Molecular cloning and further characterization of rat peroxisomal trihydroxycoprostanoyl-CoA oxidase

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The composite trihydroxycoprostanoyl-CoA oxidase cDNA sequence, derived from overlapping clones isolated via screening of two different rat liver expression libraries, consisted of 2509 bases and contained an open reading frame of 2046 bases, encoding a protein of 681 amino acids with a calculated molecular mass of 76711 Da. The reading frame and identity of the trihydroxycoprostanoyl-CoA oxidase cDNA were confirmed by the location of various tryptic peptides, obtained from the purified enzyme, in the deduced amino acid sequence. The C-terminus (His-Lys-Met) of trihydroxycoprostanoyl-CoA oxidase did not seem to interact with the C-terminal peroxisomal targeting signal 1 (PTS1) import receptor, although the tripeptide fits the rule of conserved PTS1 variants for targeting of proteins to glycosomes of Trypanosomatidae. At the protein level, trihydroxycoprostanoyl-CoA oxidase showed 45 % identical amino acids with rat palmitoyl-CoA oxidase, whereas the identity with pristanoyl-CoA oxidase was much lower (22 %). Northern analysis of multiple rat tissues revealed a signal (approx. 2.6 kb) only in liver and (although much weaker) in kidney. Dot-blot analysis of total liver RNA revealed that the mRNA for trihydroxycoprostanoyl-CoA oxidase is not induced after treatment of rats with structurally unrelated peroxisome proliferators and indicates that highly similar mRNAs are present in other mammals, including man. Immunocytochemistry showed a decrease in trihydroxycoprostanoyl-CoA oxidase protein in individual liver peroxisomes ('diluting-out effect') after treatment of rats with bezafibrate, whereas the palmitoyl-CoA oxidase labelling was significantly increased.

INTRODUCTION

In mammals peroxisomal β-oxidation is responsible for the degradation of the major portion of the very long straight-chain fatty acids, dicarboxylic fatty acids, isoprenoid-derived 2-methyl branched fatty acids (e.g. pristanic acid) and eicosanoids (e.g. prostaglandins, leukotrienes and thromboxanes). In addition, hepatic peroxisomes catalyse the β-oxidation of the side chain of cholesterol, resulting in the formation of the primary bile acids (conversion of di- and trihydroxycoprostanic acids into chenodeoxycholic and cholic acids, respectively) [1–3].

The first step of peroxisomal β-oxidation is catalysed by FAD-containing acyl-CoA oxidases that donate electrons directly to molecular oxygen, resulting in the formation of hydrogen peroxide. It has recently become clear that this step is catalysed by several enzymes, each enzyme displaying its own substrate specificity. Rat liver peroxisomes harbour three acyl-CoA oxidases: (1) palmitoyl-CoA oxidase [4,5], oxidizing the CoA-esters of straight-chain fatty acids, dicarboxylic fatty acids and eicosanoids [6]; (2) pristanoyl-CoA oxidase [7,8], oxidizing the CoA esters of 2-methyl branched fatty acids such as the naturally occurring pristanic acid and the synthetic 2-methylpalmitic acid but also displaying some activity towards the CoA-esters of straight-chain fatty acids [6]; and (3) trihydroxycoprostanoyl-CoA oxidase [5,9], oxidizing the CoA esters of the bile acid intermediates di- and trihydroxycoprostanic acids [6,9]. Enzyme activity measurements and immunoblot analysis revealed that palmitoyl-CoA oxidase and pristanoyl-CoA oxidase are present in hepatic and extrahepatic tissues [6,8], whereas trihydroxycoprostanoyl-CoA oxidase was found only in liver [5]. Similar experiments also demonstrated that treatment of rats with fibrates causes the induction of palmitoyl-CoA oxidase in liver and some extrahepatic tissues (heart, kidney and intestinal mucosa). In contrast, pristanoyl-CoA oxidase and trihydroxycoprostanoyl-CoA oxidase activities were not induced [5,7].

In human liver only two acyl-CoA oxidases have been detected: (1) palmitoyl-CoA oxidase, the substrate specificity of which resembles that of rat palmitoyl-CoA oxidase; and (2) a branched-chain acyl-CoA oxidase that oxidizes the CoA esters of 2-methyl branched fatty acids as well as those of the bile acid intermediates di- and trihydroxycoprostanic acids, which also possess a 2-methyl substitution in their side chain [10]. Thus the human branched-chain acyl-CoA oxidase combines the functions of rat pristanoyl-CoA oxidase and trihydroxycoprostanoyl-CoA oxidase. As with human palmitoyl-CoA oxidase, the human branched-chain acyl-CoA oxidase seems to be expressed not only in liver but also in kidney as revealed by enzyme activity measurements [10].

The presence of multiple acyl-CoA oxidases is reminiscent of the mitochondrial system, in which distinct acyl-CoA dehydrogenases are involved in the desaturation of different acyl-CoAs. The primary structure of these dehydrogenases has been unravelled, allowing the elucidation of the mutations present in various human diseases [11].

To characterize further the mammalian oxidases with different substrate specificities and to obtain insight in the evolvement of the corresponding genes during evolution, we decided to clone the cDNA species of rat pristanoyl-CoA oxidase [12], rat

Abbreviations used: PTS1, C-terminal peroxisomal targeting signal 1.

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The nucleotide sequence reported will appear in DDBJ, EMBL and GenBank Nucleotide Sequence Databases under the accession number X95189.

trihydroxycoprostanoyl-CoA oxidase and human branched-chain acyl-CoA oxidase. This report describes the molecular cloning and further characterization of rat trihydroxycoprostanoyl-CoA oxidase, an enzyme consisting of two identical subunits, each of approx. 70 kDa [9].

EXPERIMENTAL

Generation of polyclonal antibodies against acyl-CoA oxidases

Palmitoyl-CoA oxidase [8] and trihydroxycoprostanoyl-CoA oxidase [9] were isolated as described before. To obtain an antiserum against the native form of trihydroxycoprostanoyl-CoA oxidase, the peak fractions of the final gel-filtration column [9] were emulsified with Freund's adjuvant and injected subcutaneously in rabbits. Boosting and preparation of the antisera were done as described by Mayer and Walker [13]. The preparation of antibodies against the 53 and 21 kDa subunits (B and C subunits respectively) of rat palmitoyl-CoA oxidase [8] and against the denatured subunit of trihydroxycoprostanoyl-CoA oxidase [9] were as described.

Amino acid sequencing of tryptic peptides

For trypsin digestion of trihydroxycoprostanoyl-CoA oxidase, 4 ml of the purified enzyme preparation containing 1 mg of protein was concentrated and the buffer was replaced by 0.4 M ammonium carbonate buffer, pH 8 (Microcon-10; Amicon). An aliquot (0.1 ml containing 250 μg of oxidase) was digested with 50 μg of alkylated trypsin (Promega) for 24 h at 37 °C. Subsequently the preparation was loaded on a DeltaPak C18 column (150 mm × 2 mm; 300 Å, 5 μm; Waters). The peptides were eluted with a linear gradient of 10–90 % (v/v) acetonitrile containing 0.05 % (v/v) trifluoroacetic acid at a flow rate of 100 μl/min over a period of 120 min. Selected fractions containing pure peptides were subjected to amino acid sequencing by Edman degradation in a 477A Protein Sequencer (Applied Biosystems).

Immunoblotting

Tissues to be studied were removed from decapitated rats and homogenized in 4 vol of 0.25 M sucrose/1 mM EDTA (pH 7.2)/0.1 % (v/v) ethanol. Aliquots of the homogenates corresponding to 25 μg of tissue protein and purified enzymes (200 ng) were applied on ready-to-use gels (8–18 % ExcelGel SDS; Pharmacia). After electrophoresis (Multiphor II horizontal system; Pharmacia), the separated polypeptides were transferred to nitrocellulose by semi-dry blotting [14]. After blocking for 1 h in PBS containing 1 % (w/v) BSA and 0.05 % (v/v) Tween-20, blots were incubated overnight with antisera. Antigen–antibody complexes were detected by enhanced chemiluminescence (ECL detection reagents; Amersham) after incubation with a peroxidase-labelled goat anti-rabbit IgG. For some blots, alkaline phosphatase-labelled sheep anti-rabbit IgG and Nitro Blue Tetrazolium/5-bromo-4-chloro-3-indolyl phosphate colour development were used.

Isolation of the cDNA for rat trihydroxycoprostanoyl-CoA oxidase

For immunoscreening, XL1-Blue bacteria were transfected with 10^6 plaque-forming units of a λ-UniZap rat liver cDNA expression library [oligo(dT) primed; Stratagene]. After induction of the fusion proteins by overnight overlay with isopropyl β-D-thiogalactoside-soaked nitrocellulose membranes, the non-specific binding sites on the membranes were blocked with 10 % (v/v) newborn calf serum in Tris-buffered saline containing 0.05 % (v/v) Tween-20. Positive clones were detected by incubation overnight with the primary antibody and the immune complexes were revealed by incubation with an alkaline phosphatase-labelled sheep anti-rabbit IgG and subsequent colour detection with Nitro Blue Tetrazolium/5-bromo-4-chloro-3-indolyl phosphate.

Eight clones, which were strongly cross-reactive in the second round with both antibodies to trihydroxycoprostanoyl-CoA oxidase, were isolated and pBluescript SK(−) plasmids containing specific cDNA inserts were prepared after subcloning *in vivo*. Sequencing was done by the dideoxynucleotide chain-termination method [15] after subcloning of different parts of the cDNA into pBluescript SK(+) or by using oxidase-specific primers. Translation of the cDNA sequences and subsequent alignment with the rat palmitoyl-CoA oxidase amino acid sequence [16] revealed that the longest clone started at position 214 of rat palmitoyl-CoA oxidase. For completion of the whole open reading frame, 10^6 plaques of a random-primed and oligo(dT)-primed λ-ZAP rat liver cDNA library (Stratagene) were screened by hybridization with the radioactively labelled 5′ *Eco*RI fragment of the longest trihydroxycoprostanoyl-CoA oxidase clone (clone 3a; see Figure 1). Twenty intensely cross-hybridizing clones were further purified and their plasmid DNA was isolated after subcloning *in vivo* and characterized by PCR with a vector primer in the sense direction and trihydroxycoprostanoyl-CoA oxidase-specific primers in the reverse direction. The clones yielding the longest PCR fragments in the 5′ direction were sequenced.

Isolation of a partial cDNA for human palmitoyl-CoA oxidase

A cDNA encoding human palmitoyl-CoA oxidase (88 % identical with its rat counterpart) was isolated by immunoscreening 10^6 plaque-forming units of a λ-UniZAP human liver cDNA library [oligo(dT) primed; Stratagene] by using a polyclonal rabbit antiserum directed against the A subunit of rat palmitoyl-CoA oxidase, which also recognizes the B and C subunits. The screening was performed as described above. Six clones that in the second screening round also cross-reacted with antibodies against the 23 and 51 kDa subunits of rat palmitoyl-CoA oxidase were further purified. Their plasmid DNA was isolated after subcloning *in vivo* and sequenced. The longest clone comprised 2098 bases of the human palmitoyl-CoA oxidase cDNA sequence [17–19], starting at base 82 in the open reading frame and ending at base 2180 in the 3′ untranslated region. Plasmid DNA from this clone was used for all hybridization experiments.

RNA preparation for dot-blots

The animals (mouse, rat, hamster, guinea pig and rabbit) were anaesthetized with Nembutal; the livers were excised and immediately freeze-clamped in liquid nitrogen. Frozen liver tissue from dogs was kindly provided by Dr. Van de Werf (Department of Cardiology, University of Leuven, Leuven, Belgium). As a human sample, frozen tissue from a non-transplanted lobe of a liver prepared for transplantation was used. Approval was granted by the Institutional Ethics Committee.

For the isolation of total RNA, 100 mg of each frozen tissue was ground in liquid nitrogen and subsequently homogenized in 1 ml of TRIzol Reagent (Gibco BRL Life Technologies) with a Polytron tissue homogenizer. All subsequent steps for the extraction of total RNA were performed in accordance with the manufacturer's specifications.

The differences in mRNA content in the total RNA preparations were estimated after hybridization of dot-blots [20] (15 μg of total RNA for each dot) with a human actin cDNA probe

(Clontech) and scanning of the blot with a PhosphorImager (Molecular Dynamics). Subsequently, new dot-blots were made containing the same amount of mRNA as present in 15 μg of total RNA from a male Wistar rat. Hybridizations of the dot-blots were done in parallel with radiolabelled cDNA probes for trihydroxycoprostanoyl-CoA oxidase, palmitoyl-CoA oxidase and β-actin.

Northern analysis

For radioactive hybridization, 200 ng of trihydroxycoprostanoyl-CoA oxidase cDNA (combined plasmids derived from clones 319 and 5a) was labelled for 3 h by random priming (ReadyToGo dCTP labelling kit; Pharmacia). A rat multiple-tissue Northern blot (Clontech) was hybridized in accordance with the manufacturer's instructions except that 1.5% instead of 2% (w/v) SDS was used in the hybridization solution and that the membrane was washed under more stringent conditions (0.1 × SSC, 0.5% SDS, 68 °C) to avoid cross-hybridization with other acyl-CoA oxidases.

Post-embedding immunocytochemistry

Livers of Sprague–Dawley rats were fixed by perfusion of the anaesthetized animals via the portal vein [21] with 0.25% (v/v) glutaraldehyde/0.2 M sucrose/0.1 M Pipes buffer (pH 7.4). Tissue sections (100 μm) were cut with a microslicer (Dosaka EM Co.) and embedded in London resin white (soft grade; London Resin Co.). Ultrathin sections (50–70 nm; Reichert-Jung ultramicrotome) were collected on Formvar-coated nickel grids and processed for Protein A–gold (15 nm particles) immunogold electron microscopy [22]. For comparison, liver sections of control and bezafibrate-treated animals were incubated in parallel with the antisera directed against native trihydroxycoprostanoyl-CoA oxidase or the C subunit of palmitoyl-CoA oxidase. Incubated sections were contrasted with uranyl acetate and lead citrate and inspected in a Philips 301 electron microscope.

RESULTS AND DISCUSSION

Characterization and identification of the cDNA encoding rat liver trihydroxycoprostanoyl-CoA oxidase

The cDNA sequence for the complete open reading frame encoding rat trihydroxycoprostanoyl-CoA oxidase was obtained by three consecutive screenings of two different rat liver libraries. All oxidase clones, the restriction sites of the cDNA that were used for subcloning, and the sequencing strategy are summarized in Figure 1. The complete cloned cDNA spanned 2509 bases. Three methionine residues were present in the same reading frame at the translation start region (ATG at positions -13 to -15, 1 to 3 and 13 to 15). However, only the ATG at position 1 to 3 fitted Kozak's rule of 'conserved nucleotides' [23] with an A at -3 and a G at $+4$. The use of this start codon would result in an open reading frame of 2046 bases encoding a protein of 681 amino acids with a nominal molecular mass of 76711 Da. The consensus sequence for polyadenylation, AATAAA, was found at position 2292, 13 bases upstream of the poly(A) tail. Finally, a stretch of 20 G was found in the 3′ trailer at position 2120 (Figure 2).

The higher calculated nominal mass of the deduced trihydroxycoprostanoyl-CoA oxidase protein than that of rat palmitoyl-CoA oxidase (74.6 kDa) was confirmed by SDS/PAGE and immunoblotting of the purified enzymes. In 10% homogeneous gels, the protein (estimated molecular mass 77.6 kDa) ran just behind the A subunit of palmitoyl-CoA oxidase (estimated molecular mass 75.8 kDa) (results not shown). The previously reported molecular mass of 69.6 kDa for the trihydroxycoprostanoyl-CoA oxidase subunit [9] was based on its migration in gradient gels.

The identity of the trihydroxycoprostanoyl-CoA oxidase protein with the translated open reading frame of the cloned cDNA was confirmed by mapping the trihydroxycoprostanoyl-CoA oxidase peptides to the translated cDNA sequence (Figure 2). All peptide sequences obtained after trypsin cleavage of the purified enzyme were found in the deduced amino acid sequence.

Does trihydroxycoprostanoyl-CoA oxidase interact with the peroxisomal targeting signal 1 (PTS1) receptor?

The last three residues of trihydroxycoprostanoyl-CoA oxidase are His-Lys-Met, in comparison with Ser-Lys-Leu in palmitoyl-CoA oxidase. Most peroxisomal matrix proteins contain a C-terminal tripeptide that functions as a peroxisomal targeting signal. The tripeptide consists of Ser-Lys-Leu or a conserved variant in which Ser can be replaced by Ala or Cys, and Lys by Arg, His or Gln [12,24,25]. Although for engineered luciferase constructs His can also substitute for Ser, and Met for Leu, in glycosomal (trypanosomes) protein import experiments [26], the combination His-Lys-Met does not match any of the known C-terminal targeting sequences in mammals. Also our experimental results suggest that the C-terminus of trihydroxycoprostanoyl-CoA oxidase does not interact with the PTS1 receptor. Neither the purified oxidase nor a synthetic peptide consisting of the C-terminal 13 amino acids (CYIRPLMLGWRHKM; the underlined cysteine residue was introduced to allow coupling of the peptide to albumin) bound to the bacterially expressed biotinylated human PTS-1 receptor [27]. In contrast with trihydroxycoprostanoyl-CoA oxidase, purified rat palmitoyl-CoA oxidase (Ser-Lys-Leu) [16] or pristanoyl-CoA oxidase (Ser-Gln-Leu) [12] bound to the receptor (results not shown). To study the interaction of trihydroxycoprostanoyl-CoA oxidase and the PTS1 receptor *in vivo*, the C-terminal portion of trihydroxycoprostanoyl-CoA oxidase (starting at position 366), fused to the Gal4 DNA-binding domain, was tested in a two-hybrid system. After co-transformation of the yeasts with a plasmid encoding a fusion protein of the activation domain linked to the PTS1 receptor [27], no blue colonies emerged. In control experiments, with the C-terminal part of palmitoyl-CoA oxidase fused to the Gal4 DNA-binding domain, blue colonies were obtained (results not shown). Taken together, these results indicate that His-Lys-Met is most probably not a PTS1 targeting signal and suggest that trihydroxycoprostanoyl-CoA oxidase is targeted to peroxisomes by hitherto unidentified N-terminal or internal sequences or by co-translocation with another peroxisomal matrix protein [28,29].

Two separate gene duplication events in the mammalian acyl-CoA oxidase tree

Computer alignment of the deduced amino acid sequence of trihydroxycoprostanoyl-CoA oxidase with those of palmitoyl-CoA oxidase [16] and pristanoyl-CoA oxidase [12] revealed that trihydroxycoprostanoyl-CoA oxidase shares considerably more amino acid identity with palmitoyl-CoA oxidase (45%) than with pristanoyl-CoA oxidase (22%) (Figure 3), despite the fact that trihydroxycoprostanoyl-CoA oxidase and pristanoyl-CoA oxidase are both active on 2-methyl branched compounds, the bile acid intermediates and 2-methyl-substituted fatty acids respectively [6]. These observations, together with the finding that pristanoyl-CoA oxidase shares an amino acid identity with the other mammalian oxidases that is as low as with the yeast acyl-CoA oxidases [12], indicates that the pristanoyl-CoA oxidase

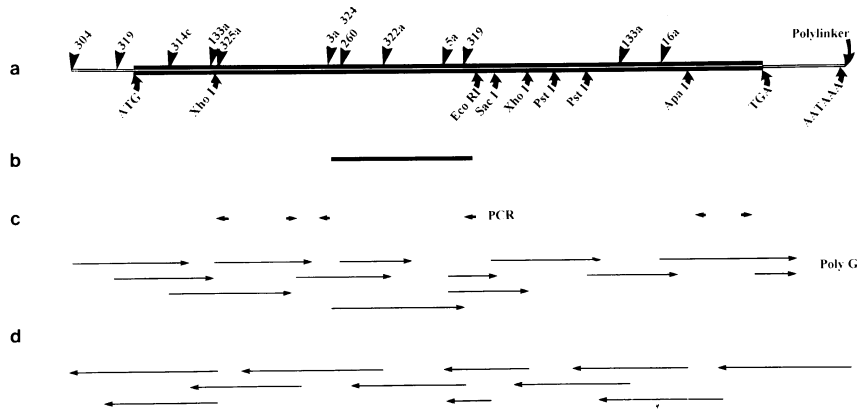

Figure 1 Cloning and sequencing strategy of rat trihydroxycoprostanoyl-CoA oxidase

The complete cDNA sequence, shown in Figure 2, was generated by the alignment of sequences obtained from distinct clones. The starting position of the clones, indicated by numbers in (**a**), is shown by arrowheads (for clones 319 and 133a both start and end positions are marked). The polylinker, which connects the oligo(dT)-primed clones to the multicloning site of the pBluescript SK(−) phagemid vector, is also indicated. The combined sequence spans 2509 bases with 197 bases in the 5′ leader sequence, an open reading frame of 2046 bases (double line) and a 3′ trailer sequence of 266 bases. Start (ATG) and stop (TGA) codons, the cleavage sites of restriction enzymes, which were used for subcloning, and the polyadenylation site (AATAAA) are also shown in (**a**). (**b**) The size and position of the *Eco*RI fragment of clone 3a, used for plaque hybridization to obtain the missing 5′ end. (**c**) The positions of specific PCR primers; (**d**) the sequences obtained in the sense and anti-sense directions. Because of a stretch of 20 G bases (Poly G) near the polyadenylation site, the polymerase reaction in the sense direction was halted.

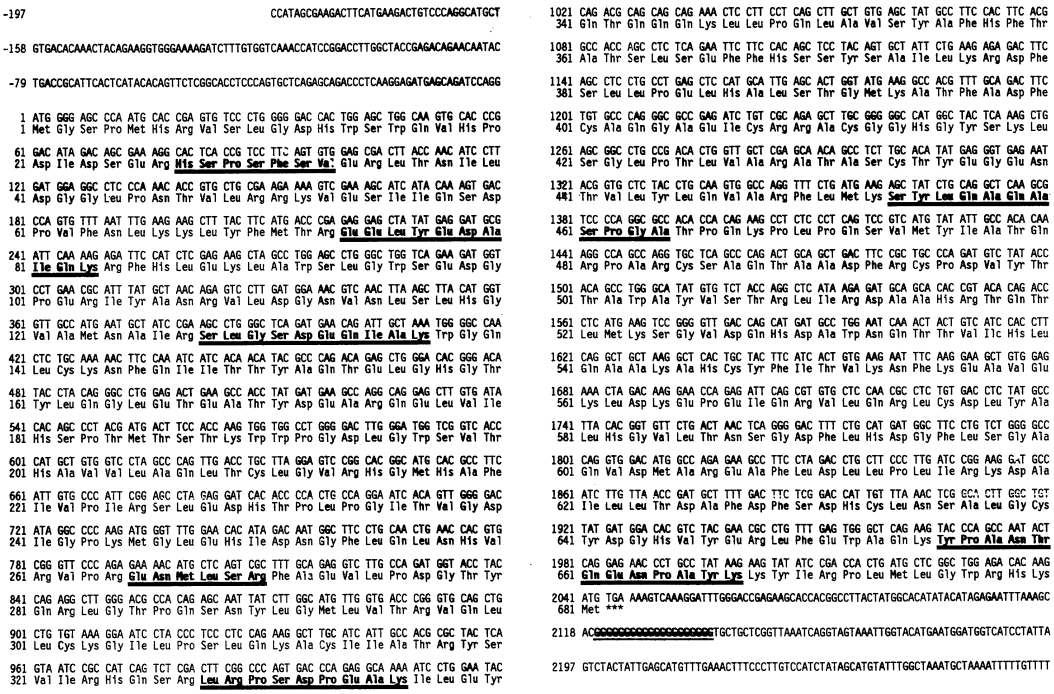

Figure 2 Nucleotide and deduced amino acid sequence of rat trihydroxycoprostanoyl-CoA oxidase

The nucleotide sequence, numbered in the 5′ to 3′ direction and starting at the methionine start codon, was generated from overlapping sequences as depicted in Figure 1. Numbers at the left of the sequences indicate the appropriate nucleotide or amino acid position. Nucleotides in the 5′ non-coding region are indicated by negative numbers. Amino acid residues are numbered starting at the start codon; the stop codon is marked ***. The regions for which peptide sequences were obtained are doubly underlined. The polyadenylation signal (AATAAA) and the poly-G region are singly underlined.

gene diverged early in evolution from a common ancestral gene followed later by a divergence of the palmitoyl-CoA oxidase and trihydroxycoprostanoyl-CoA oxidase genes.

Stretches of very conserved sequences between trihydroxycoprostanoyl-CoA oxidase and palmitoyl-CoA oxidase were found mainly in the middle part of the proteins (trihydroxycoprostanoyl-CoA oxidase residues 146–280, 308–360, 384–396, 406–418 and 434–453). Because this part of palmitoyl-CoA oxidase corresponds to the B subunit (51 kDa), this probably explains why the antibody raised against the denatured form of

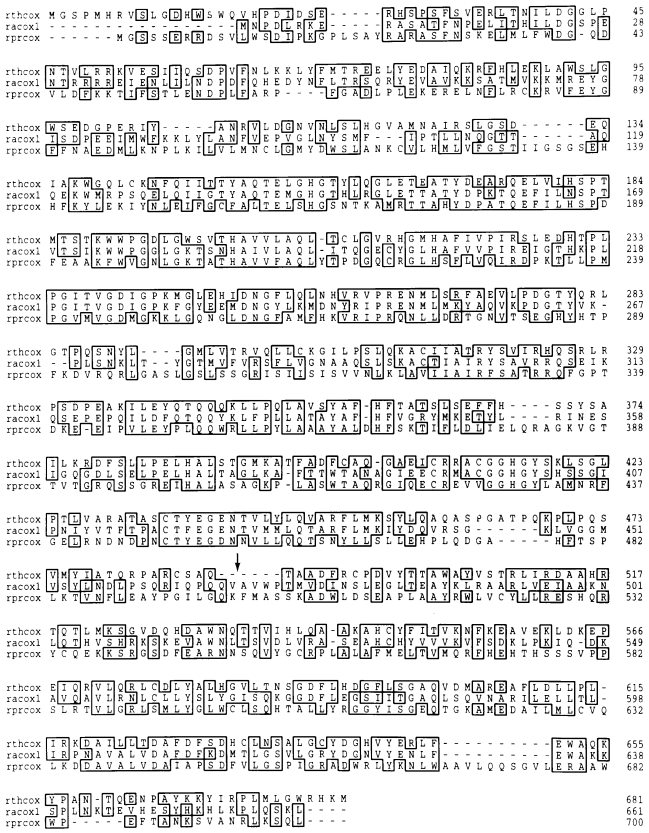

Figure 3 Alignment of the three peroxisomal rat acyl-CoA oxidases

The amino acid sequences of rat trihydroxycoprostanoyl-CoA oxidase (rthcox), rat palmitoyl-CoA oxidase (racox1) [16] and rat pristanoyl-CoA oxidase (rprcox) [12] were aligned by computer [Pile Up program (gap penalty of 3) at the Belgian EMBL Node in Brussels]. Identical and homologous amino acids are boxed. The numbers at the right of each row indicate the amino acid positions of the individual oxidases. The palmitoyl-CoA oxidase, corresponding to mRNA type II [16], was not included in the alignment because it is derived by differential splicing of exon $3_{I/II}$ from the same palmitoyl-CoA oxidase gene [40]. The site of proteolytic cleavage of palmitoyl-CoA oxidase is marked by an arrow.

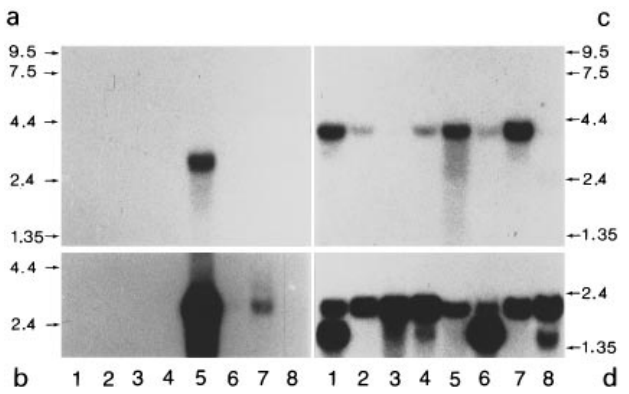

Figure 4 Rat multiple-tissue Northern blot analysis

A rat multiple-tissue Northern blot (Clontech), containing approx. 1 μg of mRNA per lane, was hybridized with a probe for trihydroxycoprostanoyl-CoA mRNA (**a**, **b**; exposure times of 2.5 and 12 h respectively). Lane 1, heart; lane 2, brain; lane 3, spleen; lane 4, lung; lane 5, liver; lane 6, skeletal muscle; lane 7, kidney; lane 8, testis. After being stripped, the membrane was reprobed for palmitoyl-CoA oxidase mRNA (**c**; exposure time 24 h) and actin mRNA (**d**; exposure time 1 h). Signals corresponding to palmitoyl-CoA oxidase mRNA became visible in spleen (lane 3) and in testis (lane 8) after a longer exposure (3 days) of the blot (results not shown). As revealed by the levels of actin mRNA (**d**) the signals for trihydroxycoprostanoyl-CoA oxidase and palmitoyl-CoA oxidase in liver (lane 5) are underestimated. The two distinct bands on the Northern blot after hybridization with the actin probe (1.8 and 2.0 kb) represent different mRNAs of distinct β-actin isoforms. The smaller isoform is present mainly in muscle tissue. The positions of markers (sizes given in kb) are shown at both sides of the blots.

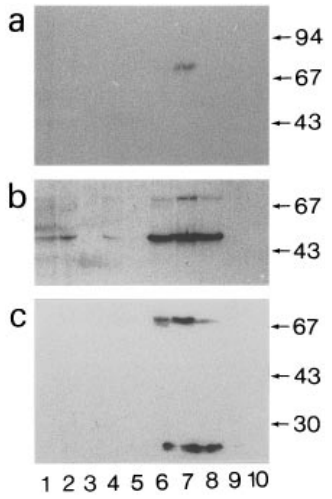

Figure 5 Immunoblot analysis of different rat tissues

Proteins (50 μg), present in homogenates of different rat tissues, were separated by SDS/PAGE followed by immunoblotting with the antiserum against the native trihydroxycoprostanoyl-CoA oxidase as described in the Experimental section (**a**). For comparison, duplicate blots were incubated with antiserum against palmitoyl-CoA oxidase, either against the 51 kDa B subunit (**b**) or against the 23 kDa C subunit (**c**). Lane 1, spleen; lane 2, lung; lane 3, testis; lane 4, cerebellum; lane 5, cerebrum; lane 6, intestinal mucosa; lane 7, liver; lane 8, kidney; lane 9, heart; lane 10, skeletal muscle. The migration of the molecular mass standards, indicated in kDa, is given at the right of the figure.

trihydroxycoprostanoyl-CoA oxidase cross-reacts weakly with the B subunit of palmitoyl-CoA oxidase (results not shown) and vice versa [9]. Interestingly, the region where the palmitoyl-CoA oxidase subunit A is cleaved into subunits B and C (cleavage between Val^{468} and Ala^{469}) [16] was not homologous. It should be noted that in contrast with palmitoyl-CoA oxidase, trihydroxycoprostanoyl-CoA oxidase (and also pristanoyl-CoA oxidase) is not cleaved after import into peroxisomes, because immunoblotting experiments with rat liver homogenates or isolated peroxisomes revealed only a single band, corresponding to the uncleaved protein. A stretch of amino acids was also conserved in the C-terminal part of trihydroxycoprostanoyl-CoA oxidase (residues 610–657), but the extreme C-terminus was completely different (see above).

Tissue distribution of trihydroxycoprostanoyl-CoA oxidase mRNA

Northern analysis of a multiple tissue blot from rat revealed that the size of trihydroxycoprostanoyl-CoA oxidase mRNA is approx. 2.6 kb (Figure 4a), suggesting that the cloned trihydroxycoprostanoyl-CoA oxidase cDNA (2509 bases) is almost complete. Because all clones for trihydroxycoprostanoyl-CoA oxidase, which were obtained from the oligo(dT)-primed library, ended at the same position (base 2310) and a polyadenylation signal (AATAAA) was located 13 bases upstream of a poly(A) sequence (see above), the missing part of the cDNA must be from the 5′ leader sequence. The size of the rat palmitoyl-CoA oxidase

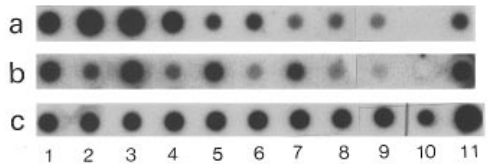

Figure 6 Dot-blot analysis of hepatic RNA from different species

Total RNA was isolated from livers of the animals listed below and aliquots, each containing the same amount of mRNA relative to the amount present in 15 μg of total RNA from male Wistar rat (lane 2), were dot-blotted in triplicate as described in the Experimental section. The dot-blots were hybridized with a probe for the mRNA for trihydroxycoprostanoyl-CoA oxidase (**a**), palmitoyl-CoA oxidase (**b**) and β-actin (**c**). The following animals were used: male Sprague–Dawley rat (lane 1), male Wistar rat (lane 2), male Wistar rat given a diet containing 0.3% (v/w) clofibrate for 14 days (lane 3), female Wistar rat (lane 4), hamster (lane 5), mouse (lane 6), rabbit (lane 7), guinea pig (lane 8), dog (lane 9) and man (lanes 10 and 11). The calculation of the mRNA content of the human hepatic RNA preparation was complicated by the fact that its mRNA content, estimated via the actin mRNA levels, was more than 100-fold higher than that of the liver of a male Wistar rat. This might be because a human β-actin cDNA probe was used for the hybridization. Therefore two different amounts of total RNA were spotted for the human preparation, one according to the calculations for the β-actin signal (lane 10) and one corresponding to 15 μg of total RNA according to absorbance measurements (lane 11). Trihydroxycoprostanoyl-CoA oxidase mRNA and palmitoyl-CoA oxidase mRNA were present in all species investigated, but in different relative quantities. Apparently the palmitoyl-CoA oxidase mRNA levels vary between distinct rat strains.

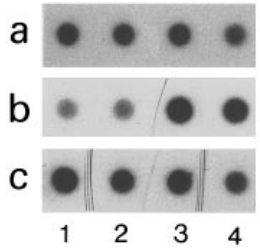

Figure 7 Dot-blot analysis of hepatic RNA from control rats and rats treated with peroxisome proliferators

Total hepatic RNA was isolated from control male Sprague–Dawley rats (lane 1) or rats treated via gastric intubation for 14 days with 75 mg/day per kg of BM 15.766 (lane 2), of bezafibrate (lane 3) or of BM 17.0249 (lane 4) and dot-blotted as described in the legend to Figure 6. After hybridization with probes for trihydroxycoprostanoyl-CoA oxidase (**a**), palmitoyl-CoA oxidase (**b**) and β-actin (**c**), blots were exposed to film.

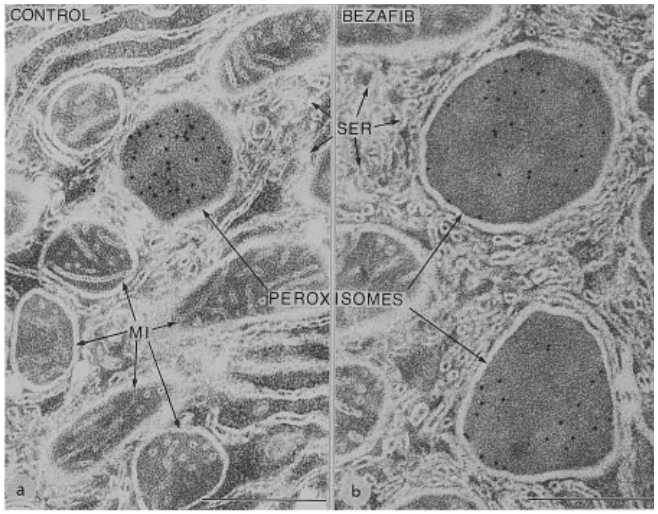

Figure 8 Immunoelectron microscopic localization of trihydroxycoprostanoyl-CoA oxidase in rat liver sections

Ultrathin liver sections from a control rat (**a**) and a bezafibrate-treated rat (**b**) were processed in parallel with a post-embedding Protein A–gold method and the antibody raised against the native form of trihydroxycoprostanoyl-CoA oxidase. Selected areas (shown at the same magnification; scale bars represent 250 nm) of pericentral hepatocytes are presented showing specific labelling of the peroxisomal matrix with gold particles. Peroxisomal cores or other cell organelles such as mitochondria (MI), smooth endoplasmic reticulum (SER), lysosomes and nuclei are negative. Most peroxisomes in the control liver exhibit a high labelling density (**a**). After bezafibrate treatment, a strong proliferation of peroxisomes and SER was seen. Individual peroxisomes were enlarged and a decrease in overall labelling density for trihydroxycoprostanoyl-CoA oxidase was observed (**b**).

mRNA was approx. 3.7 kb, which is in agreement with the results obtained by Miyazawa et al. [16]. The difference in length between the palmitoyl-CoA oxidase and trihydroxycoprostanoyl-CoA oxidase mRNA species is mainly due to the shortening of the 3′ untranslated region, which extends as much as 1681 bases for palmitoyl-CoA oxidase, whereas it is only 266 bases for trihydroxycoprostanoyl-CoA oxidase.

Trihydroxycoprostanoyl-CoA oxidase mRNA was present in large quantities in liver (exposure time 2.5 h; Figure 4a). Only after longer exposure (12 h; Figure 4b) of the blot was a weak signal observed in kidney, but not in the other tissues (exposure times up to 3 days). These results suggest that the trihydroxycoprostanoyl-CoA oxidase gene is not transcribed in extrahepatic tissues with the exception of kidney. The results agree with earlier enzyme activity determinations [5,7,30] and the immunoblot analysis of tissue homogenates shown in Figure 5a. The palmitoyl-CoA oxidase gene, in contrast, was transcribed in most tissues examined (Figure 4c). The highest mRNA levels were found in liver, heart and kidney. On immunoblot analysis liver, kidney and intestinal mucosa displayed the strongest signals (Figures 5b and 5c), in agreement with previous reports [8,31].

Dot-blot analysis of total liver RNA with trihydroxycoprostanoyl-CoA oxidase cDNA probes revealed cross-hybridization in all species examined, including man (Figure 6a).

Trihydroxycoprostanoyl-CoA oxidase mRNA is not induced by treatment of rats with different hypolipidaemic agents

Whereas palmitoyl-CoA oxidase mRNA was markedly induced in liver by treatment of male rats with clofibrate, trihydroxycoprostanoyl-CoA oxidase mRNA levels remained unchanged (Figure 6). In a second series of experiments, we tested whether other structurally unrelated peroxisome proliferators known to induce different subsets of (peroxisomal) proteins [32,33] would exert any effect on the transcription of the trihydroxycoprostanoyl-CoA oxidase gene. No increases in hepatic trihydroxycoprostanoyl-CoA oxidase mRNA levels were visible after treatment of rats with bezafibrate (an analogue of clofibrate), BM 15.766 (a hypocholesterolaemic piperazine derivative inhibiting 7-dehydrocholesterol-5,7-reductase [32]) or BM 17.0249 (2,2,13,13-tetrachlorotetradecanedioic acid [33]). In contrast, palmitoyl-CoA oxidase mRNA was strongly induced by bezafibrate and BM 17.0249, whereas treatment with BM 15.766 did not lead to higher palmitoyl-CoA oxidase mRNA levels (Figure 7). The lack of an increase in palmitoyl-CoA oxidase mRNA despite marked peroxisome proliferation after BM 15.766 treatment is in agreement with earlier results obtained by enzyme activity measurements and immunoblotting [34].

Immunocytochemistry with the post-embedding Protein A–gold method revealed a significant labelling of peroxisomes for trihydroxycoprostanoyl-CoA oxidase (Figure 8). Other cell

organelles such as nuclei, mitochondria, lysosomes or endoplasmic reticulum remained negative. After bezafibrate treatment, peroxisomes were enlarged and increased in number. A decrease in the labelling density for trihydroxycoprostanoyl-CoA oxidase was observed (Figure 8). In contrast, the labelling density for palmitoyl-CoA oxidase was significantly increased (results not shown), confirming earlier results by Beier et al. [35].

Conclusion

The present findings show: (1) that the amino acid sequence of trihydroxycoprostanoyl-CoA oxidase is more related to the sequence of palmitoyl-CoA oxidase than to that of pristanoyl-CoA oxidase, despite the fact that both trihydroxycoprostanoyl-CoA oxidase and pristanoyl-CoA oxidase are active towards 2-methyl branched carboxylates (the bile acid intermediate trihydroxycoprostanic acid and 2-methyl branched fatty acids such as pristanic acid respectively) [6]; and (2) at the mRNA level that the trihydroxycoprostanoyl-CoA oxidase gene is transcribed only in liver and – to a smaller extent – in kidney and that it is not induced by treatment of rats with structurally unrelated peroxisome proliferators. Because bile acid synthesis is essentially a hepatic function, it is not surprising that trihydroxycoprostanoyl-CoA oxidase is not expressed in extrahepatic tissues. The significance of a possible low expression in kidney remains to be elucidated. Further studies of the promoter regions of the acyl-CoA oxidase genes will help to clarify the differences in tissue-specific expression. A study of the trihydroxycoprostanoyl-CoA oxidase promoter (and pristanoyl-CoA oxidase promoter) will also be required to explain why, in contrast with rat palmitoyl-CoA oxidase, trihydroxycoprostanoyl-CoA oxidase (and also pristanoyl-CoA oxidase) is not induced by treatment of the animals with peroxisome proliferators. Peroxisome proliferators stimulate the transcription of responsive genes [36,37] via the peroxisome proliferator activated receptor, which binds as a heterodimeric complex with the retinoic X receptor to direct repeat sequences in the promoter region of the genes [38,39]. The absence of such responsive elements in the promoter might be one of the possible explanations for the non-inducibility of trihydroxycoprostanoyl-CoA oxidase (and pristanoyl-CoA oxidase).

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