Identification of the peroxisomal β-oxidation enzymes involved in the degradation of long-chain dicarboxylic acids

Sacha Ferdinandusse,[1,*] Simone Denis,[*] Carlo W. T. van Roermund,[*] Ronald J. A. Wanders,[*,†] and Georges Dacremont[§]

Departments of Clinical Chemistry* and Pediatrics,† Emma Children's Hospital, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands; and Department of Pediatrics,§ University of Ghent, Ghent, Belgium

Abstract **Dicarboxylic acids (DCAs) are ω-oxidation products of monocarboxylic acids. After activation by a dicarboxylyl-CoA synthetase, the dicarboxylyl-CoA esters are shortened via β-oxidation. Although it has been studied extensively where this β-oxidation process takes place, the intracellular site of DCA oxidation has remained controversial. Making use of fibroblasts from patients with defined mitochondrial and peroxisomal fatty acid oxidation defects, we show in this paper that peroxisomes, and not mitochondria, are involved in the β-oxidation of C16DCA. Additional studies in fibroblasts from patients with X-linked adrenoleukodystrophy, straight-chain acyl-CoA oxidase (SCOX) deficiency, D-bifunctional protein (DBP) deficiency, and rhizomelic chondrodysplasia punctata type 1, together with direct enzyme measurements with human recombinant L-bifunctional protein (LBP) and DBP expressed in a *fox2* deletion mutant of *Saccharomyces cerevisiae*, show that the main enzymes involved in β-oxidation of C16DCA are SCOX, both LBP and DBP, and sterol carrier protein X, possibly together with the classic 3-ketoacyl-CoA thiolase. This is the first indication of a specific function for LBP, which has remained elusive until now.**—Ferdinandusse, S., S. Denis, C. W. T. van Roermund, R. J. A. Wanders, and G. Dacremont. **Identification of the peroxisomal β-oxidation enzymes involved in the degradation of long-chain dicarboxylic acids. *J. Lipid Res.* 2004.** 45: **1104–1111.**

Supplementary key words straight-chain acyl-CoA oxidase • L-bifunctional protein • D-bifunctional protein

Dicarboxylic acids (DCAs) are ω-oxidation products of monocarboxylic acids. Fatty acids are first converted into ω-hydroxymonocarboxylic acids by a microsomal cytochrome P450 and then further oxidized to ω-ketomonocarboxylic acids and finally DCAs through the sequential action of cytosolic long-chain alcohol and aldehyde dehydrogenases. After activation by a dicarboxylyl-CoA synthetase present in microsomes, at least in rat liver (1), the dicarboxylyl-CoA esters are shortened via β-oxidation (**Fig. 1**). Where this β-oxidation of dicarboxylyl-CoA esters takes place, in peroxisomes or mitochondria, has been studied extensively in rats. However, no unequivocal answer has been found for this question.

In vivo studies in rats have shown that both mitochondria and peroxisomes are involved in the catabolism of DCAs (2, 3), and several in vitro studies with isolated organelles have indicated that both mitochondria and peroxisomes are capable of oxidizing DCAs (4–6). However, the contribution of the two organelles differed among these studies, with some authors concluding that the oxidation of DCAs is mainly peroxisomal (6, 7) and others concluding that this is an exclusively peroxisomal process (3, 8). The role of mitochondria seems to be substrate and tissue dependent (5) and is probably determined by the carnitine dicarboxylyltransferase activity (4–6, 8), because intact mitochondria are active with dicarboxylylcarnitine esters and, in addition, mitochondria permeabilized with digitonin are capable of oxidizing dicarboxylyl-CoA esters (3, 5).

The finding of dicarboxylic aciduria in patients with a fatty acid oxidation disorder does not make it clear either whether DCAs are oxidized in peroxisomes or mitochondria. Both patients with a peroxisomal fatty acid oxidation

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Abbreviations: ALDP, adrenoleukodystrophy protein; BCOX, branched-chain acyl-CoA oxidase; CACT, carnitine acylcarnitine translocase; CPT, carnitine palmitoyltransferase; DBP, D-bifunctional protein; DCA, dicarboxylic acid; LBP, L-bifunctional protein; MTP, mitochondrial trifunctional protein; PBD, peroxisome biogenesis disorder; RCDP, rhizomelic chondrodysplasia punctata; SCOX, straight-chain acyl-CoA oxidase; SCPx, sterol carrier protein X; VLCAD, very long-chain acyl-CoA dehydrogenase; XALD, X-linked adrenoleukodystrophy.

[1] To whom correspondence should be addressed.
e-mail: s.ferdinandusse@amc.uva.nl

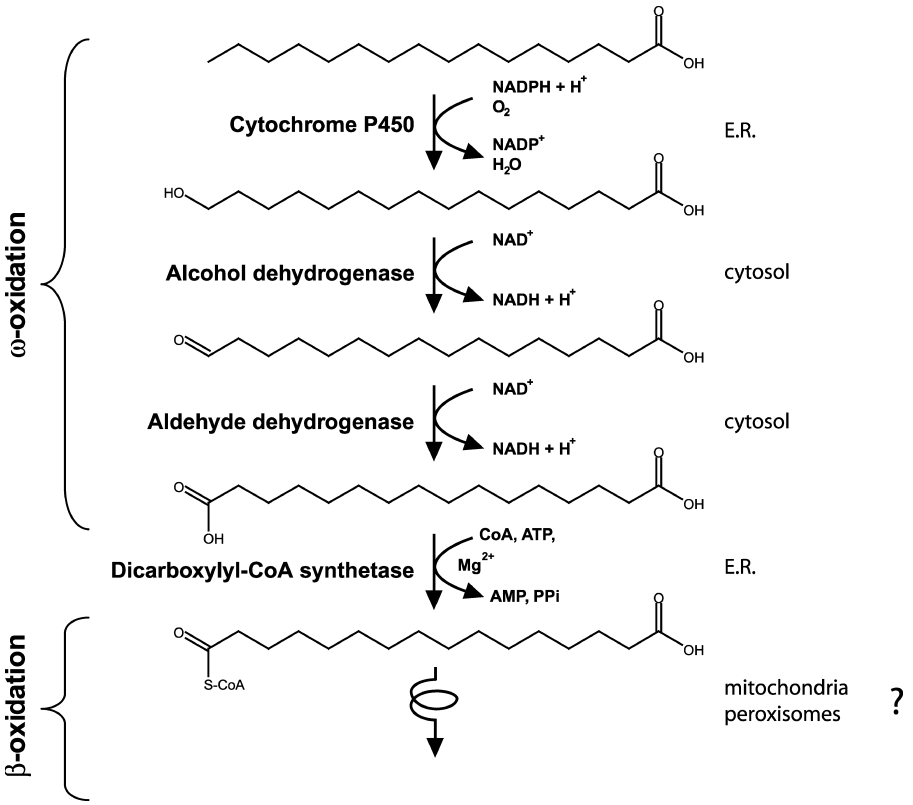

Fig. 1. Pathway of the formation of dicarboxylic acids (DCAs) from monocarboxylic acids via ω-oxidation and their subsequent breakdown via β-oxidation. E.R., endoplasmic reticulum.

disorder [i.e., patients with a peroxisome biogenesis disorder (PBD)] (9) and patients with a mitochondrial fatty acid oxidation disorder (10) display dicarboxylic aciduria. In a recent study by Rizzo et al. (11), hexadecanedioyl- and octadecanedioyl-carnitine were identified in plasma of PBD patients by tandem mass spectrometry. It has been hypothesized that peroxisomes can only shorten DCAs, because the peroxisomal β-oxidation system cannot handle DCAs shorter than six carbon atoms (2, 4). These shortened DCAs can then either move to the mitochondrion, where they can be β-oxidized to completion, producing succinate, or be excreted in urine. In this study, we set out to investigate the role of peroxisomes and mitochondria, and their fatty acid oxidation systems, in the breakdown of DCAs.

Figure 2 shows a schematic representation of the peroxisomal β-oxidation system. There are two complete sets of β-oxidation enzymes present in the peroxisome (12). Straight-chain acyl-CoA oxidase (SCOX) is responsible for the initial oxidation of very long-chain fatty acyl-CoAs, whereas branched-chain acyl-CoA oxidase (BCOX) oxidizes branched-chain fatty acyl-CoAs. The enoyl-CoA esters of both straight- and branched-chain fatty acids are then hydrated and subsequently dehydrogenated by the same enzyme: D-bifunctional protein (DBP). The function of the second multifunctional protein present in the per-

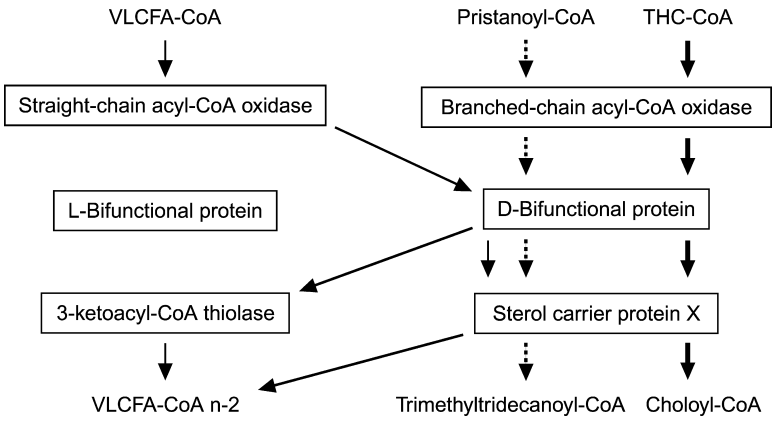

Fig. 2. Schematic representation of the fatty acid β-oxidation machinery in human peroxisomes catalyzing the oxidation of very long-chain fatty acyl-CoAs (VLCFA-CoA) and branched-chain fatty acyl-CoAs (pristanoyl-CoA and trihydroxycholestanoyl-CoA). The oxidation of VLCFA-CoAs (C24:0 and C26:0) involves straight-chain acyl-CoA oxidase (SCOX), D-bifunctional protein (DBP), and both 3-ketoacyl-CoA thiolase and sterol carrier protein X (SCPx), whereas the oxidation of branched-chain fatty acyl-CoAs involves branched-chain acyl-CoA oxidase, DBP, and SCPx [see ref. (12) for review].

oxisome, L-bifunctional protein (LBP), is still unknown. The last step of the β-oxidation process, the thiolytical cleavage, is performed by sterol carrier protein X (SCPx) in case of the branched-chain substrates, whereas straight-chain substrates most likely are handled by both SCPx and the classic 3-ketoacyl-CoA thiolase. To date, the only single enzyme deficiencies of the peroxisomal β-oxidation system that have been identified are SCOX deficiency and DBP deficiency. In addition, patients suffering from rhizomelic chondrodysplasia punctata (RCDP) type 1 lack 3-ketoacyl-CoA thiolase in their peroxisomes (13–15).

To elucidate the role of both the peroxisome and the mitochondrion in the breakdown of long-chain DCAs, we studied the β-oxidation of [16-^{14}C]16DCA in fibroblasts from patients with a PBD and from patients with a deficiency of one of the following mitochondrial enzymes: carnitine palmitoyltransferase (CPT) I, CPT II, carnitine acylcarnitine translocase (CACT), very long-chain acyl-CoA dehydrogenase (VLCAD), and mitochondrial trifunctional protein (MTP) (16, 17). In addition, we investigated the role of the various peroxisomal β-oxidation enzymes in the degradation of long-chain DCAs by measuring the β-oxidation of [16-^{14}C]16DCA in fibroblasts of patients with a deficiency of SCOX and DBP and from a patient suffering from RCDP type 1 and X-linked adrenoleukodystrophy (XALD). For further investigation of the role of LBP and DBP in the metabolism of C16DCA, we performed direct enzyme activity measurements with human recombinant LBP and DBP expressed in a *fox2* deletion mutant of *Saccharomyces cerevisiae*.

MATERIALS AND METHODS

Patient cell lines

Cell lines were used from several patients suffering from various peroxisomal and mitochondrial fatty acid β-oxidation disorders. The patients with a PBD all had the clinical and biochemical abnormalities described for PBD patients, including deficient C26:0 and pristanic acid β-oxidation and phytanic acid α-oxidation and the absence of peroxisomes, as assessed by immunofluorescence microscopy analysis using antibodies against catalase (18). The fibroblasts from the XALD patient had impaired C26:0 β-oxidation, which is caused by a mutation in the gene encoding the peroxisomal membrane protein adrenoleukodystrophy protein (ALDP) (19). The SCOX- and DBP-deficient patients all had mutations in the encoding gene, and no enzyme activity could be measured in fibroblasts of these patients (20, 21). Peroxisomes from the patient with RCDP type 1 lack 3-ketoacyl-CoA thiolase as a result of a mutation in the PEX7 gene encoding the peroxisomal targeting signal 2 receptor. Immunoblot studies performed with an antibody raised against 3-ketoacyl-CoA thiolase revealed that only the unprocessed protein of 44 kDa is present in fibroblast homogenates. It is known that 3-ketoacyl-CoA thiolase is synthesized as a precursor protein and is proteolytically cleaved to its mature form of 41 kDa inside the peroxisome (22). The fibroblasts from patients with a mitochondrial β-oxidation disorder used in this study were from patients with a confirmed deficiency of CPT I, CACT, CPT II, VLCAD, or MTP attributable to mutations in the encoding genes [see ref. (17) for review]. These mutations result in a deficiency of mitochondrial fatty acid oxidation, as established by individual enzyme activity measurements in cultured skin fibroblasts.

All patient cell lines used in this study were taken from the cell repository of the Laboratory for Genetic Metabolic Diseases, University of Amsterdam, Academic Medical Center, and were derived from patients diagnosed at this center. Informed consent was obtained from parents or guardians of the patients whose fibroblasts were studied.

Synthesis of [16-^{14}C]16DCA

[16-^{14}C]16DCA was prepared from 15-hydroxypentadecanoic acid (Sigma-Aldrich Chemie BV, Zwijndrecht, The Netherlands) by a one-carbon elongation procedure at C15. To enhance solubility and protect the carboxyl group, 15-hydroxypentadecanoic acid was first converted to its corresponding methylester with two equivalents of (trimethylsilyl)diazomethane in hexane-methanol (3:1, v/v). After removal of the solvent, the C15 alcohol was dissolved in dichloromethane and converted to its mesylate by stirring with 1.25 equivalents of methane sulfonyl chloride and 1.25 equivalents of triethylamine for 24 h at room temperature. After washing the reaction mixture with saturated sodium chloride and removal of the solvent, the crude mesylate was purified by silica gel column chromatography using dichloromethane as eluent. Subsequently, the purified mesylate was reacted with one equivalent of [^{14}C]KCN (55 mCi/mmol) in dimethyl sulfoxide at 70°C for 48 h to produce the 16-^{14}C-labeled nitrile. The nitrile was extracted with hexane, taken to dryness, and hydrolyzed in 1:1 ethanol/10% (w/v) aqueous sodium hydroxide (v/v) at 80°C for 48 h to yield [16-^{14}C]16DCA, which after acidification was extracted with diethylether and purified by silica gel column chromatography with hexane-ether-acetic acid (60:40:1) as eluent. Thin layer chromatography of the purified acid showed only one labeled band with the same relative mobility value as commercial hexadecanedioic acid. GC-MS analysis of the dimethylester showed only one peak with a molecular ion at *m/z* 288 ([C^{14}]M$^+$ 5.5%) 286 ([C^{12}]M$^+$ 0.3%). The overall yield of the elongation from the C15-mesylate was 79%. The synthesized [16-^{14}C]16DCA had a specific activity of 50 mCi/mmol.

β-Oxidation measurements

[16-^{14}C]16DCA β-oxidation was measured as described before (23) with some minor modifications described here. Incubations were carried out for 6 h in minimal essential medium supplemented with penicillin/streptomycin and containing 50 mM MES (pH 5.25) as well as 10 μM [16-^{14}C]16DCA and 30 μM C16DCA as substrate. The radioactivity recovered as ^{14}C-labeled acid-soluble products was taken as a measure of fatty acid oxidation. Parallel incubations were performed to determine the amount of protein.

Analysis of C16DCA oxidation products in cultured skin fibroblasts

Incubations were carried out at 37°C in minimal essential medium supplemented with penicillin/streptomycin containing 0.4% BSA, 400 μM L-carnitine, and 300 μM C16DCA as substrate. Parallel incubations were performed to determine the amount of protein. After 96 h of incubation, the culture medium was removed and lipids were extracted for analysis. To 100 μl of medium, 10 μl of C7DCA (500 μM) and 20 μl of a mixture of fatty acids (100 μM C9, C13, C17, and C23) were added as internal standards. One milliliter of acetonitrile/37% hydrochloric acid (23:1, v/v) was added to each sample, and hydrolysis was allowed to proceed for 2 h at 90°C. After cooling to room temperature, 1 ml of saturated NaCl solution was added and the lipids were extracted twice with 2 ml of ether-ethylacetate (1:1, v/v). The combined organic layers were dried with a small amount of

Na_2SO_4 and centrifuged for 10 min at 1,600 *g* to remove the Na_2SO_4. The organic phase was taken to dryness under a stream of nitrogen, and the residue was resuspended in 100 μl of chloroform. To 75 μl of this sample, 75 μl of methanol-water (45:5, v/v) containing 0.01% of a 25% aqueous ammonia solution was added, and the samples were analyzed by electrospray ionization mass spectrometry as described (24), with a cone voltage of 35 V instead of 45 V as the only modification.

Synthesis of C16dicarboxylyl-CoA and C16:1dicarboxylyl-CoA

The CoA ester of C16DCA (Sigma-Aldrich Chemie BV) was synthesized using *Pseudomonas* acyl-CoA synthetase (25). Five hundred micromolar C16DCA was incubated in 50 mM HEPES (pH 7.5), 120 mM KCl, 1 mM DTT, 5 mM ATP, 2 mM CoA, 12 mM $MgCl_2$, 0.1 mM EGTA, and 0.1% Triton X-100 with 1 U/ml *Pseudomonas* acyl-CoA synthetase (Sigma-Aldrich Chemie BV) for 4 h at 37°C. The reaction was terminated by adding 0.3 M HCl and 0.2 M KCl, followed by centrifugation for 10 min at 20,000 *g* at 4°C. The supernatant was removed and the pellet resuspended in 500 μl of ethanol, resulting in a stock solution of 10 mM C16DC-CoA. Subsequently, 2-ene-C16DC-CoA (C16:1DC-CoA) was produced by incubating 100 μM C16DC-CoA in 50 mM HEPES (pH 7.5) and 50 μM flavin adenine dinucleotide with 1.25 U/ml acyl-CoA oxidase (*Arthrobacter* species; Boehringer Mannheim) for 2 h at 37°C. The reaction was terminated with 0.5 M HCl followed by centrifugation for 10 min at 20,000 *g* at 4°C. The supernatant was removed and the pellet resuspended in 20 mM MES. The pH was adjusted to a value between 4 and 6. 2-ene-C16-CoA (C16:1-CoA) was synthesized from C16-CoA (Sigma-Aldrich Chemie BV) as described for C16:1DC-CoA.

Disruption of the FOX2 gene

The *fox2*Δ deletion mutant was generated by one-step PCR-mediated gene disruption using *kan*MX4 (26) as a selectable marker. PCR-derived disruption constructs comprised the *kan*MX4 gene flanked by short regions of homology (50 bp) corresponding to the *FOX2* (YKR009C) 5′–3′ noncoding region. The resulting PCR fragments were introduced into *S. cerevisiae* BJ1991 cells. G418-resistant clones were selected by growth on yeast extract-peptone-dextrose plates containing 200 mg/l G418 (GIBCO Invitrogen) (26).

Expression of human recombinant LBP and DBP

The construction of the plasmid for the expression of human DBP in yeast has been described (27). The coding sequence of human LBP cDNA was amplified by PCR using cloning primers (*Bam*HI-LBP-F, 5′-CGA TGG ATC CAT GGC CGA GTA TAC GCG GCT GC-3′; and *Sal*I-LBP-R, 5′-CGA TGT CGA CTC ACA ATT TAC TGC TAG GGG AGC CTG-3′) and subsequently cloned into the yeast expression vector pEL26 under transcriptional control of the oleate-inducible *CTA1* promoter (28). The subcloned PCR fragment was sequenced to exclude errors introduced by *Taq* polymerase. *S. cerevisiae* strain BJ1991, in which the *fox2* gene (*fox2*Δ) is disrupted, was transformed with the expression plasmids containing the cDNA encoding either LBP or DBP using the lithium acetate method (29). Transformants were selected and grown at 28°C on minimal medium containing 6.7 g/l yeast nitrogen base without amino acids, 3 g/l glucose, and 20 mg/l of the appropriate amino acids. Protein expression was induced by shifting the cells to a rich medium containing oleic acid (5 g/l potassium phosphate buffer, pH 6, 3 g/l yeast extract, 5 g/l peptone, and 1 g/l oleic acid plus 2 g/l Tween 40). The cells were harvested and stored as pellet at −80°C. Before analysis, the pellets were resuspended in PBS containing complete protease inhibitor cocktail. To prepare cell lysates, 200 μl glass beads were added and the suspension was vortexed 10 times for 15 s with a 45 s interval at 4°C. The lysates were subsequently homogenized by sonication, and cell debris was removed by centrifugation at 10,000 *g* for 30 s The supernatant was used for enzyme activity measurements.

Enzyme activity measurements

The activities of LBP and DBP were measured with C16:1DC-CoA and C16:1-CoA. Incubations consisted of 1 μg of lysate of *fox2*Δ yeast expressing LBP or DBP, 50 mM Tris (pH 8.5), 150 mM KCl, 1 mM NAD, 5 mM pyruvate, and 18 U/ml LDH in a final volume of 100 μl. Measurements with C16:1-CoA as substrate were performed in the presence of 10 μM BSA. Reactions were started by the addition of either C16:1DC-CoA or C16:1-CoA. After an incubation period of 15 min at 37°C, reactions were terminated by the addition of 2 M HCl to a final concentration of 0.18 M. The reaction mixture was then neutralized using 0.6 M MOPS plus 2 M KOH followed by the addition of acetonitrile [final concentration 28% (v/v)]. After centrifugation for 10 min at 20,000 *g* at 4°C, the supernatant (140 μl) was applied to a reverse-phase C18 column (Supelcosil SPLC-18-DB, 250 mm × 4.6 mm, 5 μm; Supelco). Resolution between the different CoA esters was achieved by elution with a linear gradient of acetonitrile [40–58% (v/v) for incubations with C16:1-CoA and 22–40% (v/v) for incubations with C16:1DC-CoA] in 16.9 mM sodium phosphate buffer (pH 6.9) at a flow rate of 1 ml/min under continuous monitoring of the absorbance at 254 nm. The amount of ketoacyl-CoA formed was calculated from the ratio of ketoacyl-CoA over the total amount of substrate and products, e.g., enoyl-CoA, hydroxyacyl-CoA, and ketoacyl-CoA (with a correction for different absorption coefficients), and was used to calculate the enzyme activity.

RESULTS

Oxidation of [16-^{14}C]16DCA in cultured skin fibroblasts

β-Oxidation of [16-^{14}C]16DCA was measured in cultured skin fibroblasts from patients with various peroxisomal and mitochondrial fatty acid β-oxidation disorders (**Table 1**). Fibroblasts from patients with a mitochondrial fatty acid oxidation defect (at the level of CPT I, CACT, CPT II, VLCAD, or MTP) revealed normal β-oxidation activities compared with the activity measured in control fibroblasts. In contrast, fibroblasts from patients with a PBD showed strongly reduced oxidation activities, suggesting a major role of peroxisomes in C16DCA oxidation. Subsequently, we studied the involvement of different peroxisomal β-oxidation enzymes in the oxidation of [16-^{14}C]16DCA and found normal activities in fibroblasts from patients suffering from XALD, RCDP type 1, and DBP deficiency but strongly reduced oxidation activities in fibroblasts from patients with SCOX deficiency (Table 1).

Analysis of C16DCA oxidation products in cultured skin fibroblasts

Cultured skin fibroblasts were incubated with C16DCA to study the formation of partial oxidation products, which are not detected as acid-soluble radiolabeled products in the β-oxidation measurements described above if β-oxidation occurs at the unlabeled C-1 end of the DCA. After a 96 h incubation of control human skin fibroblasts,

TABLE 1. β-Oxidation of [16-^{14}C]16 dicarboxylic acid in human skin fibroblasts

Sample	n	Specific Activity
		pmol/h/mg
Controls	6	36.0 ± 8.5[a]
Peroxisome biogenesis disorder	6	5.7 ± 5.4
X-linked adrenoleukodystrophy	1	57.2
Straight-chain acyl-CoA oxidase	5	5.5 ± 7.3
D-bifunctional protein	7	41.1 ± 18.3
Rhizomelic chondrodysplasia punctata type 1	1	47.7
Carnitine palmitoyltransferase I	1	40.1
Carnitine acylcarnitine translocase	2	37.3/44.4
Carnitine palmitoyltransferase II	1	57.3
Very long-chain acyl-CoA dehydrogenase	1	40.9
Mitochondrial trifunctional protein	1	40.5

n = number of different cell lines; all incubations were performed in duplicate.

[a] Mean value ± SD.

formation of C14DCA and C12DCA was observed (**Fig. 3**). Fibroblasts from a patient with a CACT deficiency also formed C14DCA and C12DCA in similar amounts as the control fibroblasts; however, fibroblasts from PBD patients revealed hardly any formation of C14DCA and C12DCA (Fig. 3).

Enzyme activity measurements with human recombinant LBP and DBP

Because normal β-oxidation of [16-^{14}C]16DCA was found in DBP-deficient fibroblasts, we wanted to investigate whether the other peroxisomal multifunctional protein, LBP, could be responsible for the oxidation of DCAs. Because no patients with LBP deficiency have been identified, we expressed human LBP and DBP separately in the *S. cerevisiae fox2*Δ deletion mutant to perform direct enzyme measurements with C16:1DC-CoA as substrate. The expression of both proteins was checked by immunoblotting experiments with antibodies raised against purified LBP and DBP, respectively, which revealed proteins of the correct molecular weight in both cases (data not shown). *fox2*Δ cells have a deletion of the *FOX2* gene, which is the yeast ortholog of the human DBP. Unlike mammals, *S. cerevisiae* does not have a second multifunctional protein. This was confirmed by the observation that there was no formation of ketoacyl-CoA esters after incubation of *fox2*Δ cell lysates with either C16:1DC-CoA or C16:1-CoA as substrate. In contrast, both human recombinant LBP and DBP expressed in *fox2*Δ cells formed keto-C16DC-CoA from the corresponding enoyl-CoA ester. Subsequently, we determined the Michaelis-Menten constants of human LBP and DBP expressed in *fox2*Δ cells for C16:1DC-CoA. LBP had an apparent K_m of 0.3 μM for C16:1DC-CoA, and the apparent K_m of DBP for this substrate was 0.9 μM (**Fig. 4**). For comparison, these parameters were also determined for C16:1-CoA. The apparent K_m of LBP was 10.4 μM, and the apparent K_m of DBP for this substrate was 12.8 μM (Fig. 4). The measurements with C16:1-CoA were performed in the presence of 10 μM albumin, because in the absence of albumin enzyme activity was inhibited with increasing substrate concentrations, which is a known phenomenon especially for C16-CoA (30). For this reason, the kinetic parameters for C16:1DC-CoA and C16:1-CoA cannot be compared directly.

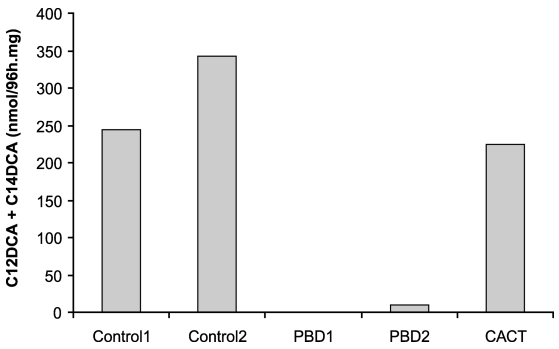

Fig. 3. Formation of C14DCA and C12DCA by cultured skin fibroblasts from two control subjects, two peroxisome biogenesis disorder (PBD) patients, and one carnitine acylcarnitine translocase (CACT)-deficient patient after incubation with 300 μM C16DCA for 96 h. Lipids were extracted from the incubation medium and were analyzed by electrospray ionization mass spectrometry.

DISCUSSION

Although many studies about the site of degradation of DCAs have been published in the past, the role of peroxisomes and mitochondria in this process has remained unclear and is therefore still the subject of discussion. Many different experimental setups have been used to obtain a definite answer and to get insight into why both patients with peroxisomal and patients with mitochondrial fatty acid oxidation defects have dicarboxylic aciduria. The differences in the obtained results are most likely attributable to the use of many different experimental conditions [e.g., different substrates (C12DCA vs. C16DCA), different materials (isolated organelles vs. tissue homogenates or hepatocytes), different methods to measure β-oxidation (measurement of acyl-CoA oxidase activity vs. metabolite formation), and the use of different inhibitors of the mitochondrial β-oxidation]. In addition, in all studies, rats, or tissues or cultured cells derived from rats, were used. To circumvent many of these problems, cultured skin fibroblasts of patients with defined genetic defects causing either a peroxisomal or a mitochondrial fatty acid oxidation defect were used in this study. Our results show that C16DCA is broken down in the peroxisome, because the β-oxidation of [16-^{14}C]16DCA was deficient in fibroblasts from patients lacking functional peroxisomes and normal in fibroblasts from patients with a mitochondrial fatty acid oxidation defect (at the level of CPT I, CACT, CPT II, VLCAD, or MTP). In addition, normal formation of the partial oxidation products C14DCA and C12DCA was found after incubation of fibroblasts from a CACT-deficient patient with C16DCA compared with control fibroblasts, whereas hardly any C14DCA and C12DCA was formed in fibroblasts from PBD patients. These results are in good agreement with the recent identification of hexa-

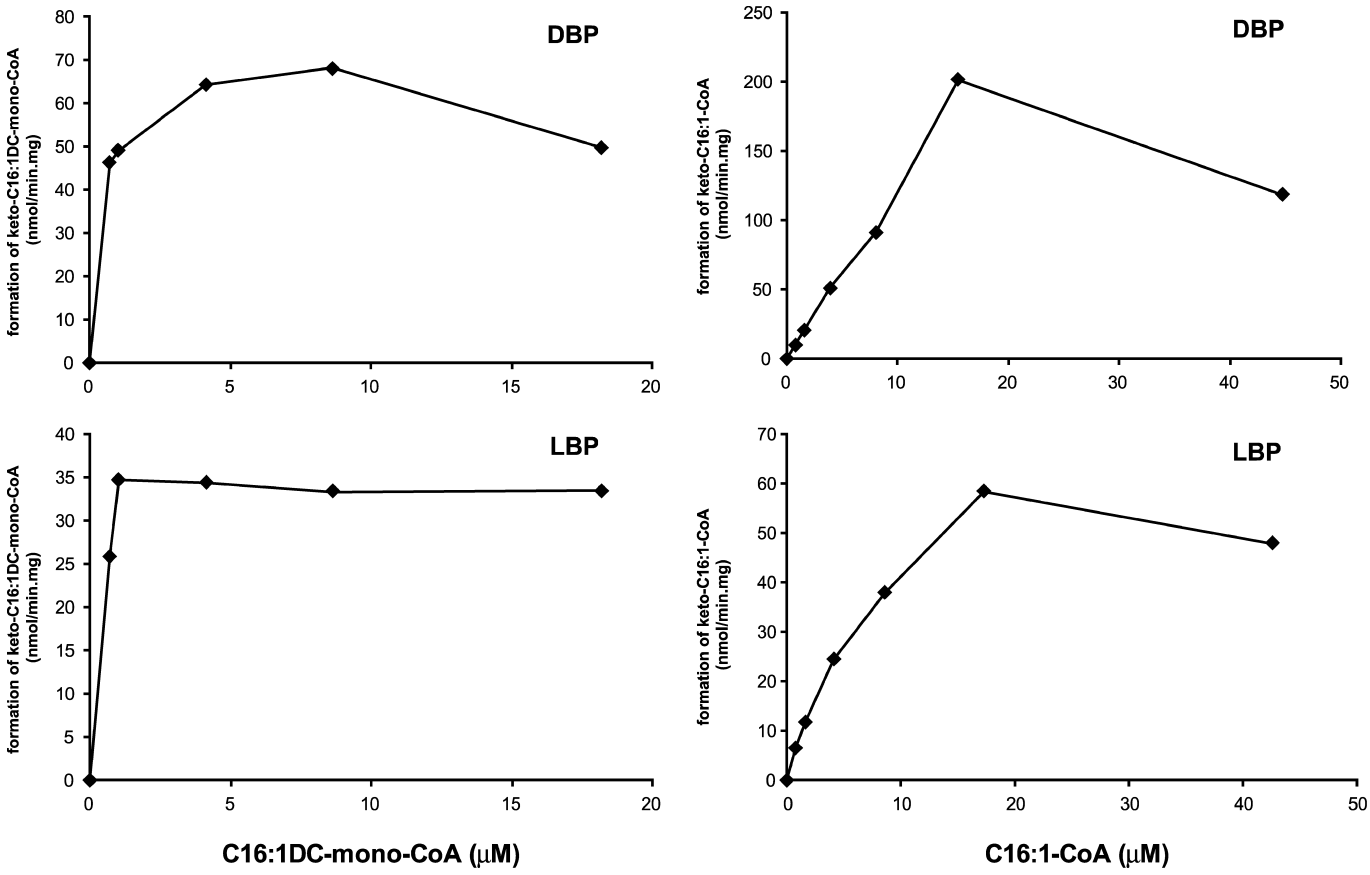

Fig. 4. Kinetic analysis of human DBP (upper panels) and L-bifunctional protein (LBP; lower panels) expressed separately in the *S. cerevisiae fox2*Δ deletion mutant with C16:1DC-CoA (left panels) and C16:1-CoA (right panels), respectively, as substrate. The results are means of two independent experiments.

decanedioyl- and octadecanedioyl-carnitine in plasma of PBD patients by tandem mass spectrometry (11) and the excretion of C16DCA in urine of PBD patients (9). The small residual activity found could be either mitochondrial or result from peroxisomal enzymes mislocalized in the cytosol.

Our results support the hypothesis that long-chain DCAs are shortened in the peroxisome and that the shortened products are either excreted in the urine or move to the mitochondrion, where they can be β-oxidized to completion (2, 4). The mitochondrial β-oxidation system itself is most likely capable of oxidizing DC-CoAs, although the affinity is much lower than for monocarboxylic CoA esters. DC-CoAs, however, are very poor substrates for CPT I; therefore, long-chain DCAs do not enter the mitochondrion (5). Medium- or short-chain DCAs produced from longer chain DCAs in peroxisomes may be converted to carnitine esters by the peroxisomal carnitine octanoyltransferase or carnitine acetyltransferase and therefore do not need CPT I to enter the mitochondrion. Alternatively,

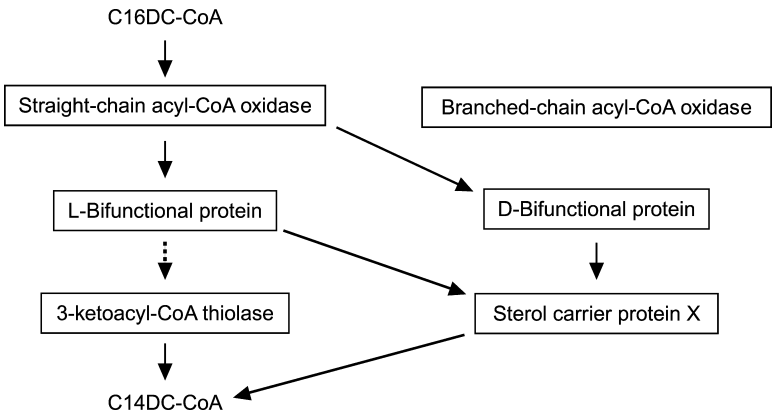

Fig. 5. Schematic representation of the fatty acid β-oxidation machinery in human peroxisomes catalyzing the oxidation of C16DC-CoA. The oxidation of C16DC-CoA involves *1*) SCOX, *2*) both LBP and DBP, and *3*) SCPx and possibly 3-ketoacyl-CoA thiolase.

they might not need the mitochondrial carnitine shuttle at all because they are able to enter the mitochondrion as free fatty acids. This hypothesis is also in agreement with medium-chain dicarboxylic aciduria in patients with a mitochondrial fatty acid oxidation defect (10). In light of this hypothesis, however, the finding of such a medium-chain dicarboxylic aciduria in PBD patients is unexpected and perhaps can be explained by mitochondrial abnormalities described in these patients.

Because our results revealed that peroxisomal β-oxidation is essential for the degradation of C16DCA, we wanted to know which peroxisomal β-oxidation enzymes are involved in this process. Current evidence holds that oxidation of the straight-chain fatty acids, C24:0 and C26:0, involves SCOX, DBP, and both 3-ketoacyl-CoA thiolase and SCPx, whereas oxidation of the branched-chain fatty acids, pristanic acid and the bile acid intermediates, involves BCOX, DBP, and SCPx. The function of the second multifunctional protein, LBP, is still unknown (12). Our measurements in fibroblasts from patients with a single peroxisomal enzyme deficiency showed that SCOX is the acyl-CoA oxidase involved in the β-oxidation of C16DCA, because a strongly reduced rate of [16-^{14}C]16DCA β-oxidation was found in fibroblasts of patients with SCOX deficiency. Because no SCOX protein can be detected when performing immunoblot analysis in any of the SCOX-deficient cell lines used in this study, it is not likely that the deficient SCOX protein is responsible for the residual activity. This suggests that either the mitochondrial β-oxidation system is responsible for this residual activity, as in fibroblasts of PBD patients, or that the other peroxisomal oxidase, BCOX, can also handle this substrate but that its activity is not sufficient for normal β-oxidation of [16-^{14}C]16DCA. The latter hypothesis is supported by enzymatic studies with purified peroxisomal oxidases. Van Veldhoven et al. (31) showed that in rat liver palmitoyl-CoA oxidase (corresponding to human SCOX) is responsible for 70% of the oxidase activity with C16DC-CoA, whereas pristanoyl-CoA oxidase was responsible for the remaining 30%. It is very important for the interpretation of these results, however, to keep in mind that in humans only one oxidase is present that handles all branched-chain substrates, whereas in rats both a pristanoyl-CoA oxidase and a trihydroxycoprostanoyl-CoA oxidase are present (32).

With regard to the second and third steps of the β-oxidation of long-chain DCAs, our measurements in fibroblasts of DBP-deficient patients revealed that LBP is not only able to handle this substrate but can maintain normal β-oxidation in case of a DBP deficiency. This was also demonstrated in the study of Rizzo et al. (11) by the normal amounts of hexadecanedioyl- and octadecanedioyl-carnitine in the plasma of a DBP patient, whereas increased levels of these metabolites were identified in the plasma of PBD patients. Because this is the first indication of a function for LBP [all other known peroxisomal substrates, including very long-chain fatty acids (12), branched-chain fatty acids (12), the precursor of docosahexaenoic acid (C24:6n-3) (33, 34), and leukotrienes (35), are handled by DBP], we set out to further investigate the role of LBP in the metabolism of C16DCA. Because no patients with LBP deficiency have been identified, we could not perform similar β-oxidation experiments to establish the role of LBP. To resolve this problem, we expressed human LBP and DBP separately in the *S. cerevisiae fox2*Δ deletion mutant to enable direct enzyme measurements with C16:1DC-CoA as substrate. Our results show that both LBP and DBP are active with C16:1DC-CoA and have comparable affinities for this substrate [LBP had an apparent K_m of 0.3 μM and DBP had an apparent K_m of 0.9 μM (Fig. 4)]. In addition, for comparison we performed measurements with C16:1-CoA as substrate and found that also for this substrate LBP and DBP have comparable affinities. These results strongly suggest that both LBP and DBP are involved in the oxidation of long-chain DCAs, because they can handle C16:1DC-CoA equally well, and that in case of a deficiency of DBP, LBP can take over its function. The contribution of both enzymes to the oxidation of C16DCA depends most likely on the content of LBP and DBP in different tissues, which has been shown to differ (36). It would be interesting to study whether DBP can also take over the function of LBP completely by investigating whether the LBP knockout mouse (37) accumulates hexadecanedioyl-carnitine in plasma.

Our measurements in fibroblasts of a patient with RCDP type 1 have shown that the presence of the classic peroxisomal 3-ketothiolase is not crucial for the normal β-oxidation of [16-^{14}C]16DCA. This suggests that either the other thiolase, SCPx, is responsible for the oxidation of this substrate or that, as is the case for the very long-chain fatty acids, both thiolases are able to perform the last step of C16DCA β-oxidation and can maintain normal activity by themselves in the absence of the other thiolase. In addition, we found that ALDP, the protein that is defective in patients with XALD, who have an impaired β-oxidation of very long-chain fatty acids, does not appear to be involved in the degradation of long-chain DCAs, because we found normal oxidation of [16-^{14}C]16DCA in an XALD patient.

In conclusion, our results show that C16DCA is primarily oxidized in the peroxisome by the sequential action of SCOX, both LBP and DBP, and SCPx, possibly together with the classic 3-ketothiolase (**Fig. 5**).

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