Peroxisomal D-hydroxyacyl-CoA dehydrogenase deficiency: Resolution of the enzyme defect and its molecular basis in bifunctional protein deficiency

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ABSTRACT   Peroxisomes play an essential role in a number of different metabolic pathways, including the β-oxidation of a distinct set of fatty acids and fatty acid derivatives. The importance of the peroxisomal β-oxidation system in humans is made apparent by the existence of a group of inherited diseases in which peroxisomal β-oxidation is impaired. This includes X-linked adrenoleukodystrophy and other disorders with a defined defect. On the other hand, many patients have been described with a defect in peroxisomal β-oxidation of unknown etiology. Resolution of the defects in these patients requires the elucidation of the enzymatic organization of the peroxisomal β-oxidation system. Importantly, a new peroxisomal β-oxidation enzyme was recently described called D-bifunctional protein with enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase activity primarily reacting with α-methyl fatty acids like pristanic acid and di- and trihydroxycholestanoic acid. In this patient we describe the first case of D-bifunctional protein deficiency as resolved by enzyme activity measurements and mutation analysis. The mutation found (Gly^{16}Ser) is in the dehydrogenase coding part of the gene in an important loop of the Rossman fold forming the NAD$^+$-binding site. The results show that the newly identified D-bifunctional protein plays an essential role in the peroxisomal β-oxidation pathway that cannot be compensated for by the L-specific bifunctional protein.

Although peroxisomes were initially believed to play only a minor role in mammalian metabolism, it is now clear that they catalyze essential reactions in a number of different metabolic pathways and thus play an indispensable role in intermediary metabolism.

The importance of peroxisomes in humans is made apparent by the existence of a group of inherited diseases, the peroxisomal disorders, caused by an impairment in one or more peroxisomal functions. The cerebro-hepato-renal (Zellweger) syndrome is generally considered to be the prototype of this group of diseases. Patients with this disease lack morphologically distinguishable peroxisomes leading to the loss of virtually all peroxisomal functions. Clinically, patients with Zellweger syndrome show a large variety of severe abnormalities often leading to early death (1, 2).

The metabolic pathways in which peroxisomes are involved include the biosynthesis of ether phospholipids and bile acids, the β-oxidation of fatty acids, the α-oxidation of phytanic acid, the synthesis of cholesterol and other isoprenoids, the detoxification of glyoxylate, and the synthesis of docosahexaenoic acid (3, 4).

One of the most important functions of peroxisomes is the β-oxidation of fatty acids and fatty acid derivatives (4, 5). Fatty acid oxidation in peroxisomes differs from that in mitochondria in many respects, although the overall β-oxidative mechanisms are the same. In both organelles, fatty acid oxidation proceeds via a sequence of four steps involving α,β-dehydrogenation, hydration, 3-hydroxyacyl-CoA dehydrogenation, and, finally, thiolytic cleavage. An important difference is that the two β-oxidation systems display different substrate specificities. Indeed, the major dietary fatty acids including palmitate, oleate, and linoleate are oxidized in mitochondria, whereas peroxisomes are involved in the β-oxidation of a range of minor fatty acids including very-long-chain fatty acids (notably C26:0), and 2-methyl branched-chain fatty acids like pristanic acid. Furthermore, peroxisomes are the sole site of di- and trihydroxycholestanoic acid (DHC and THC) β-oxidation. β-Oxidation of the latter two cholestanoic acids results in the formation of the primary bile acids chenodeoxycholic acid and cholic acid, respectively (4, 5).

Several peroxisomal disorders have been described in which peroxisomal fatty acid β-oxidation is defective. These include X-linked adrenoleukodystrophy (6), the most common peroxisomal disorder, as well as pseudo-neonatal adrenoleukodystrophy due to straight-chain acyl-CoA oxidase deficiency (7), L-bifunctional protein deficiency (8), and pseudo-Zellweger syndrome due to a deficiency of 41-kDa peroxisomal thiolase (9, 10). Apart from these disorders with a defined defect in peroxisomal β-oxidation, many patients have been described with a defect in peroxisomal β-oxidation of unknown etiology (see references 52–59 in ref. 1).

One of the major reasons for the difficulty in resolving the underlying defect in these patients has been our insufficient knowledge about the functional organization of the peroxisomal β-oxidation system. Recent studies, however, have shed new light on the enzymology of the peroxisomal system. Indeed, it is now clear that multiple enzymes are present for each of the β-oxidation steps. The existence of multiple acyl-CoA oxidases had already been established (see ref. 4 for

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Abbreviations: DHC, dihydroxycholestanoic acid; THC, trihydroxycholestanoic acid.
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review). Recently, however, a new peroxisomal β-oxidation enzyme was discovered containing both enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase activity (11–15), which was first identified as a 17β-estradiol dehydrogenase (16, 17). Structural analysis of this protein has revealed three functional domains. The N-terminal part (amino acids 1–323) contains the 3-hydroxyacyl-CoA dehydrogenase activity, whereas the central part (amino acids 324–596) harbors the 2-enoyl-CoA hydratase activity. The C-terminal part (amino acids 597–737) shows strong homology with sterol carrier protein and catalyzes the *in vitro* transfer of 7-dehydrocholesterol and phosphatidylcholine between membranes (11).

In contrast to the well known L-bifunctional protein (18) that converts trans-enoyl-CoA thioesters to their 3-keto forms via the L-hydroxy-stereoisomer, this new bifunctional enzyme catalyzes these same transformations via D-hydroxyacyl-CoAs, which has prompted Hashimoto and coworkers (14, 15) to name it D-bifunctional enzyme.

An important finding is that the two bifunctional proteins have different substrate specificities. The D-bifunctional protein catalyzes the formation of 3-ketoacyl-CoA intermediates from both straight-chain and 2-methyl-branched-chain fatty acids, whereas the L-specific bifunctional protein is incapable of forming 3-ketoacyl-CoA intermediates from 2-methyl-branched substrates because the dehydrogenase component is virtually inactive toward the 2-methyl-3-hydroxy diastereomer formed in the previous hydration reaction (12–15).

These new findings have guided the way toward unraveling the defect in some patients suffering from a disorder of peroxisomal β-oxidation of unknown origin. In this paper, we describe a patient with a defect in the 3-hydroxyacyl-CoA-dehydrogenase component of the D-bifunctional protein. We also describe the molecular basis of this enzyme defect in this new type of peroxisomal disorder.

CASE REPORT

The patient, a boy, is the first child of Caucasian, nonconsanguineous parents and has not been described in literature before. He was delivered at 36 weeks of gestation after an uncomplicated pregnancy. At birth his weight was 2,745 g and his head circumference was at the 50th percentile, but macrocephaly developed during the first year of life. Physical examination revealed several dysmorphic features: a high forehead with frontal bossing, low-set ears, and a large fontanel. The liver was palpable 2.5 cm below the costal margin. In addition, a long, small thorax, hypospadias, muscle wasting (atrophy–dystrophy limb-girdle-type), and general hypotonia were noted. Neurological examination showed also negative traction and Moro response, with maximal headlag (at 4 weeks). In vertical suspension, there was slipping through. At the age of 2 months, the patient became cyanotic and developed epileptic seizures leading to aspiration. MRI of the brain showed white matter abnormalities consistent with dysmyelination. The patient died at the age of 16 months from an aspiration pneumonia.

EXPERIMENTAL PROCEDURES

Fibroblast Cultures. Fibroblasts were grown from skin biopsy specimens according to established procedures described in detail before (19).

Determination of Plasma Metabolites and Erythrocyte Plasmalogens. Very-long-chain fatty acids, bile acids, phytanic acid, and pristanic acid were measured in plasma by using gas chromatographic procedures as described in detail in (19). Erythrocyte plasmalogen levels were measured as described by Björkhem *et al.* (20).

Peroxisomal Functions in Cultured Skin Fibroblasts. *De novo* plasmalogen synthesis, peroxisomal C26:0 and pristanic

acid β-oxidation, DHAPAT-activity, catalase immunofluorescence, and very-long-chain fatty acids were measured as described before (19).

Measurement of the Enoyl-CoA Hydratase and 3-Hydroxyacyl-CoA Dehydrogenase Components of D-Bifunctional Protein. The combined activity of the enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase components of the D-bifunctional protein were measured in a medium of the following composition: 50 mM Tris·HCl (pH 8.5), 1 mM NAD$^+$, 150 mM KCl, 0.1 mM (24E)-3α,7α,12α-trihydroxy-5β-cholest-24-enoyl-CoA (24-ene-THC-CoA; prepared as described in ref. 21), 5 mM pyruvate, and 18 units/ml lactate dehydrogenase. Reactions were allowed to proceed for 60 min at 37°C by using a protein concentration of 150 μg/ml. Reactions were terminated by addition of 2 M HCl to a final concentration of 0.18 M followed by neutralization to a pH of about 5.0 by using 0.6 M Mes plus 2 M KOH. Resolution of the different CoA-esters was achieved essentially as described by the method of Xu and Cuebas (21).

RNA Isolation and cDNA Synthesis. Total RNA was isolated either from cultured skin fibroblasts (stored at −80°C) or from freshly prepared lymphocytes by using the acid guanidinium thiocyanate-phenol-chloroform extraction procedure described by Chromczynski and Sacchi (22) and subsequently used to prepare cDNA (23).

PCR and Sequencing. The cDNA encoding the D-bifunctional protein was amplified in three overlapping fragments by using primers with −21M13 (−21M) or universal M13 (M13R) extensions. Fragment 1 corresponds to bases −48–806 of the cDNA (ATG = 1; ref. 24) and was amplified with primers −21MHSD-48 (5′-tgt aaa acg acg gcc agt GGC CAG CGC GTC TGC TTG TTC-3′) and M13RHSD806 (5′-cag gaa aca gct atg acc ACT GCC TCA GGA GTC ATT GG-3′). Fragment 2, corresponding to bases 675-1543 of the cDNA, was amplified with primers −21MHSD675 (5′-tgt aaa acg acg gcc agt TTG TCA CGA GAG TTG TGA GG-3′) and M13RHSD1543 (5′-cag gaa aca gct atg acc GTA AGG GAT TCC AGT CTC CAC-3′). Amplification of fragment 3, corresponding to bases 1489–2313 of the cDNA, was done with primers −21MHSD1489 (5′- tgt aaa acg acg gcc agt ACC TCT CTT AAT CAG GCT GC-3′) and M13RHSD2313 (5′- cag gaa aca gct atg acc CCC TGC ATC TTA GTT CTA ATC AC-3′).

PCR mixtures (25 μl final volume) contained 2–5 μl cDNA, 10 mM Tris·HCl (pH 8.4), 50 mM KCl, 0.1 mg/ml BSA, oligonucleotide primers as indicated (12.5 pmol each), 1.5–2.0 mM MgCl$_2$, and 2.5 units *Taq* polymerase. Thermocycling conditions consisted of 120 s at 96°C initial to cycling, 30 cycles of 30 s at 94°C, 30 s at 55°C, and 90 s at 72°C, followed by 10 min of extension at 72°C.

Sequence analysis of PCR fragments was performed after purification of the fragments from the PCR mixtures by using Prep-A-Gene DNA purification system (Bio-Rad). Both sense and antisense strands were sequenced by using −21M13 and universal M13 (M13rev) fluorescent primers, respectively, on an Applied Biosystems 377A automated DNA sequencer following standard protocols of the manufacturer.

Construction of the Expression Plasmids. Two expression plasmids were constructed for the expression of wild-type and G46A mutated D-bifunctional protein in *Saccharomyces cerevisiae*. The complete cDNA encoding D-bifunctional protein was obtained either from control fibroblasts (wild-type sequence) or from fibroblasts of the index patient homozygous for the G46A mutation, by means of PCR using a low error rate mixture of DNA polymerases (Expand High Fidelity, Boehringer). The following oligonucleotide primers were used for the amplification reaction: sense: 5′-ttt tct aga ATG GGC TCA CCG CTG AGG TTC-3′ (position 1–21 of the cDNA sequence; *Xba*I extended) (24) and antisense: 5′-ttt tct gca gCT TCA GAG CTT GGC GTA GTC-3′ (position 2213–2194; *Pst*I extended). The PCR-amplified cDNA containing a *Xba*I and

*Pst*I restriction site at 5′ and 3′ ends, respectively, was digested with *Xba*I and *Pst*I and cloned into the *Xba*I and *Pst*I restriction sites downstream of the catalase promoter of pEL 26 (25). Both plasmids containing the wild-type (pDBF-WT) and the G46A mutated sequence (pDBF-G46A) of D-bifunctional protein were sequenced completely to exclude PCR artifacts.

Yeast Culture and Expression. The yeast strain used in this study, *S. cerevisiae* BJ1991 (*MAT α, leu2, ura3–251, prb1–1122, pep4–3*), was transformed with pEL26, pDBF-WT, and pDBF-G46A and grown on minimal essential medium containing 0.3% glucose and 0.67% yeast nitrogen base without amino acids (Difco) and appropriate amino acids at 30°C.

To induce expression, the medium was shifted to rich oleic acid medium containing 0.12% oleic acid, 0.2% Tween-40, 0.5% potassium phosphate buffer (pH 6.0), 0.3% yeast extract, and 0.5% peptone. The culture on rich oleic acid medium was inoculated at an OD_{600} of 0.1 and incubated for 17 h at 30°C.

For the measurement of the enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase activities of D-bifunctional protein, cells were resuspended in phosphate-buffered saline (pH 7.4) containing 1 μg/ml leupeptin, 1 mM EDTA, and 1 mg/ml Pefabloc and disrupted by agitation at 4°C for 15 min with a vortex mixer in the presence of glass beads (∅ = 0.45 mm). The homogenates were centrifuged at 2,000 × *g* at 4°C for 2 min, and the supernatants were used for enzyme activity measurements.

RESULTS

Peroxisomal Abnormalities in Plasma and Fibroblasts from the Patient. The index patient showed a variety of clinical signs and symptoms suggestive of a peroxisomal disorder. This included facial dysmorphism, hypotonia, and hepatomegaly (see *Case Report*).

To substantiate the clinical diagnosis, plasma very-long-chain fatty acids and erythrocyte plasmalogen levels, the two principal parameters in the diagnosis of peroxisomal disorders, were measured (see ref. 1 for review). The data in Table 1 show that plasma very-long-chain fatty acids were clearly abnormal both in absolute (C26:0 in μM) and relative (C26:0/C22:0-ratio) terms. Erythrocyte plasmalogen levels were completely normal, suggesting that peroxisomal abnormalities were not

generalized as in Zellweger syndrome but restricted to a defect in peroxisomal β-oxidation.

In line with this notion, we found the accumulation of other fatty acids and fatty acid derivatives known to be oxidized in peroxisomes. This includes the bile acid intermediates di- and trihydroxycholestanoic acid. The latter two cholestanoic acids are β-oxidized in peroxisomes to produce the primary bile acids chenodeoxycholic and cholic acid, respectively (4, 5). In addition, plasma pristanic acid was elevated whereas plasma phytanic acid was normal.

Peroxisomal functions were subsequently studied in fibroblasts from the patient. The results of Table 2 show normal values for *de novo* plasmalogen biosynthesis and dihydroxyacetone phosphate acyltransferase, a peroxisomal enzyme catalyzing the first step in etherphospholipid synthesis. For comparison, Table 2 contains data obtained in fibroblasts of Zellweger patients, which are characterized by the absence of peroxisomes.

β-Oxidation activity, measured with hexacosanoic (C26:0) and pristanic acid as substrates, was clearly deficient in fibroblasts from the patient, especially with pristanic acid as substrate.

Immunofluorescence microscopy studies on the patient's fibroblasts by using antibodies directed against human catalase revealed a punctate pattern of immunofluorescence, suggesting the normal presence of peroxisomes in these cells. This contrasts with the diffuse fluorescence pattern in Zellweger cells reflecting the cytosolic localization of catalase in these cells. Remarkably, the number of immunofluorescent particles was found to be reduced in the patient's cells. Furthermore, the size of these particles is enlarged, which is in line with data from other patients with an isolated β-oxidation defect (7, 9, 26).

Identification of Varanic Acid (3α,7α,12α,24-Tetrahydroxy-5β-Cholestan-27-oic Acid) in Serum from the Patient. Gas chromatographic analysis of plasma bile acids not only revealed the presence of bile acids typically found in Zellweger patients (di- and trihydroxycholestanoic acids) (Table 1) but also an unusual bile acid, at a concentration that exceeded that of DHC and THC. Careful analysis by using gas chromatography/mass spectrometry led to the identification of varanic acid (see ref. 27 for details), which is the 24-hydroxy derivative of 3α,7α,12α-trihydroxy-5β-cholestan-27-oic acid.

Table 1. Biochemical findings in plasma and erythrocytes of the index patient and controls

Parameter measured	Index patient	Controls, mean (5–90% range)
Plasma		
VLCFA		
C26:0, μM	4.60	0.78 (0.27–1.56) $n = 109$
C26:0/C22:0 ratio	0.24	0.01 (0.00–0.02) $n = 109$
Pristanic acid, μM	6.03	<3 $n = 30$
Phytanic acid, μM	4.10	<9 $n = 30$
Bile acid intermediates		
DHC, μM	0.30	ND
THC, μM	1.26	ND
Varanic acid, μM	10.42	ND
Erytrocytes		
% plasmalogens		
C16:0	10.0	10.2 (6.8–11.9) $n = 30$
C18:0	23.4	22.7 (10.6–24.9) $n = 30$

ND, not detectable; VLCFA, very-long-chain fatty acids; THC, trihydroxycholestanoic acid; DHC, dihydroxycholestanoic acid.

Table 2. Biochemical findings in fibroblasts of the index patient, Zellweger patients, and controls

Parameter measured	Index patient	Zellweger (*n*)	Controls (*n*)
De novo plasmalogen biosynthesis			
[³H]/[¹⁴C] in PE	1.3	>6.4 (46)	1.5 ± 0.9 (38)
[³H]/[¹⁴C] in PC	0.7	>3.1 (46)	1.1 ± 0.7 (38)
DHAP-AT activity, nmol/2 h·mg	7.1	0.6 ± 0.5 (23)	8.1 ± 2.5 (78)
VLCFA			
C26:0, nmol/mg protein	1.94	1.44 (0.53–3.05) $n = 66$	0.10 (0.06–0.22) $n = 101$
C26:0/C22:0 ratio	0.83	0.52 (0.21–1.07) $n = 66$	0.03 (0.02–0.05) $n = 101$
Peroxisomal β-oxidation, pmol/h·mg			
C26:0	169	316 ± 384 (28)	902 ± 430 (81)
Pristanic acid	0	16 ± 19 (31)	1030 ± 475 (74)

PE, total phosphatidylethanolamine; PC, total phosphatidylcholine; DHAPAT, dihydroxyacetonephosphate acyltransferase; VLCFA, very-long-chain fatty acids. The control and Zellweger values listed in the table represent mean ± SD (*n* − 1) except for VLCFA, for which the mean and the ranges are given.

Biochemistry: van Grunsven *et al.*

Proc. Natl. Acad. Sci. USA 95 (1998)   2131

Table 3. Analysis of the hydratase and dehydrogenase components of D-bifunctional protein in fibroblasts of the index patient compared with fibroblasts from Zellweger patients and control fibroblasts

	Enzyme activity, nmol/min·mg		
	Index patient	Zellweger	Controls (*n*)
Enoyl-CoA hydratase	0.45	ND	0.28 ± 0.02 (10)
D-hydroxyacyl-CoA			
dehydrogenase	ND	ND	0.11 ± 0.02 (10)

ND, not detectable. The control values listed in the table represent mean ± SD (*n* − 1).

The 3-Hydroxyacyl-CoA Dehydrogenase Component of the Newly Identified D-Specific Bifunctional Protein but Not the Hydratase Component Is Deficient in the Patient as Concluded from Enzyme Activity Measurements in Fibroblasts. The accumulation of varanic acid in the patient's plasma suggested the possibility that the defect in peroxisomal β-oxidation in the patient would be at the level of the 3-hydroxyacyl-CoA dehydrogenase responsible for the conversion of 24R,25R-varanoyl-CoA to 24-keto-THC-CoA. Recent studies have shown that in contrast to the generally held view, the conventional L-specific bifunctional enzyme is not capable of catalyzing the formation of 24-keto-THC-CoA from 24-ene-THC-CoA (15, 21), which is in fact catalyzed by the newly identified D-specific bifunctional protein (12, 15). We have studied the hydration and subsequent dehydrogenation of 24-ene-THC-CoA to its 24-keto ester in cultured skin fibroblasts by using HPLC, which allows discrimination between the α,β-unsaturated, 24-OH- and 24-keto-acyl-CoA esters of THC (21). The data are shown in Table 3. In control fibroblasts, 24R,25R-varanoyl-CoA and 24-keto-THC-CoA are readily formed from the corresponding enoyl-CoA ester. In the absence of NAD$^+$, no 24-keto-THC-CoA was formed as expected (results not shown). In fibroblasts from a Zellweger patient, formation of both 24-OH- and 24-keto-THC-CoA was deficient. The enzyme activity in fibroblasts from the index patient showed normal formation of 24R,25R-varanoyl-CoA but *no* production of 24-keto-THC-CoA, suggesting that the hydratase part of the D-specific bifunctional protein was normally active, whereas the 3-hydroxyacyl-CoA dehydrogenase component was completely inactive.

Identification of the Molecular Defect in the Patient. To find conclusive evidence for a deficiency of the 3-OH-acyl-CoA


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TACTGG TCACCGGCGCGGGGGCA
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control

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TACTGG TCACCAGCGCGGGGGCA
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patient

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TACTGG TCACCRGCGCGGGGGCA
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father

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TACTGG TCACCRGCGCGGGGGCA
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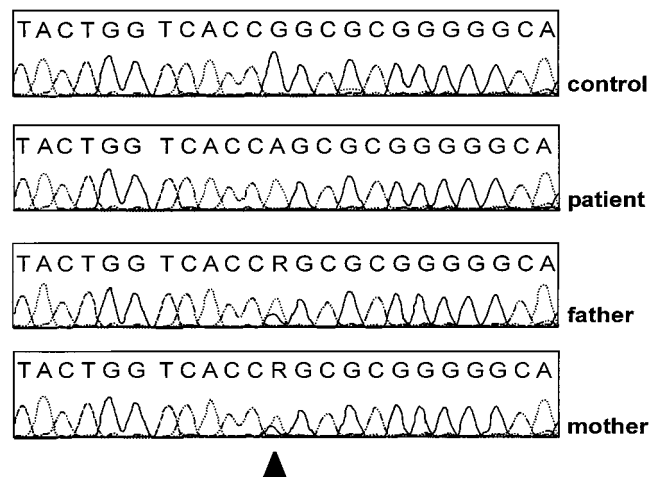mother

FIG. 1. Mutation analysis of D-bifunctional protein. Electropherograms show that the patient is homozygous for the G-to-A transition at base 46. Both the father and the mother are heterozygous for this mutation.

Table 4. Expression of D-bifunctional protein in *S. cerevisiae*

	Enzyme activity, nmol/min·mg		
	pEL26	pDBF-WT	pDBF-G46A
Enoyl-CoA hydratase	0.59	6.29	1.55
D-hydroxyacyl-CoA			
dehydrogenase	ND	8.49	ND

ND, not detectable; pEL26, expression plasmid; pDBF-WT, pEL26 containing the open reading frame of the wild-type D-bifunctional protein gene; pDBF-G46A, pEL26 containing the open reading frame of the mutant D-bifunctional protein gene.

dehydrogenase component of D-bifunctional protein, cDNA was prepared from total RNA isolated from human skin fibroblasts. The cDNA encoding the D-bifunctional protein was amplified by PCR by using three primer sets based on the published human cDNA sequence (24). The resulting overlapping PCR fragments were of the predicted size, showed no gross abnormalities, and were subsequently sequenced.

Sequence analysis of the patient's cDNA revealed a single G-to-A mutation at base 46 (Fig. 1). The G46A mutation changes the codon for Gly to Ser at amino acid 16. The analysis of cDNA suggested that the patient was homozygous for the G46A mutation. To study whether the patient was truly homozygous for the G46A mutation or heterozygous with the other allele being a null allele, sequence analysis of cDNA prepared from blood cells from the parents was subsequently carried out. Fig. 1 shows that both parents are heterozygous for the Gly[16]Ser mutation.

Expression of Wild-Type and Mutant D-Bifunctional Protein in *S. cerevisiae*. To establish whether the G46A mutation results in an inactive enzyme, both wild-type (pDBF-WT) and mutant enzyme (pDBF-G46A) were expressed in *S. cerevisiae* (Table 4). Wild-type yeast [transformed with the empty expression plasmid (pEL26)] was found to contain a small amount of enoyl-CoA hydratase activity as measured with 24-ene-THC-CoA as substrate, whereas no 3-hydroxyacyl-CoA dehydrogenase activity could be measured. This activity most likely results from the FOX2 protein (28). Upon expression of the wild-type D-bifunctional protein, a strong increase of both the enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase activity was found with 24-ene-THC-CoA as substrate. When the mutated D-bifunctional protein carrying the Gly[16]Ser mutation was expressed, there was clear 24-ene-THC-CoA hydratase activity, whereas formation of 24-keto-THC-CoA was completely lacking. These data show that the 3-hydroxyacyl-CoA dehydrogenase component of D-bifunctional protein is inactive as a result of the Gly[16]Ser mutation with preservation of the activity of the enoyl-CoA hydratase component of D-bifunctional protein. The lower rate of varanoyl-CoA formation observed with the mutated D-bifunctional protein (Table 4) turned out to be due, at least in part, to a lower level of expression of the mutated D-bifunctional protein.

DISCUSSION

Recent studies have shown that the peroxisomal β-oxidation system is much more complex than originally envisaged. It was already known that peroxisomes contain multiple acyl-CoA oxidases with distinct substrate specificities. These findings have now been extended and have led to the identification of a new bifunctional protein harboring enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase activity, which differs from the conventional bifunctional enzyme in many respects. First, the two bifunctional enzymes have different stereospecificities regarding the β-hydroxyacyl-CoA intermediate. The conventional enzyme is L(S)-specific, whereas the new enzyme is D(R)-specific (12–15). Second, the enoyl-CoA intermediates

generated from α-methyl-branched chain fatty acids such as pristanic and di- and trihydroxycholestanoic acids appear to be metabolized via D-hydroxyacyl-CoA to β-ketoacyl-CoA esters exclusively by the action of the newly identified bifunctional enzyme (12, 15, 21).

In this paper we have used this newly acquired knowledge to resolve the defect in a patient with an unknown defect in peroxisomal β-oxidation. The increased levels of varanic acid suggested that the 3-hydroxyacyl-CoA-dehydrogenase component of the D-bifunctional protein likely could be defective. Subsequent measurements of enzyme activity in fibroblasts from the patient showed no conversion of 24R,25R-varanoyl-CoA to 24-keto-THC-CoA, whereas the enoyl-CoA hydratase activity of D-bifunctional protein was normal (Table 3).

Furthermore, sequence analysis of cDNA showed that the patient carried a point mutation at position 46 (G to A) leading to a Gly[16]Ser substitution. The fact that both parents are heterozygous for this mutation is consistent with the patient being homozygous. The glycine at position 16 is located in an important loop of the Rossman fold forming the NAD^+-binding site of the dehydrogenase coding region of D-bifunctional protein (17). The consensus motive for the NAD^+-binding site is well characterized, and the mutated glycine is best conserved in the dehydrogenase protein family (29). Recent structure–function relationships and crystallization studies confirmed the functionality of this fold (30, 31). A BLAST search using the NAD^+-binding motive in the SWISS-PROT database revealed many different dehydrogenases and oxidases, whereas no dehydrogenase was found with Gly replaced by Ser. This strongly suggests that the glycine at position 16 is important for binding of NAD^+ and cannot be replaced by serine without affecting the 3-hydroxyacyl-CoA dehydrogenase activity. Indeed, expression studies showed that the G46A mutation causes the loss of the 3-hydroxyacyl-CoA dehydrogenase activity, whereas the enoyl-CoA hydratase activity is not affected (Table 4).

Several studies have indicated that L-bifunctional protein is involved in the degradation of straight-chain fatty acids (12, 13). Therefore, it is very striking that this patient, who is deficient in D-bifunctional enzyme, accumulates straight-chain fatty acids (VLCFA) both in plasma and fibroblasts (Table 1 and 2). A possible explanation could be that there is competitive inhibition of L-bifunctional protein by the accumulating intermediates, notably 24-ene-THC-CoA and/or varanoyl-CoA. Furthermore, CoA depletion caused by the accumulation of D-hydroxyacyl-CoA esters could be the underlying reason for the insufficient degradation of straight-chain fatty acids.

It should be noted that the newly identified enzyme is also highly active toward the enoyl-CoA esters of straight-chain fatty acids. Accordingly, it might well be that the D-specific enzyme is also the main enzyme involved in the β-oxidation of very-long-chain fatty acids. Such studies are now underway in our laboratories.

Using the approach described in this paper, including enzymatic analysis of the D-specific bifunctional protein in fibroblasts followed by mutation analysis in cDNA, we are now studying all patients with a defect in peroxisomal β-oxidation of unknown etiology. Preliminary studies show that D-bifunctional protein deficiency is very frequent among these patients. Furthermore, of the four patients with deficiency of the 3-hydroxyacyl-CoA component of D-bifunctional protein, all were found to be heterozygous for the G46A mutation, with another mutation on the other allele. Resolution of the full molecular basis is in progress.

In the past many patients with bifunctional enzyme deficiency have been identified by means of complementation analysis (32–38) by using fibroblasts from the patient described by Watkins *et al.* (8) with L-bifunctional protein deficiency as the reference cell line. We have also performed complemen-

tation analysis and found that cells from the patient described in this paper and the bifunctional protein-deficient patient do *not* complement. This is a remarkable and unexpected finding. Although one has to be extremely careful in the interpretation of results from complementation studies, these data suggest that the original patient described by Watkins *et al.* (8) is deficient in the D-bifunctional protein rather than the L-bifunctional protein. This is now under active study in collaboration with the group of Watkins and Moser.

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