Dicarboxylic acids are formed by \( \alpha \)-oxidation of fatty acids in the endoplasmic reticulum and degraded as the CoA ester via \( \beta \)-oxidation in peroxisomes. Both synthesis and degradation of dicarboxylic acids occur mainly in kidney and liver, and the chain-shortened dicarboxylic acids are excreted in the urine as the free acids, implying that acyl-CoA thioesterases (ACOTs), which hydrolyze CoA esters to the free acid and CoASH, are needed for the release of the free acids. Recent studies show that peroxisomes contain several acyl-CoA thioesterases with different functions. We have now expressed a peroxisomal acyl-CoA thioesterase with a previously unknown function, ACOT4, which we show is active on dicarboxylyl-CoA esters. We also expressed ACOT8, another peroxisomal acyl-CoA thioesterase that was previously shown to hydrolyze a large variety of CoA esters. ACOT4 and ACOT8 are both strongly expressed in kidney and liver and are also target genes for the peroxisome proliferator-activated receptor \( \alpha \). Enzyme activity measurements with expressed ACOT4 and ACOT8 show that both enzymes hydrolyze CoA esters of dicarboxylic acids with high activity but with strikingly different specificities. Whereas ACOT4 mainly hydrolyzes succinyl-CoA, ACOT8 preferentially hydrolyzes longer dicarboxylyl-CoA esters (glutaryl-CoA, adipyl-CoA, suberyl-CoA, sebacyl-CoA, and dodecanedioyl-CoA). The identification of a highly specific succinyl-CoA thioesterase in peroxisomes strongly suggests that peroxisomal \( \beta \)-oxidation of dicarboxylic acids leads to formation of succinate, at least under certain conditions, and that ACOT4 and ACOT8 are responsible for the termination of \( \beta \)-oxidation of dicarboxylic acids of medium-chain length with the concomitant release of the corresponding free acids.

Acyl-CoA thioesterases (ACOTs)\(^2\) are a growing family of enzymes that catalyze the hydrolysis of the CoA esters of various lipids to the free acids and coenzyme A (CoASH), thereby regulating levels of these compounds (for review, see Ref. 1). The thioesterase activity with fatty acyl-CoAs was found in several cellular compartments, including peroxisomes (2–4). At that time, the broad acyl-CoA chain length specificity of these enzymes was noted. Nowadays, however, the search for peroxisomal \( \beta \)-oxidation has been focused on the identification of specific acyl-CoA thioesterases. With the advent of new techniques, such as proteomics and metabolomics, we have identified several genes encoding acyl-CoA thioesterases that are conserved family that is tentatively named the type I acyl-CoA thioesterase gene family (5, 6). This gene family contains two further acyl-CoA thioesterase genes, both located in a cluster on mouse chromosome 12 D3. The genes of this family are named Acot1 to Acot6 and encode acyl-CoA thioesterases with localization in cytosol (7, 8), mitochondria (9), and peroxisomes (5, 6). These enzymes were formerly known as CTE-I (ACOT1), MTE-I (ACOT2), PTE-Ia (ACOT3), PTE-Ib (ACOT4), PTE-Ic (ACOT5), and PTE-IId (ACOT6). However, a new nomenclature system for acyl-CoA thioesterases/hydrolases has been agreed on by the Human Genome Organization (HUGO) Genome Nomenclature Committee and Mouse Genomic Nomenclature Committee, and from now on we suggest the use of the ACOT nomenclature system (10).

The genes of this family are named Acot1 to Acot6 and encode acyl-CoA thioesterases (10). The highly conserved COX-2 protein (11) and the mouse enzyme (12). Later, this enzyme was characterized in rat as a branched chain acyl-CoA thioesterase (13). Mouse Acot8 was shown to be widely expressed in various tissues, and the enzyme hydrolyzed all tested CoA esters including bile acid-CoAs, branched-chain acyl-CoAs, and short-, medium-, and long-chain acyl-CoAs. We therefore hypothesized that the multiplicity of peroxisomal acyl-CoA thioesterases may reflect the diverse functions that peroxisomes play in lipid metabolism and that identification of specific acyl-CoA thioesterases may give new insights into metabolic pathways in peroxisomes.

Fatty acid oxidation is an important source of energy, especially during fasting and diabetes. Although mitochondria are considered the primary site for \( \beta \)-oxidation of fatty acids for energy utilization, it is now well established that peroxisomes play a key role in the metabolism of a variety of lipids such as very long-chain fatty acids, branched-chain fatty acids, dicarboxylic fatty acids, bile acid intermediates, prostaglandins, leukotrienes, thromboxanes, pristanic acid, and xenobiotic carboxylic acids (for reviews, see Refs. 19–21). The functional importance of peroxisomes is underscored by the severity of peroxisomal disorders such as Zellweger syndrome, X-linked adrenoleukodystrophy, and Refsum disease (for review, see (22)). Most lipids that undergo \( \beta \)-oxidation in peroxisomes are apparently only chain-shortened, and recent data suggest that octanole and longer chain fatty acids undergo 1–3 cycles of \( \beta \)-oxidation in peroxisomes (23–25). The apparent lack of correlation between acyl chain length and the number of cycles of \( \beta \)-oxidation in peroxisomes suggests that the incomplete oxidation of fatty acids is due to the presence of acyl-CoA thioesterases that “terminate” \( \beta \)-oxidation, which would release free fatty acids that can exit the peroxisome. These free fatty acids may subsequently be transported to the mitochondria for...
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further oxidation, be subject to ω-oxidation, or be excreted as free carboxylic acids in the urine. One example is the peroxisomal metabolism of dicarboxylic acids, which results in the production of the medium-chain dicarboxylic acids adipic acid, suberic acid, and sebacic acid, which are excreted in the urine. Dicarboxylic acids are formed by an initial hydroxylation of the ω-carbon by microsomal CYP4A enzymes, followed by further oxidations by alcohol dehydrogenase to form the ω-oxo-fatty acid and by aldehyde dehydrogenase that finally converts the ω-oxo-fatty acid into a dicarboxylic acid (26). It has been suggested that medium-chain fatty acids are the substrates for the initial ω-hydroxylation (27–29) and that medium-chain dicarboxylic acids are chain-shortened in peroxisomes to adipic acid (C6). In this study we have cloned, expressed, and characterized ACOT4 and show it to be a peroxisomal succinyl-CoA thioesterase. Succinate is excreted in urine, but the origin of urinary succinate is poorly understood. Therefore, these data suggest that succinyl-CoA may be an end product of peroxisomal β-oxidation of dicarboxylic fatty acids and that urinary succinate may at least in part originate from peroxisomes. As discussed above, ACOT8 is a peroxisomal acyl-CoA thioesterase that apparently acts as a “nonspecific” acyl-CoA thioesterase hydrolyzing all acyl-CoAs (14). We also expressed ACOT8 and investigated the activity of ACOT8 with dicarboxylyl-CoA esters. We show that ACOT8 also hydrolyzes CoA esters of dicarboxylic acids but with a different specificity than ACOT4. These data suggest that peroxisomes contain acyl-CoA thioesterases that function in the release of free dicarboxylic acids for excretion in urine.

EXPERIMENTAL PROCEDURES

Subcellular Localization of ACOT4—The open reading frame encoding Acot8 was amplified by reverse transcription PCR (RT-PCR) as described below, cloned into the pcDNA3.1/NT-GFP expression vector (Invitrogen), and fully sequenced. Cloning of ACOT4 into the pcDNA3.1/NT-GFP vector results in the expression of ACOT4 in fusion with N-terminal green fluorescent protein (GFP). Cell culture, transfection, and fluorescence microscopy were performed as described earlier (14).

Animals and Treatments—Adult male wild-type (+/+) mice or PPARα-null (−/−) mice on a pure Sv/129 genetic background (kindly provided by Dr. Frank Gonzalez and Dr. Jeffrey Peters or purchased from the Jackson Laboratory) were used in this study. The mice were fed either a standard chow diet or a diet containing 0.1% Wy-14,643 (Calbiochem-Novabiochem International) for 1 week or were fasted for 24 h before sacrifice. Following sacrifice by CO2 asphyxiation and cervical dislocation, tissues were excised, frozen in liquid nitrogen, and stored at −70°C.

Isolation of Total RNA—Total RNA was isolated with TRIzol® Reagent (Invitrogen) or QuickPrep® total RNA extraction reagent (Amer sham Biosciences) and DNase-treated.

Cloning and Expression—The open reading frame for Acot4 was amplified by RT-PCR using the primers 5′-CATATGGCAGCGCAGATGCAGCGAC-3′ and 5′-CTGACCAAAGCGCCCAATGTC-3′ (CyberGene AB, Huddinge, Sweden) with the addition of an Ndel site (indicated in boldface). These primers were designed based on the genomic sequence (GenBank™ accession No. NM_134247). Acot8 was cloned into the pET-16b vector (Novagen Inc.), sequenced, and transformed into BL21(DE3)pLysS cells (Novagen Inc.). Protein expression was induced by the addition of 1 mI isopropyl-1-thio-β-D-galactopyranoside for 3 h at 37°C. Recombinant protein was affinity purified on a HiTrap™ column (Amersham Biosciences) as described (6). The full-length open reading frame for Acot8 was amplified and cloned into the pET-16b vector, and protein was expressed and purified as described previously (14).

Chemical Synthesis of Medium-Chain Dicarboxylyl-CoAs—Adipyl-CoA (C6), suberyl-CoA (C8), sebacyl-CoA (C10), and dodecanedioyl-CoA (C12) were synthesized chemically from the free acid by first forming the anhydride followed by the CoA ester as described (30). Instead of adding equal amounts of dicarboxylic acid anhydride and CoASH to the reaction, a molar ratio of 4:1 was used. The products formed were purified by reversed phase high performance liquid chromatography using a C18 Ultrasphere ODS 5-μm (4.6 × 250 mm) column (Beckman) with the mobile phase containing 50 mM ammonium formate, pH 5.4, and a gradient of increasing acetonitrile starting with 5% acetonitrile for 5 min followed by 35 min of a linear increase to 50% acetonitrile and then down to 5% again after 5 min. The dicarboxylyl-CoAs purified were identified by electrospray mass spectrometry in a Quattro micro™ triple quadrupole mass spectrometer (Micromass, Wythenshawe, Manchester, UK) equipped with a nano-electrospray ion source. Mass spectra were acquired in the positive ion mode over a mass scan range of m/z 200–1200 for 1 min at a scan rate of 5 s per scan. This shows both the whole molecule and a diffracted molecule with a neutral loss of 507, confirming the identification of the correct CoA ester as described previously (31).

Acyl-CoA Thioesterase Activity Measurements—Acyl-CoA thioesterase activity was measured spectrophotometrically at 412 nm using 5,5′-dithiobis(2-nitrobenzoic acid) as described earlier (14). Recombinant ACOT4 was analyzed for activity with commercially available acyl-CoAs (Sigma), synthesized dicarboxylyl-CoAs (as described above), 4,8-dimethyl-nonanoyl-CoA, 2-trans-decenoyl-CoA and clofibrayl-CoA.

The activity of recombinant ACOT8 protein was measured with the different dicarboxylyl-CoAs. Protein was determined using the Bradford assay (32). The effect of CoASH on the enzyme activity was measured at 232 nm in phosphate-buffered saline. Enzyme kinetics were calculated using the SigmaPlot enzyme kinetics program.

Tissue Expression of Acot4 mRNA Using Quantitative PCR—The tissue expression of Acot4 mRNA was examined by quantitative PCR (Q-PCR) in various tissues from male Sv/129 mice. Pooled samples of total RNA from three individual animals were used for cDNA synthesis. The cDNA synthesis was performed using 1 μg of total RNA using TaqMan reverse transcription reagents (Applied Biosystems). Q-PCR was performed in an ABI Prism 7000 sequence detection system using TaqMan universal PCR master mix (Applied Biosystems). An Acot4 amplicon spanning over the exon 2/exon 3 boundary was amplified using the primers 5′-TTGAAGAAGCAGTGCGGTACA-3′ and 5′-AAAGCCAGAAGCCCAATGTC-3′ and a probe with 5′-carboxyfluorescein (5′-FAM) and 4′-(4-dimethylaminophenyl-azo)benzoic acid (3′-Dabcyl), 5′-CTCGCATCCAAAGGTTAAGGCGCCA-3′ (CyberGene AB). The primers were designed using the Primer Express software (Applied Biosystems), and the PCR product was analyzed by agarose gel electrophoresis and sequenced. As an endogenous control, an amplicon of 18 S was used, using the predeveloped TaqMan Assay Reagent for 18 S rRNA (Applied Biosystems). The Q-PCR was run in single-plex in triplicate for each tissue with cDNA from pooled tissue samples. Thermal cycling was performed at 50°C for 2 min and 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Data were analyzed using the ABI Prism 7000 SDS software, and the average cycle threshold (CT) value per triplicate was used to calculate the relative amounts of Acot4 mRNA using the 2^[-ΔΔCT] method.

Tissue Expression of Cyp4A10 Using RT-PCR—Tissue expression was examined by RT-PCR in various mouse tissues. A fragment of Cyp4A10 was amplified using the primers 5′-CTGGAGAAAGCCTAAGTT-
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**RESULTS**

Molecular Cloning and Sequence Analysis of Acot4—The gene for Acot4 was identified in 1999 as a member of the type I acyl-CoA thioesterase gene family (5). To characterize Acot4 further, we have now amplified the open reading frame from mouse kidney and verified the sequence to the published sequence with GenBank Accession Number NM_134247.

Subcellular Localization Experiments Show That Acot4 Is Localized in Peroxisomes—ACOT4 contains a putative consensus type I peroxisomal targeting signal (PTS1) of Cys–Arg–Leu (–CRL) at the carboxy-terminal end of the protein, which may target the protein to peroxisomes (34). To establish the cellular localization of ACOT4, we expressed the open reading frame in-frame with an N-terminal GFP in both control fibroblasts and fibroblasts from a Zellweger patient. The Zellweger syndrome is a peroxisome biogenesis disorder caused by defects in peroxisome assembly most commonly due to PEX5 deficiency (35). The ACOT4-GFP fusion protein showed a punctate pattern of expression in control fibroblasts, indicating a peroxisomal localization (Fig. 1A). However, in fibroblasts from the Zellweger patient, ACOT4 expression resulted in a diffuse labeling throughout the cells with no punctate labeling, indicating that ACOT4 remained in the cytosol (Fig. 1B). These results confirm that ACOT4 is indeed a peroxisomal protein.

Characterization of Recombinant Acot4 Shows That It Is a Specific Succinyl-CoA/Glutarlyl-CoA Thioesterase—Both ACOT4 and ACOT8 (14) were expressed as His-tagged fusion proteins and purified. The purified proteins were detected by SDS-PAGE as single bands of ~50 and ~35 kDa stained with Coomassie Brilliant Blue (Fig. 2). The recombinant ACOT4 protein was analyzed for thioesterase activity with acetyl-CoA (C2:0), propionyl-CoA (C3:0), butyryl-CoA (C4:0), heptanoyl-CoA (C7:0), hexanoyl-CoA (C6:0), octanoyl-CoA (C8:0), decanoyl-CoA (C10:0), lauroyl-CoA (C12:0), myristoyl-CoA (C14:0), palmitoyl-CoA (C16:0), palmitoleoyl-CoA (C16:1), stearoyl-CoA (C18:0), oleoyl-CoA (18:1), elaidoyl-CoA (C18:1 trans), linoleoyl-CoA (C18:2), arachidoyl-CoA (C20:0), arachidonoyl-CoA (C20:4), acetoacetyl-CoA, di-β-hydroxybutyryl-CoA, isovaleryl-CoA, crotonyl-CoA, di-3-hydroxy-3-methylglutaryl-CoA, malonyl-CoA, succinyl-CoA, and glutaryl-CoA. We also tested the activity with some non-commercially available substrates including adipyl-CoA, suberyl-CoA, sebacyl-CoA, dodecanedioyl-CoA, behenyl-CoA (C22:0-CoA), 4,8-dimethylnonanoyl-CoA, 2-trans-decenoyl-CoA, c12:0-CoA, 4,8-dimethylnonanoyl-CoA, 2-trans-decenoyl-CoA, clofibracyl-CoA, chlo7oyl-CoA, and trihydroxycoprostanoyl-CoA. However, ACOT4 was only active with succinyl-CoA and glutaryl-CoA. The activity with these substrates conformed to Michaelis-Menten kinetics with calculated $V_{\text{max}}$ values of 3.98 ± 0.16 and 1.14 ± 0.16 μmol/min/mg protein for succinyl-CoA and glutaryl-CoA, respectively (TABLE ONE). The corresponding calculated $K_m$ values were 13.3 ± 1.32 and 37.1 ± 9.05 μM, respectively, suggesting that succinyl-CoA is the preferred substrate for ACOT4.

Acot4 and Acot8 Both Hydrolyze Dicarboxylyl-CoAs but with Different Specificities—ACOT8 was recently characterized as a broad range acyl-CoA thioesterase showing activity with all acyl-CoAs tested (14). However, acyl-CoA thioesterase activity for dicarboxylyl-CoAs other than malonyl-CoA was not tested at that time, and we therefore expressed ACOT8 to reinvestigate the activity with dicarboxylyl-CoAs ranging from malonyl-CoA to dodecanedioyl-CoA. ACOT8 was active on all the dicarboxylyl-CoAs tested but preferentially hydrolyzed the longer-chain dicarboxylyl-CoAs. Enzyme kinetics were calculated (TABLE ONE) and show that whereas ACOT8 readily hydrolyzes CoA esters from glutaryl-CoA to dodecanedioyl-CoA, ACOT4 only hydrolyzes succinyl-CoA efficiently (with high $V_{\text{max}}$ and low $K_m$). The difference...
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### TABLE ONE

| Kinetic characterization of ACOT4 and ACOT8 with dicarboxylyl-CoA esters |
|--------------------------------------------------|
| Acyl-CoA thioesterase activity of three protein preparations of ACOT4 (shown as the mean ± S.E.) and one protein preparation of ACOT8 was used to calculate V\(_{\text{max}}\) and \(K_m\) using the SigmaPlot enzyme kinetics program. N.D., not detectable. |
| Structure | \(V_{\text{max}}\) (µmol/min/mg) | \(K_m\) (µM) | \(V_{\text{max}}\) (µmol/min/mg) | \(K_m\) (µM) |
|----------|-----------------|--------|-----------------|--------|
| Malonyl-CoA | N.D. | N.D. | 0.78 | 38.7 |
| Succinyl-CoA | 3.98 ± 0.16 | 13.3 ± 1.32 | 0.82 | 34.7 |
| Glutaryl-CoA | 1.14 ± 0.16 | 37.1 ± 9.05 | 2.35 | 15.9 |
| Adipyl-CoA | N.D. | N.D. | 3.40 | 21.8 |
| Suberyl-CoA | N.D. | N.D. | 4.13 | 19.8 |
| Sebacyl-CoA | N.D. | N.D. | 3.82 | 10.5 |
| Dodecanedioyl-CoA | N.D. | N.D. | 5.03 | 14.1 |

Figure 3. ACOT4 and ACOT8 both hydrolyze dicarboxylyl-CoA esters but with different specificities. Thioesterase activity of expressed ACOT4 and ACOT8 was measured with short- and medium-chain dicarboxylyl-CoAs at 25 \(\mu\)M. For ACOT4 three different protein preparations were used to measure the activity for which the mean values are shown ± S.E.; for ACOT8 one protein preparation was used for the activity measurements.

![Figure 3](image)

Figure 4. Acot4 is mainly expressed in kidney, liver, and proximal and distal intestines. Tissue expression of Acot4 was examined by single-plex Q-PCR in various tissues from male Sv/129 mice using an 18 S amplicon as an endogenous control. The Acot4 primers and probe span the exon 2/exon 3 boundary. Samples from three animals were pooled and then run in triplicate in two experiments, and the relative amounts of mRNA were calculated using the 2\(^{-}\Delta\Delta CT\) method. Prox. intest., proximal intestine (first 10 cm of the small intestine); Dist. intest., distal intestine (last 10 cm of the small intestine); BAT, brown adipose tissue; WAT, white adipose tissue.

![Figure 4](image)

Figure 5. Acot4 expression is under control of the PPAR\(\alpha\). A, regulation of expression of Acot4 mRNA by treatment with 0.1% Wy-14,643 for 1 week was examined in mouse liver using wild-type (+/+ ) and PPAR\(\alpha\)-null (−/−) animals using single-plex Q-PCR. Primers and probe were designed to cover the exon 2/exon 3 boundary, and an amplification of 18 S was used as an endogenous control. Samples were run in triplicate from three individual animals for each group, and the relative amounts of mRNA were calculated using the 2\(^{-}\Delta\Delta CT\) method. B, regulation of the expression at protein level was investigated in liver homogenates from two wild-type (+/+ ) and two PPAR\(\alpha\)-null (−/−) animals using Western blot analysis with an affinity-purified anti-ACOT4 peptide antibody.

![Figure 5](image)
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FIGURE 6. Cyp4A10 is expressed in liver, kidney, and proximal intestine. Tissue expression of Cyp4A10 was investigated by RT-PCR using β-actin as an endogenous control. A 401-bp fragment of Cyp4A10 corresponding to 1589–1990 bp of the open reading frame was amplified, and samples were analyzed by electrophoresis on a 1% agarose gel. MM, molecular marker; NC, negative control; BAT, brown adipose tissue; WAT, white adipose tissue.

Discussion

Acot4 and Acot8 Hydrolyze Dicarboxylyl-CoA Esters—In this study we have cloned, expressed, and characterized ACOT4, which is a member of the type I acyl-CoA thioesterase gene family. In GFP localization experiments using human skin fibroblasts we show that ACOT4 is indeed a peroxisomal enzyme, in line with the presence of a peroxisomal targeting signal 1 targeting signal of -CRL at the carboxyl terminal end of the protein. Kinetic characterization of recombinant ACOT4 with numerous acyl-CoA esters showed that ACOT4 was only active on succinyl-CoA and glutaryl-CoA. The much higher values with succinyl-CoA than with glutaryl-CoA strongly suggest that succinyl-CoA is the preferred substrate for ACOT4, although glutaryl-CoA may also be a physiological substrate. We reported previously that peroxisomes contain an acyl-CoA thioesterase, ACOT8, with very broad acyl-CoA substrate specificity (14). At that time we did not test ACOT8 activity with succinyl-CoA or longer chain dicarboxylyl-CoA esters. We have now re-expressed ACOT8 and show it to be active on malonyl-CoA, succinyl-CoA, adipyl-CoA, suberyl-CoA, sebacyl-CoA, and dodecanediyl-CoA, but with a strikingly different substrate specificity compared with ACOT4. Another important difference between these two enzymes is that ACOT8 is strongly regulated (inhibited) by CoASH with an IC50 of ~15 μM (14), whereas ACOT4 activity is insensitive to CoASH added up to 500 μM (this study). The consequence of the different regulation by CoASH is likely to be reflected in that ACOT4 would be constitutively active, whereas the activity of ACOT8 will depend on free intraperoxisomal CoASH levels, which may change under different physiological conditions.

The identification of an apparently specific succinyl-CoA/glutaryl-CoA thioesterase in peroxisomes strongly suggests that these CoA esters are produced or metabolized in peroxisomes. There are two well known pathways that potentially produce succinyl-CoA, including catabolism of amino acids and β-oxidation of dicarboxylic acids. Succinyl-CoA can be formed from the metabolism of methionine, valine, and isoleucine (via the formation of propionyl-CoA, which can be further converted to succinyl-CoA), but the only amino acid suggested to be metabolized in peroxisomes is lysine (for review, see Ref. 36), which produces glutaryl-CoA as an intermediate. Although pipericolic acid oxidase is the only enzyme in this pathway that has been identified as a peroxisomal enzyme, it is possible that glutaryl-CoA may be produced in peroxisomes by this pathway or, alternatively, further metabolized in peroxisomes. Glutaryl-CoA may also be produced in peroxisomes via β-oxidation of odd-numbered dicarboxylic acids. The possibility of a further chain-shortening of glutaryl-CoA to malonyl-CoA has been put forward by Sacksteder et al. (37), who demonstrated that peroxisomes contain a malonyl-CoA decarboxylase. However, the further metabolism of glutaryl-CoA in peroxisomes is not clear. Although peroxisomal straight-chain acyl-CoA oxidase can catalyze the dehydrogenation to butyryl-CoA (38), peroxisomes apparently do not decarboxylate
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FIGURE 8. Hypothetical role for acyl-CoA thioesterases in the synthesis and metabolism of medium-chain dicarboxylic acids. During normal conditions a small fraction (5–10%) of fatty acids are converted to dicarboxylic acids by \( \omega \)-oxidation. These dicarboxylic acids are then oxidized to adipyl-CoA (C6), sebacyl-CoA (C8), and suberyl-CoA (C10), which are hydrolyzed by ACOT8 and excreted in the urine. Under certain conditions when the activity of ACOT8 is inhibited, adipyl-CoA may be further oxidized to succinyl-CoA followed by hydrolysis by ACOT4 to succinate that is either excreted in the urine or possibly transported to the mitochondria. During conditions such as fasting, high fat feeding, or diabetes, oxidation of dicarboxylic acids is increased (in part due to PPAR \( \gamma \) activation). Under these conditions, ACOT8 is postulated to be more active (due to CoASH sequestration), and more adipic acid, sebacic acid, and suberic acid are excreted in the urine at the expense of succinate. In this scheme, we also postulate that the partial \( \beta \)-oxidation of fatty acids is due to the action of various thioesterases, for example ACOT3, ACOT5, and ACOT8, which will contribute to the premature termination of \( \beta \)-oxidation that will produce medium-chain fatty acids for \( \omega \)-oxidation. These medium-chain fatty acids may re-enter the peroxisome as dicarboxylic acids for further chain shortening and subsequent excretion in urine. Plus sign (+) indicates genes up-regulated by PPAR \( \gamma \). MC-DCA, medium-chain dicarboxylic acid; SCOX, straight-chain acyl-CoA oxidase; L-BP, L-specific bifunctional protein; D-BP, D-specific bifunctional protein; SCPx, sterol carrier protein \( \alpha \), possibly also involving the classic 3-ketoacyl-CoA thiolase. Although several of the enzymes involved in \( \beta \)-oxidation of dicarboxylic acids are induced by clofibrate treatment in rats and mice, this treatment is not sufficient to cause increased production of dicarboxylic acids, probably due to lack of substrate and/or lack of induction of the 3-ketoacyl-CoA synthetase, which is likely to be the rate-limiting step in \( \beta \)-oxidation of dicarboxylic acids (43, 47, 51). Studies into the tissue distribution of peroxisomal dicarboxylic acid oxidation showed that the activity is highest in kidney and no detectable activity in skeletal muscle (42, 53). In this study we show that Acot8 and Cyp4a10 are mainly expressed in liver and proximal intestine, with only very low activity detected in heart and no detectable activity in skeletal muscle (42, 53). The important role of peroxisomes was further supported by a recent study (52) employing human skin fibroblasts deficient in mitochondrial \( \beta \)-oxidation that showed that \( \beta \)-oxidation of at least C16 dicarboxylic acids is solely a peroxisomal process (52). Also, the peroxisomal \( \beta \)-oxidation enzymes show 15–50-fold lower \( K_{m} \) values for dicarboxyl-CoAs than the mitochondrial enzymes. Recently, the enzymology of the peroxisomal pathway for \( \beta \)-oxidation of dicarboxylic acids was elucidated, showing that at least C16 dicarboxylic acid is primarily oxidized by the sequential action of the straight-chain acyl-CoA oxidase, the L- and D-specific bifunctional proteins, and the sterol carrier protein \( \alpha \), possibly also involving the classic 3-ketoacyl-CoA thiolase. Although several of the enzymes involved in \( \beta \)-oxidation of dicarboxylic acids are induced by clofibrate treatment in rats and mice, this treatment is not sufficient to cause increased production of dicarboxylic acids, probably due to lack of substrate and/or lack of induction of the 3-ketoacyl-CoA synthetase, which is likely to be the rate-limiting step in \( \beta \)-oxidation of dicarboxylic acids (43, 47, 51). Studies into the tissue distribution of peroxisomal dicarboxylic acid oxidation showed that the activity is highest in kidney followed by liver and proximal intestine, with only very low activity detected in heart and no detectable activity in skeletal muscle (42, 53). In this study we show that Acot8 and Cyp4a10 are mainly expressed in liver and proximal intestine, with only very low activity detected in heart and no detectable activity in skeletal muscle (42, 53).
kidney and liver, and we showed previously that Acot8 is strongly expressed in these tissues (14), which is similar to the reported tissue distribution of dicarboxylic acid synthesis and peroxisomal β-oxidation activity of dicarboxylyl-CoA esters. Furthermore, Acot4 (this study), Acot8 (14), and Cyp4A10 (54) are all strongly up-regulated by Wy-1,4,643 treatment, similar to the straight-chain acyl-CoA oxidase, which catalyzes the initial step in the β-oxidation of dicarboxylyl-CoAs.

Dicarboxylic acids metabolized in peroxisomes are believed to originate from medium-chain monocarboxylic acids produced in mitochondria (29, 41, 51), but long- and very long-chain monocarboxylic acids have also been proposed as origins for dicarboxylic acid production. In vivo, in situ, and in vitro experiments on the oxidation of dicarboxylic acids of various chain lengths show that dodecanedioic acid is most readily oxidized followed by sebacic acid and suberic acid, whereas adipic acid is not metabolized at all. The role of mitochondria in providing medium-chain fatty acids for subsequent synthesis of medium-chain dicarboxylic acids is further supported by the fact that Zellweger patients excrete increased amounts of adipic acid, suberic acid, and sebacic acid in the urine (55–57). In particular, these patients excrete increased levels of suberic and sebacic acid, which suggest that peroxisomes normally chain-shorten these dicarboxylic acids to adipic acid and, thus, that there is a relative block in the conversion to adipic acid in Zellweger patients. It is not clear whether peroxisomes metabolize adipyl-CoA to succinyl-CoA. Feeding adipic acid to rats results in excretion of the unchanged acid, suggesting that adipic acid is not metabolized at all (42). However, this is likely due to the lack of activation of adipic acid to the corresponding CoA ester, because the dicarboxylyl-CoA synthetase is not active on short-chain dicarboxylic acids (46). If adipyl-CoA is formed in peroxisomes from suberyl-CoA, it appears plausible that adipyl-CoA can be chain-shortened to succinyl-CoA. Therefore, because succinate is found in urine and a succinyl-CoA thioesterase is not active on short-chain dicarboxylic acids (46). If adipyl-CoA is formed in peroxisomes from suberyl-CoA, it appears plausible that adipyl-CoA can be chain-shortened to succinyl-CoA. Therefore, because succinate is found in urine and a succinyl-CoA thioesterase in peroxisomes has been identified, our data suggest that peroxisomal β-oxidation of dicarboxylic fatty acids may proceed to succinyl-CoA, followed by hydrolysis to succinate by ACOT4 for excretion in urine. It is also possible that succinate formed in peroxisomes can be transferred to mitochondria to enter the Krebs cycle. It has been shown that catabolism of dicarboxylic acids has an anti-ketogenic effect (41, 49, 50, 58), which has been attributed to succinate entering the Krebs cycle and thereby lowering acetyl-CoA levels and ketone body production. However, odd carbon number dicarboxylic acids also exhibit the same anti-ketogenic effect, which should not produce succinate (58). It is possible that the anti-ketogenic effect by dicarboxylic acid metabolism is not necessarily related to the breakdown of dicarboxylic acids but rather to peroxisomal β-oxidation in general, which produces acetyl-CoA (49, 50). This acetyl-CoA is hydrolyzed to acetate and then transported out of the peroxisome. Several studies suggest that acetate/acetyl-CoA produced in peroxisomes is not transported to mitochondria but rather used to synthesize malonyl-CoA (23–25, 49, 50) or for biosynthetic purposes such as, for example, fatty acid and cholesterol biosynthesis.

Possible Role for Peroxisomes in Synthesis and Metabolism of dicarboxylic Acids—Several studies provide evidence that peroxisomes partially β-oxidize fatty acids longer than hexanoate, and octanoate is β-oxidized 1–2 cycles whereas longer-chain fatty acids have been suggested to be chain-shortened to C12 and C14 fatty acids (23–25). As outlined in Fig. 8, we propose that peroxisomal acyl-CoA thioesterases (mainly ACOT3, ACOT5, and ACOT8) are responsible for the incomplete β-oxidation of fatty acids in peroxisomes by premature hydrolysis of the CoA esters, which removes acyl-CoAs from the β-oxidation system. By this mechanism, medium-chain fatty acids are produced that can leave the peroxisome and become accessible for β-oxidation in the endoplasmic reticulum, mainly in kidney and liver. The dicarboxylic acids formed by β-oxidation are activated to the corresponding CoA esters and can re-enter the peroxisomal β-oxidation system for further chain shortening. Under conditions when the peroxisomal β-oxidation system is not saturated, that is, under conditions when CoASH is not completely sequestered ACOT8 is inhibited by CoASH and β-oxidation may proceed to succinyl-CoA, which is hydrolyzed by ACOT4. However, under ketotic conditions when the peroxisomal β-oxidation system may be saturated, ACOT8 is activated and hydrolyzes longer chain dicarboxylyl-CoA esters to release more adipic acid and suberic acid. We are not aware of data demonstrating actual production of succinate from peroxisomal β-oxidation of dicarboxylic acids, and although one study reported that adipic acid was the shortest chain length detected (50), other studies have indicated that succinate may be formed during peroxisomal β-oxidation of dicarboxylic acids (49). However, the identification of an apparently specific succinyl-CoA thioesterase in peroxisomes strongly suggests that succinyl-CoA is formed in peroxisomes. This possibility is further supported by our recent finding that the human ortholog for ACOT4 shows a similar substrate specificity.3 Succinate formed by ACOT4 can be excreted into urine and, at least in part, contribute to the succinate that is found in urine normally or be transported to the mitochondria.

In conclusion, in this study we have identified a novel peroxisomal thioesterase, ACOT4, which is highly specific for succinyl-CoA, with tissue expression and regulation of expression being compatible with a role in metabolism of dicarboxylic acids. We also characterized ACOT8 and show that it is also highly active on CoA esters of dicarboxylic acids but demonstrates a substrate specificity that is distinct from ACOT4. These data suggest that ACOT4 and ACOT8 regulate β-oxidation of dicarboxylic acids in peroxisomes, which may under certain conditions proceed to succinyl-CoA.

Acknowledgments—We thank Dr. Ronald Wanders, Dr. Kavro Hiltnun, and Dr. Rolf Berge for the kind gifts of a number of CoA esters that are not commercially available and Ulla Anderson for expertise in high performance liquid chromatography and mass spectrometry.

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