New insights in peroxisomal beta-oxidation
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Reinvestigation of peroxisomal 3-ketoacyl-CoA thiolase deficiency: identification of the true defect at the level of D-bifunctional protein

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Abstract
So far only one single patient with a deficiency of peroxisomal 3-ketoacyl-CoA thiolase has been reported. The patient accumulated very long-chain fatty acids and the bile acid intermediate trihydroxycholestanolic acid in body fluids. At the time, these abnormalities were believed to be the logical consequence of the assumption that 3-ketoacyl-CoA thiolase was the only thiolase involved in the peroxisomal β-oxidation of all fatty acyl-CoAs. Recent studies have shown, however, that peroxisomes contain two sets of β-oxidation enzymes, including a second peroxisomal thiolase, i.e. sterol carrier protein X, responsible for the β-oxidation of branched-chain fatty acids but also of bile acid intermediates. Since the reported biochemical aberrations could no longer be explained by a deficiency of 3-ketoacyl-CoA thiolase, we reinvestigated the previously reported patient. In this paper, we show that the true defect in this patient is at the level of D-bifunctional protein (DBP) and not at the level of 3-ketoacyl-CoA thiolase. Immunoblot analysis revealed the absence of DBP in post-mortem brain of the patient, whereas 3-ketoacyl-CoA thiolase was normally present. In addition, we found that the patient had a homozygous deletion of part of exon 3 and intron 3 of the DBP gene, resulting in skipping of exon 3 at the cDNA level. Our findings have great implications, since they imply that the group of identified single peroxisomal β-oxidation enzyme deficiencies is limited to straight-chain acyl-CoA oxidase, DBP and α-methylacyl-CoA racemase deficiency and that there is no longer evidence for the existence of 3-ketoacyl-CoA thiolase deficiency as a distinct clinical entity.

Introduction
In 1986, Goldfischer et al. (1) described a patient with clinical features similar to those of patients with Zellweger syndrome. In contrast to patients with Zellweger syndrome who lack functional peroxisomes, however, this patient had apparently normal peroxisomes in liver and kidney. There was an accumulation of very long-chain fatty acids (VLCFAs) in plasma and of 3α,7α,12α-trihydroxycholestanolic acid (THCA) in duodenal aspirate of the patient (1). Later studies by Clayton et al. (2) showed that 3α,7α,12α,24-tetrahydroxycholestanolic acid (varanic acid), an intermediate in the formation of cholic acid from THCA, was present in body fluids of the patient. Immunoblot experiments by Schram et al. (3) revealed the absence of 3-ketoacyl-CoA thiolase in post-mortem liver of the patient, whereas normal levels were found for other peroxisomal matrix enzymes.
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(acyl-CoA oxidase, bifunctional protein and catalase). These results led to the conclusion that the strongly reduced rate of peroxisomal β-oxidation measured in liver of the patient and the accumulation of VLCFAs and THCA in body fluids were caused by a deficiency of 3-ketoacyl-CoA thiolase. Following the identification of the gene encoding human 3-ketoacyl-CoA thiolase in 1991, molecular studies in this patient were performed but no large DNA rearrangements involving the thiolase gene were observed in Southern blot experiments (4).

At the time the patient was described, it was believed that the peroxisomal β-oxidation system consisted of only a single set of enzymes: an acyl-CoA oxidase catalyzing the first step, a bifunctional protein catalyzing the second and third step, and a thiolase responsible for the last step of the β-oxidation process. In the last few years, however, a number of studies have shed new light on the enzymology of the peroxisomal β-oxidation system (see for recent reviews (5-7)). These studies have shown that peroxisomes contain two sets of β-oxidation enzymes which differ in substrate specificity (Fig. 1). In addition to

![Diagram of fatty acid β-oxidation machinery](attachment:image.png)

**Fig. 1** 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 THC-CoA). Oxidation of VLCFA-CoAs (C24:0 and C26:0) involves straight-chain acyl-CoA oxidase, D-bifunctional protein (DBP) and both 3-ketoacyl-CoA thiolase and sterol carrier protein X (SCPx), while oxidation of branched-chain fatty acyl-CoAs involves branched-chain acyl-CoA oxidase, DBP and SCPx (see (6) for review).

the original acyl-CoA oxidase, which is now called straight-chain acyl-CoA oxidase (SCOX), a second oxidase was identified, called branched-chain acyl-CoA oxidase (BCOX). SCOX is responsible for the oxidation of VLCFAs such as C26:0 and C24:0, whereas BCOX is involved in the β-oxidation of pristanic acid and the bile acid intermediates THCA and dihydroxycholestanolic acid (DHCA) (Fig. 1). The second bifunctional protein that was identified, has been named D-bifunctional protein (DBP) because it forms and dehydrogenates D-3-hydroxyacyl-CoAs, in contrast to the original
protein, L-bifunctional protein (LBP), which produces L-hydroxy intermediates. Both in vitro studies performed with the purified bifunctional proteins and the identification of patients with a deficiency of DBP (8-11) has provided unequivocal evidence that DBP is involved in the degradation of VLCFAs as well as the branched-chain fatty acids, pristanic acid and DHCA/THCA. The physiological function of LBP remains elusive at this moment. Both peroxisomal thiolases are believed to be involved in VLCFAs degradation. In addition, sterol carrier protein X (SCPx), the second peroxisomal thiolase that was identified, which contains both a thiolase domain and a sterol carrier protein domain, is the key enzyme in the β-oxidation of pristanic acid and DHCA/THCA.

Since the new insights into the peroxisomal β-oxidation system and the physiological function of the different β-oxidation enzymes no longer provided an explanation for the biochemical findings in the reported patient, we reinvestigated this unique case. In this paper, we describe the unraveling of the true enzymatic and genetic defect in this patient.

Materials and Methods

Patient L.C.
The patient's clinical and biochemical characteristics have been described in (1). Skin fibroblasts were obtained from the patient's parents, who were first cousins.

Immunoblot analysis
Homogenates of post-mortem brain and kidney material (100 and 5-50 μg of protein, respectively) were subjected to electrophoresis on a 10% (w/v) SDS-polyacrylamide gel essentially as described by Laemmli (12) and transferred to a nitrocellulose sheet. After blocking of non-specific binding sites with 50 g/L Profstar and 10 g/L BSA in 1 g/L Tween-20/PBS for 1 h, the blot was incubated for 2 h with different antibodies against peroxisomal matrix enzymes. The antibodies used were: anti-3-ketoacyl-CoA thiolase (diluted 1:2,000 in 3 g/L BSA) (13), anti-SCPx (diluted 1:1,000) (14), anti-SCOX (diluted 1:3,000) (13), anti-LBP (diluted 1:5,000) (13) and anti-DBP (diluted 1:10,000) (15). Goat anti-rabbit IgG antibodies conjugated to alkaline phosphatase were used for detection, according to the manufacturer's instructions (Bio-Rad, CA).

DNA isolation
DNA was isolated from post-mortem brain and kidney material from patient L.C. and from fibroblasts of the patient’s parents using the Wizard® Genomic DNA purification kit, according to the manufacturer’s instructions (Promega, WI).

RNA isolation and cDNA synthesis
Total RNA was isolated from brain and kidney material from patient L.C. and from fibroblasts of the patient’s mother by the acid guanidium thiocyanate-phenol-chloroform extraction procedure described by Chomczynski and Sacchi (16) and subsequently used to prepare cDNA (17).
**PCR**

**3-ketoacyl-CoA thiolase**
The cDNA encoding 3-ketoacyl-CoA thiolase was amplified by PCR in two overlapping fragments. The first fragment (bases -58 to 848) was amplified with the primers THIOF-58 (5'-TGT TAA CTC CGC GGT CAG TTC CCG GAC TGG-3') and THIOR 484 (5'-CCA GGG TTC CCT CTG TCA GCC AGG GAC ATG-3'), and the second fragment (403-1326) was amplified with the primers THIOF 403 (5'-GTG GCA TCA GAA ATG GGT CTT ATG ACA TTG-3') and THIOR 1326 (5'-GCT GCT AGA GCA GCA GGA CTG TCT GCG TAG-3').

**DBP**
The cDNA encoding DBP was amplified by PCR in three overlapping fragments by means of three primer sets tagged with either -21M13 (5'-tgtaaacgacgcagt-3') or universal M13rev (5'-cagaaacagt acc-3') extensions. The first fragment (bases -48 to 806) was amplified with the primers -21MDBP -48 (5'-[21M13]-GGC CAG CGC GTC TGG TTC TT-3') and M13RDBP 806 (5'-[M13rev]-ACT GCC TCA GGA GTC ATT GG-3'), the second fragment (bases 675 to 1543) was amplified with the primers -21MDBP 675 (5'-[21M13]-TTG TCA CGA GAG TTG TGA GG-3') and M13RDBP 1543 (5'-[M13rev]-GTA AGG GAT TCC AGT CTC CAC-3') and the third fragment (bases 1489 to 2313) was amplified with the primers -21MDBP 1489 (5'-[21M13]-ACC TCT CTT AAT CAG GCT GC-3') and M13RDBP 2313 (5'-[M13rev]-CCC TGC ATC TTA GTT CTA ATC AC-3').

For sequence analysis, the 3' end of intron 2, exon 3 and intron 3 were amplified by PCR with the primers -21MDBPIS2 -55F (5'-[21M13]-CAC ATT TTG AAA GTC TAG AA-3') and M13DBPIS3+E4 (5'-[M13rev]-CAC CTA TTC TTC CAA AAG CAT CC-3').

**Sequencing**
PCR fragments were sequenced in both directions either by means of -21M13 and M13rev fluorescent primers or by means of big dye-deoxy terminators (Applied Biosystems, CA) on an Applied Biosystems 377A automated DNA sequencer according to the manufacturer's protocol (Perkin Elmer, CA).

**Enzyme activity measurements**
The activity of DBP in cultured skin fibroblasts of the patient's parents were measured as described in (9).

**Results**

**Molecular analysis of peroxisomal 3-ketoacyl-CoA thiolase**
Patient L.C. is the only patient reported to suffer from a deficiency of peroxisomal 3-ketoacyl-CoA thiolase (3). This was concluded from immunoblot experiments which revealed the normal presence of SCOX and LBP, but no 3-ketoacyl-CoA thiolase in post-
mortem liver material from the patient. To determine whether this thiolase deficiency is caused by mutations in the gene encoding 3-ketoacyl-CoA thiolase, we sequenced the cDNA amplified by RT-PCR from RNA isolated from post-mortem brain and kidney material from the patient and a control subject. Unfortunately no liver material from the patient could be used for this study, because the liver samples used for the studies described in Schram et al. (3) were not available anymore. No mutations were identified by sequence analysis of the cDNA encoding the thiolase from both brain and kidney in the patient.

![Table and Images]

**Fig. 2** Immunoblot analysis in post-mortem kidney and brain of a control subject (indicated by C), a patient suffering from Zellweger syndrome (indicated by Z) and patient L.C. (indicated by P). Antibodies were used against (A) peroxisomal 3-ketoacyl-CoA thiolase (THIO), (B) D-bifunctional protein (DBP), (C) L-bifunctional protein (LBP), (D) straight-chain acyl-CoA oxidase (SCOX), and (E) sterol carrier protein X (SCPx). In (A) the arrowheads indicate the 44 kDa precursor form and the 41 kDa mature form of 3-ketoacyl-CoA thiolase. In (B) the arrowheads indicate the 79 kDa full-length protein, the 45 kDa enoyl-CoA hydratase component of DBP and the 35 kDa 3-hydroxyacyl-CoA dehydrogenase component of DBP. In (C) the arrowhead indicates the 79 kDa full-length LBP. In (D) the arrowheads indicate the 70, 50 and 20 kDa components of SCOX. In (E) the arrowheads indicate the 58 kDa full-length protein and the 46 kDa thiolase component of SCPx.

**Biochemical reinvestigation**

The absence of mutations in the cDNA encoding the thiolase in conjunction with the current view that peroxisomes contain two sets of β-oxidation enzymes, prompted us to reinvestigate the patient at the biochemical level. To this end, we performed immunoblot experiments using antibodies against the different enzymes, except BCOX since no antibody against this enzyme was available. The results are shown in **Fig. 2**. In contrast to the previous data in liver, the mature 41 kDa form of 3-ketoacyl-CoA thiolase was normally present in both brain and kidney from patient L.C. (Fig. 2A). Also the other peroxisomal thiolase, SCPx, as well as the 70, 50 and 20 kDa components of SCOX and
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LBP were normally present. DBP, however, was deficient in brain from patient L.C., while it was normally present in brain of the control subject. The full-length protein of 79 kDa was not detectable, as well as the two proteolytically processed polypeptides: the 45 kDa band corresponding to the enoyl-CoA hydratase component of DBP and the 35 kDa band corresponding to the 3-hydroxyacyl-CoA dehydrogenase component of DBP. In kidney, no DBP could be detected in both the control subject and patient L.C. (Fig. 2B).

Since no skin fibroblasts of patient L.C. were available, we measured DBP activity in fibroblasts of the patient's parents and found a partially reduced activity (Table 1), which is in agreement with heterozygosity for DBP deficiency.

Table 1 Activity measurements of the enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase component of D-bifunctional protein in fibroblasts of the patient’s parents and control subjects.

<table>
<thead>
<tr>
<th>Activity measured</th>
<th>Mother of patient L.C.</th>
<th>Father of patient L.C.</th>
<th>Controls (n = 24)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pmol/min/mg</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hydratase (formation of 24-OH-THC-CoA)</td>
<td>86</td>
<td>127</td>
<td>240 ± 65</td>
</tr>
<tr>
<td>Dehydrogenase (formation of 24-keto-THC-CoA)</td>
<td>10</td>
<td>26</td>
<td>73 ± 33</td>
</tr>
</tbody>
</table>

n = number of controls; *mean value ± SD

Resolution of the molecular basis of DBP deficiency

To confirm the apparent DBP deficiency in patient L.C. at the molecular level, we amplified the cDNA encoding DBP from brain and kidney by PCR in three overlapping fragments and subsequently sequenced the PCR products. We found a homozygous deletion of base pair 113 through base pair 220, corresponding to exon 3 of the DBP gene (18). We also analyzed the cDNA encoding DBP in fibroblasts of the patient's mother and found a heterozygous deletion of exon 3. To determine the cause of skipping of exon 3 in patient L.C., the 3' end of intron 2, exon 3 and intron 3 of the DBP gene was amplified from brain and kidney DNA and the PCR products subsequently sequenced. This revealed a deletion of 138 base pairs, encompassing base pair 145 through base pair 220 of exon 3 and the first 63 base pairs of intron 3 (Fig. 3). In fibroblasts from the parents of the patient the same deletion was identified in heterozygous form.

Discussion

The data presented in this paper show that the true defect in the only patient documented with a deficiency of 3-ketoacyl-CoA thiolase is at the level of DBP. No DBP protein could be detected by immunoblot analysis in brain of the patient, whereas 3-ketoacyl-CoA thiolase was normally present. These results were confirmed by cDNA analysis in brain and kidney. The cDNA encoding 3-ketoacyl-CoA thiolase was completely normal, whereas the patient had a homozygous deletion of exon 3 in DBP cDNA. Studies at the genomic level revealed that skipping of exon 3 in this patient is caused by a deletion of part of exon 3 and the 5' end of intron 3. The parents of the patient, who were
consanguineous, are heterozygous for this deletion, which results in a partially reduced DBP activity as measured in their fibroblasts. Exon 3 consists of 108 base pairs and skipping of this exon leads to an in-frame deletion of 36 amino acids. Since neither the full-length 79 kDa band nor the 45 and 35 kDa bands, corresponding to the enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase components of DBP respectively, were present in brain material from the patient, the mutated protein is probably unstable and rapidly degraded.

![Diagram](image)

Fig. 3 (A) Schematic representation of exon 2-4 of D-bifunctional protein (DBP) and the intervening intron sequences. The deletion in the DBP gene in patient L.C. is indicated (from bp 145 in exon 3 through the first 63 bps of intron 3). This was determined by amplifying part of the DBP gene with the primers -21MDBPIVS2 -55F and M13DBPIVS3+E4, which are depicted, and subsequent sequencing of the PCR products. The deletion on the genomic level results in skipping of exon 3 at the cDNA level. (B) Products of amplification of the DBP gene with the primers -21MDBPIVS2 -55F and M13DBPIVS3+E4 in brain of a control subject (C), the patient’s father (F), the patient’s mother (M) and patient L.C. (P).

Our immunoblot experiments showed the normal presence of 3-ketoacyl-CoA thiolase in kidney and brain from patient L.C., which is in contrast with the earlier data from Schram et al. (3) showing the absence of thiolase in liver. The most likely explanation for these discrepant results is that the quality of the liver material used by Schram et al. (3), which was obtained post-mortem, was very poor. Unfortunately, this possibility cannot be investigated, since this liver material is no longer available.

The first patient with a deficiency of DBP was described in 1997, 10 years after the reported thiolase deficiency in patient L.C. (8). Since then several other cases of DBP deficiency have been reported in literature (reviewed in (5)) and to date DBP deficiency constitutes one of the most frequently occurring single peroxisomal enzyme deficiency disorders. The clinical as well as the biochemical abnormalities in patient L.C. were similar to those reported in patients with an established DBP deficiency. The mutation identified
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in patient L.C. has not been reported before. Our findings have great implications, since they imply that the group of single peroxisomal β-oxidation enzyme deficiencies is limited to SCOX (19), DBP (8-11) and α-methylacyl-CoA racemase deficiency (20), and that 3-ketoacyl-CoA thiolase deficiency is no longer a distinct disease entity. To conclude, this study stresses the importance of reinvestigation of patients that have been described in literature with an unknown defect of peroxisomal β-oxidation now that the knowledge of the peroxisomal β-oxidation system and the enzymes involved has improved greatly in recent years. The elucidation of the true defect in these patients will further increase our understanding of the peroxisomal β-oxidation system and its substrates, and will be important for prenatal diagnosis in this group of patients.

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References


