6 kilobase pairs (Fig. 8). A much weaker signal was obtained from rabbit kidney, while no signal was detected from other organs tested (not shown). Signals were also obtained with mouse and rat liver (Fig. 8). Fasting had no significant effect on mRNA level in any of the three species tested.

Expression of THCA-CoA Oxidase Activity in COS Cells—To express THCA-CoA activity in eukaryotic cells, a plasmid vector, pCAGGS, carrying a modified chicken β-actin promoter (35) was used. A cDNA fragment including the coding sequence of the oxidase was prepared from the λ clone 10 by polymerase chain reaction (Gene Amp XL polymerase chain reaction kit; Perkin-Elmer). The primers contained a restriction site for the XbaI, and the fragment obtained numbered 2081 bp, corresponding to positions 46–2126 in the cDNA. This fragment was ligated into the XbaI site of the expression vector pCAGGS, and the construct was propagated in Epicurian Coli XLI-Blue MR-F’Kan supercompetent cells (Stratagene, La Jolla, CA). The insert could be cleaved out of the vector by digestion with XbaI, and the orientation was established by DNA sequencing. An estimated quantity of 20 μg of plasmid DNA with the cDNA inserted the correct way was used to transfect approximately 2 × 10⁶ cells. In a control experiment, a similar quantity of plasmid DNA with the cDNA inserted in the opposite direction was allowed to transfect the same number of cells.

When incubating a homogenate of the transfected cells with tritium-labeled THCA-CoA as described peroxisomal targeting signals (33). In particular, the sequence is different from HKM (His-Lys-Met) found in rat THCA-CoA oxidase (14) and SKL (Ser-Lys-Leu) found in human branched chain acyl-CoA oxidase (15).

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When incubating a homogenate of the transfected cells with tritium-labeled THCA-CoA as described under “Experimental Procedures,” a low but significant THCA-CoA oxidase activity was detected with the assay based on radio-HPLC (Fig. 9). Two independent experiments resulted in 3 and 2.3% total conversion of (25R)- and (25S)-THCA-CoA into Δ⁷⁴-THCA. A homogenate of cells transfected with a construct with the coding region inversely inserted had no detectable activity. It should be noted
that it is only (25S)-THCA-CoA that is a substrate of the enzyme (19). It could be calculated that the conversion of this stereoisomer into D24-THCA was 13 and 16%, respectively, in the above two experiments with cells transfected with the cDNA inserted the correct way. (cf. Fig. 9). Combined gas chromatography-mass spectrometry allows a considerably more sensitive and accurate assay than the above HPLC method. Fig. 10 shows analysis by this method of hydro-

**FIG. 6.** Rabbit THCA-CoA oxidase cDNA and the sequence of the encoded polypeptide. The sequences of the five peptides obtained by in-gel digestion are underlined.
FIG. 7. Alignment of rabbit THCA-CoA oxidase with other peroxisomal oxidases. Comparison of the whole peptide sequence (oryctolagus THCCox; oTHCCox) with human branched chain acyl-CoA oxidase (hBRCACox) (15), rat THCA-CoA oxidase (rTHCCox) (14), and rat palmitoyl-CoA oxidase (rACox) (33). Residues matching the whole peptide sequence are shaded.
lyzed extracts of incubations of (25R- and (25S)-THCA-CoA with the same amount of cells transfected by either a complete or a truncated insert. The ion at m/z 500, corresponding to the molecular ion of the methyl ester trimethylsilyl derivative of THCA, and the ion at m/z 498, corresponding to the molecular ion of the same derivative of Δ24-THCA, were followed through the gas chromatography. There was no separation of the R- and S-stereoisomers of THCA on the column used. In the analysis of the incubation with cells transfected with the complete insert (Fig. 10A), a conversion of 1.2% of the substrate (25R)- and (25S)-THCA-CoA, was obtained. In addition, there was another product peak, corresponding to a conversion of 0.3%, occurring in the tracing of the ion at m/z 498 (Fig. 10A). This peak had a retention time identical to that of the methyl ester trimethylsilyl ether of 24-OH-THCA. This compound has a prominent ion at m/z 498 in its mass spectrum, corresponding to the M-90 ion. Formation of 24-OH-THCA is most probably due to the presence of hydratase activity in the COS cells, converting the newly synthesized Δ24-THCA-CoA into the corresponding 24-hydroxylated product. A cDNA fragment devoid of the N-terminal sequence corresponding to the region up to the second methionine (in position 18) was also used in a separate expres-
sion experiment. A very small product peak calculated to represent a conversion of about 0.3% of the substrate was observed (Fig. 10B). Most probably, this represents a small endogenous THCA-CoA oxidase activity in the COS cells.

When using unlabeled DHCA-CoA as a substrate and analyzing the product by gas chromatography-mass spectrometry as above, no conversion into Δ^{24}-DHCA could be detected in incubations with the transfected cells. In these analyses, the ion at m/z 412, corresponding to the molecular ion of the methyl ester trimethylsilyl derivative of DHCA, and the ion at m/z 410, corresponding to the molecular ion of the same derivative of Δ^{24}-DHCA, were used. A significant conversion was obtained, however, in incubations with either purified rabbit liver peroxisomes or with the crude enzyme from the gel filtration step. The rates of these reactions were about half of those obtained with THCA-CoA as substrate.

**DISCUSSION**

In the present work, we have cloned a THCA-CoA oxidase from rabbit liver peroxisomes. That the cloned protein truly represents this enzyme was demonstrated by the expression of THCA-CoA oxidase activity after transfection in COS cells. Even if the activity was low, it exceeded severalfold that in mock-transfected cells. Furthermore, the product of the oxidation reaction was verified both by HPLC and by gas chromatography-mass spectrometry. During the preparation of this manuscript, the cloning of two proteins closely related to the one described here was published, a rat peroxisomal THCA-CoA oxidase (14) and a human peroxisomal branched chain acyl-CoA oxidase (15). Expression of the branched chain acyl-CoA oxidase cDNA was demonstrated by the use of antibody against the protein. For neither of the two has enzyme activity been demonstrated in an expression system, however. Formal proof of the correct protein being cloned was thus not presented. The high degree of homology between the protein described here and both rat peroxisomal THCA-CoA oxidase and human peroxisomal branched chain acyl-CoA oxidase (73.6 and 81%, respectively) makes it clear that these three proteins represent the same enzyme from the three different species.

When compared with peroxisomal rat acyl-CoA oxidase, the similarity was only 41.8%. Of special interest was the presence of two methionines at the N-terminal part of rabbit THCA-CoA oxidase, the second of which (at position 18) corresponds to the start of rat acyl-CoA oxidase. Obviously, the initial 17 amino acid residues are essential for catalytic activity, since a clone lacking this portion was unable to elicit a significant THCA-CoA oxidase activity after transfection in COS cells. The more specific function of this part of the protein is unknown; possibly, it is required for correct binding of the substrate.

The C-terminal tripeptide of the protein was found to be SNL (Ser-Asn-Leu). This is different from the previously reported C-terminal tripeptides considered to be peroxisomal targeting signals. From site-directed mutagenesis experiments it is known that certain variations of the tripeptide motif are tolerated for it to function as a peroxisomal targeting signal (36). A substitution of Asn in SNL for Lys in SKL (Ser-Lys-Leu), the C-terminal tripeptide considered to be peroxisomal targeting required for correct binding of the substrate, Baumgart et al. (14) could not confirm the interaction of the C terminus of rat THCA-CoA oxidase with the human peroxisomal targeting signal receptor, in contrast to the situation with rat palmitoyl-CoA oxidase and pristanoyl-CoA oxidase, and suggested that THCA-CoA oxidase was targeted to peroxisomes by a hitherto unknown mechanism. In view of the finding that SKL and SNL are the C termini of the counterparts of this enzyme in human and rabbit liver, respectively, this suggestion does not appear to be true for THCA-CoA oxidases of other species.

It has generally been considered that both THCA and DHCA are chain-shortened by the same peroxisomal enzymes. The crude enzyme preparation that eluted from the gel filtration column (Fig. 1) converted both THCA-CoA and DHCA-CoA to the respective unsaturated derivatives, although DHCA-CoA was converted at a considerably lower rate than THCA-CoA. This may not be surprising in view of the fact that chenodeoxycholic acid is normally found only in trace amounts in rabbit bile (37). After transfection of COS cells with the appropriate clone, we were not able to detect any activity toward DHCA-CoA. Whether this is due to the activity being too low for detection by the method used or to the existence of a separate oxidase for DHCA-CoA can only be speculated on.

As expected, the enzyme was expressed in livers from both rat and mouse in addition to rabbit. As previously demonstrated (14), kidney also gave a weak signal. Formation of bile acids is considered to take place only in the liver. The function of the enzyme in the kidney is not known. The possibility cannot be excluded that small amounts of cholesterol hydroxylated in the 27-position in other organs may eventually be further oxidized and chain-shortened in the kidneys.

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