methods

A novel HPLC-based method to diagnose peroxisomal D-bifunctional protein enoyl-CoA hydratase deficiency

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Abstract D-bifunctional protein (D-BP) plays an indispensable role in peroxisomal β-oxidation, and its inherited deficiency in humans is associated with severe clinical abnormalities. Three different subtypes of D-BP deficiency can be distinguished: *1*) a complete deficiency of D-BP (type I), *2*) an isolated D-BP enoyl-CoA hydratase deficiency (type II), and *3*) an isolated D-BP 3-hydroxyacyl-CoA dehydrogenase deficiency (type III). In this study, we developed a method to measure D-BP dehydrogenase activity independent of D-BP hydratase (D-BP HY) activity to distinguish between D-BP deficiency type I and type II, which until now was only possible by mutation analysis. For this assay, the hydratase domain of D-BP was expressed in the yeast *Saccharomyces cerevisiae*. After a coincubation of yeast homogenate expressing D-BP HY with fibroblast homogenate of patients using the enoyl-CoA ester of the bile acid intermediate trihydroxycholestanoic acid as substrate, D-BP dehydrogenase activity was measured. Fibroblasts of patients with a D-BP deficiency type II displayed D-BP dehydrogenase activity, whereas type I and type III patients did not. This newly developed assay to measure D-BP dehydrogenase activity in fibroblast homogenates provides a quick and reliable method to assign patients with deficient D-BP HY activity to the D-BP deficiency subgroups type I or type II.—Gloerich, J., S. Denis, E. G. van Grunsven, G. Dacremont, R. J. A. Wanders, and S. Ferdinandusse. **A novel HPLC-based method to diagnose peroxisomal D-bifunctional protein enoyl-CoA hydratase deficiency. *J. Lipid Res.* 2003.** 44: **640–644.**

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In humans, both mitochondria and peroxisomes are able to β-oxidize fatty acids. Peroxisomal β-oxidation is essential for the oxidation of a distinct set of substrates that cannot be broken down by the mitochondrial β-oxidation system. Substrates of the peroxisomal β-oxidation system include *1*) very long chain fatty acids, *2*) α-methyl-branched chain fatty acids such as pristanic acid, and *3*) the bile acid intermediates dihydroxycholestanoic acid and trihydroxycholestanoic acid (THCA) [as reviewed in ref. (1)]. Peroxisomal β-oxidation is impaired in peroxisomal biogenesis disorders, as well as in patients with a single enzyme defect in the peroxisomal β-oxidation system (2). One of the single enzyme defects in peroxisomal β-oxidation is a deficiency of D-bifunctional protein (D-BP; also known as MFE-2, MFP-2, D-PBE). The enoyl-CoA hydratase (D-BP HY) and 3-hydroxyacyl-CoA dehydrogenase (D-BP DH) domains of D-BP catalyze the second and third step of peroxisomal β-oxidation, respectively. D-BP deficiency can be divided into three subgroups. In the first group, the patients have a deficiency in both the hydratase and dehydrogenase component of D-BP (type I) (3, 4). In the second group, only the hydratase component of D-BP (type II) is deficient (5), and in the third group, only the dehydrogenase component of D-BP is deficient (type III) (3).

In the assay that is currently used to diagnose D-BP deficiency (6), D-BP activity is measured by incubating fibroblast homogenates with the enoyl-CoA ester of the bile acid intermediate THCA (THC:1-CoA). From this substrate, 24-hydroxy-THC-CoA is produced by the action of D-BP HY, which is subsequently converted into 24-keto-THC-CoA via the dehydrogenase component of D-BP. With this assay, however, no distinction can be made between D-BP deficiency type I and II, since the substrate for D-BP DH cannot be synthesized by the defective hydratase component of the patient's D-BP.

Until now, the only way to differentiate between these two types of D-BP deficiency was by mutation analysis. To be able to distinguish between these two types of D-BP deficiency in a less time-consuming and laborious way, we have developed a method to measure D-BP DH activity in

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Abbreviations: D-BP, D-bifunctional protein; D-BP DH, D-BP 3-hydroxyacyl-CoA dehydrogenase; D-BP HY, D-BP enoyl-CoA hydratase; THCA, trihydroxycholestanoic acid; PTS, peroxisomal targeting signal.

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fibroblast homogenates of patients with a defective D-BP HY based on the original D-BP assay described above. In our newly developed assay, the inability to form 24-hydroxy-THC-CoA because of the patient's defective D-BP HY is overcome by adding wild-type D-BP HY expressed in yeast to the reaction mixture. The 24-hydroxy-THC-CoA formed is used as substrate for the patient's D-BP DH. This way, formation of 24-keto-THC-CoA by D-BP DH can be measured despite of D-BP HY deficiency. In this paper, we have assigned several patients with D-BP deficiency to the D-BP deficiency subgroups type I or type II with the help of our newly developed method.

MATERIALS AND METHODS

Patient cell lines

All patient cell lines used in this study were taken from the cell repository of the laboratory for Genetic Metabolic Diseases (Academical Medical Center, University of Amsterdam, The Netherlands) and were derived from patients diagnosed in this Center. Informed consent was obtained from parents or guardians of the patients whose fibroblasts were studied in this article.

D-BP enoyl-CoA hydratase expression in yeast

An expression vector containing the coding sequence of the human wild-type enoyl-CoA hydratase domain of D-BP was used for the D-BP HY expression in yeast. This plasmid (pHY-WT) was constructed as described (5). The yeast strain used in this study, *Saccharomyces cerevisiae* (*MATα*, *leu2*, *ura3-251*, *prb1-1122*, *pep4-3*, *gal2*), was transformed with pHY-WT using the lithium acetate procedure (7). Transformed yeast cells were grown at 28°C on minimal essential medium [0.67% yeast nitrogen base without amino acids (YNB-WO, Difco Laboratories Inc., Detroit, MI), 0.3% glucose and amino acids (20–30 μg/ml) as required]. To induce expression, cells were shifted to rich oleic acid medium (50 mM potassium phosphate, pH 6.0, 0.3% yeast extract, 0.5% peptone, 0.12% oleic acid, and 0.2% Tween-80).

Cells were harvested by centrifugation and washed twice with water. Yeast homogenates were made by resuspending cells in lysis buffer containing 40% (v/v) glycerol and 1 tablet-10 ml Complete mini protease inhibitor cocktail (Roche, Basel, Switzerland) in PBS, and subsequently disrupting cells by agitation at 4°C for 15 min on a vortex mixer in the presence of glass beads (Ø = 0.45 mm). The homogenate was centrifuged at 2,000 *g* at 4°C for 2 min and the supernatant was kept at −20°C until used for enzyme activity measurements.

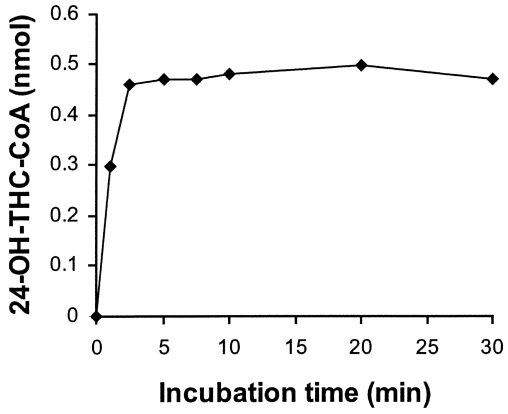

Fig. 1. Activity of D-BP enoyl-CoA hydratase (D-BP HY) in homogenates of yeast cells transformed with the wild-type D-BP HY domain. The total amount of 24-hydroxy-THC-CoA formed (nmol) is depicted against reaction time in minutes.

D-BP activity measurements

The activity of the enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase components of D-BP were measured as described by Van Grunsven, with minor modifications (6). Briefly, fibroblast homogenates were incubated for 30 min at 37°C in a reaction mixture (total volume 100 μl) containing 50 mM Bis-Tris Propane (BTP) pH 9.0, 1 mM NAD^+, 150 mM KCl, 0.1 mM 3α,7α,12α-trihydroxy-5β-cholest-24-enoyl-CoA [THC:1-CoA, prepared as described by Xu and Cuebas (8)], 0.5 mM oxaloacetate, and 0.5 U/ml malate dehydrogenase. The final protein concentration was 0.3 mg/ml. Reactions were terminated by the addition of 2 M HCl to a final concentration of 0.18 M, followed by neutralization to a pH of ∼5 by addition of 0.6 M MES plus 2 M KOH. After addition of acetonitril to the samples, resolution of the different CoA-esters was achieved by High Performance Liquid Chromatography (HPLC).

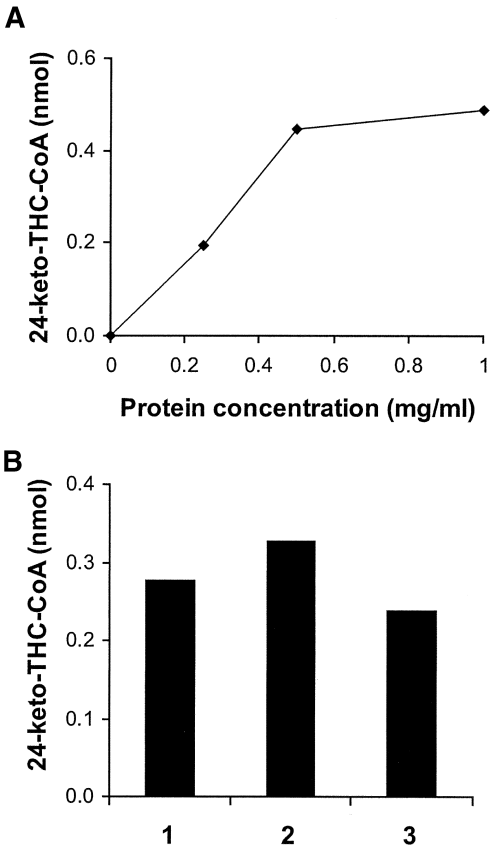

Fig. 2. The formation of keto-THC-CoA by the action of D-BP 3-hydroxyacyl-CoA dehydrogenase (D-BP DH) in fibroblasts of a control subject (A) measured after a preincubation of 15 min with 0.3 mg/ml D-BP HY yeast homogenate and an incubation of 30 min with different concentrations of fibroblast homogenate, and (B) measured after *1*) a coincubation of 30 min, *2*) a coincubation of 45 min with both 0.3 mg/ml yeast D-BP HY and 0.5 mg/ml fibroblast homogenate of a control subject, and *3*) a preincubation of 15 min with 0.3 mg/ml D-BP HY yeast homogenate and an incubation of 30 min with 0.5 mg/ml fibroblast homogenate of a control subject.

TABLE 1. Specific activity of D-BP 3-hydroxyacyl-CoA dehydrogenase in fibroblast homogenates of controls and patients suffering from D-BP deficiency type I and type II

Mutation	Specific Activity D-BP DH	References
	pmol/min/mg	
Control (n = 3)	125 ± 30	
D-BP deficiency type I		
281–302 del (22 bp)	n.d.	10
281–622 del (342 bp)	n.d.	11
422–423 del (2 bp)	n.d.	5
869–881 del (13 bp)	n.d.	3
1211–1262 del (52 bp)	n.d.	4
D-BP deficiency type II		
N457Y	70	5
1439–1504 del (66 bp)	13	11
Patient 1	101	
Patient 2	90	
Patient 3	39	
Patient 4	39	
Patient 5	31	
Patient 6	30	
Patient 7	19	

D-BP DH, D-BP 3-hydroxyacyl-CoA dehydrogenase; n.d., not detectable.

For measurement of 3-hydroxyacyl-CoA dehydrogenase activity in fibroblasts of D-BP enoyl-CoA hydratase deficient patients, a D-BP DH assay was developed based on the D-BP assay described above. In the D-BP DH assay, 0.3 mg/ml yeast homogenate expressing D-BP HY was coincubated with fibroblast homogenate to synthesize the substrate for the dehydrogenase component of D-BP, 3α,7α,12α,24-tetrahydroxy-5β-cholestan-26-oyl-CoA (24-hydroxy-THC-CoA). The assay was optimized for protein concentration and incubation time.

Immunoblot analysis

Patient fibroblast homogenates used for measuring D-BP DH activity were also used for immunoblot analysis of D-BP. Fifty micrograms of fibroblast homogenate was separated on a 10% (w/v) SDS-polyacrylamide gel, essentially as described by Laemmli (9), and transferred to a nitrocellulose membrane (Schleicher and Shuell, Keene, NH). D-BP was detected using an antiserum raised against D-BP (3). Goat anti-rabbit IgG secondary antibody conjugated to alkaline phosphatase was used for detection, according to the manufacturer's instructions (Bio-Rad, Hercules, CA). Densitometric analysis of the immunoblot was performed using NIH Image 1.61.

RESULTS AND DISCUSSION

Hydratase activity in yeast homogenates

In order to measure the activity of D-BP DH independent of D-BP HY activity, the substrate for D-BP DH, 24-hydroxy-THC-CoA, is needed. To synthesize this substrate, wild-type D-BP HY was expressed in the yeast *S. cerevisiae*. Activity of D-BP HY in yeast homogenate was measured in time, using the D-BP activity assay (**Fig. 1**). Optimal levels of 24-hydroxy-THC-CoA were reached within 3 min of incubation. Increasing the amount of yeast homogenate in the reaction mixture from 0.3 mg/ml to 0.9 mg/ml did not lead to an increase in the amount of 24-hydroxy-THC-CoA formed. In homogenates of yeast cells transformed with the empty plasmid, no D-BP HY activity could be detected. D-BP HY yeast homogenates displayed no D-BP DH activity.

D-BP DH assay

To be able to distinguish between patients with either D-BP deficiency type I or D-BP deficiency type II without having to sequence the gene, we developed a method to determine D-BP DH activity in fibroblast homogenates. We used D-BP HY expressed in yeast to generate 24-hydroxy-THC-CoA, the substrate for the DH component. To determine optimal conditions for this assay, fibroblast homogenates of control subjects were used.

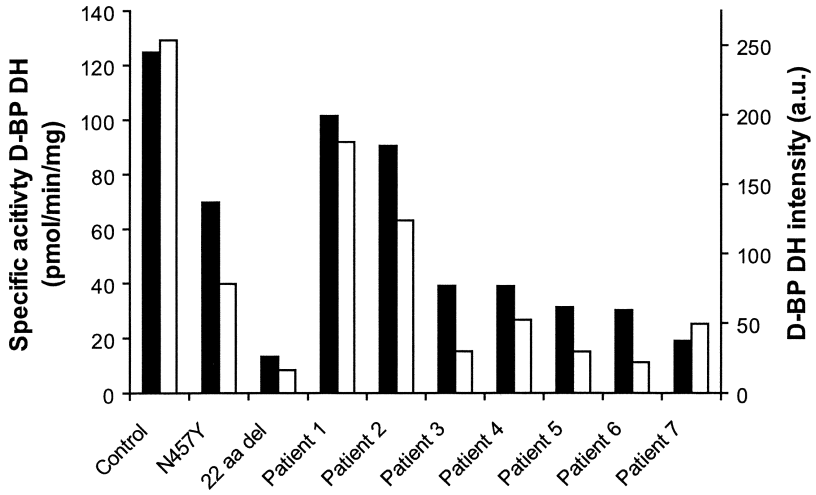

Fig. 3. Specific activity of D-BP DH measured in controls and D-BP deficiency type II patients (two patients with described mutations, others determined by D-BP DH assay) are depicted as black bars and the intensity of the D-BP DH bands on immunoblot (the 79 kDa band of full length D-BP plus the 35 kDa band of the D-BP DH component) are depicted as white bars.

First, the optimal protein concentration of fibroblast homogenate was determined by measuring the formation of 3α,7α,12α-trihydroxy-24-keto-5β-cholestanoyl-CoA (24-keto-THC-CoA) at different protein concentrations after a preincubation of 15 min with 0.3 mg/ml D-BP HY yeast homogenate (**Fig. 2A**). Formation of 24-keto-THC-CoA was linear with protein concentration up to a concentration of 0.5 mg/ml. Based on these findings, a concentration of 0.5 mg/ml was used in future experiments.

Second, the optimal reaction time was determined. Two different assay set-ups were tested: *1*) a preincubation of 15 min with D-BP yeast homogenate before addition of the fibroblast homogenate, and *2*) a coincubation of D-BP HY yeast homogenate and fibroblast homogenate. Results are shown in Fig. 2B. Because sufficient 24-keto-THC-CoA was formed to be readily detectable, a coincubation of fibroblast and D-BP HY yeast homogenate of 45 min was chosen for standard assay condition.

D-BP DH activity in D-BP deficient patients

To determine the specificity of our newly developed assay, D-BP DH activities were measured in fibroblast homogenates of D-BP deficient patients who were assigned to the three different subgroups by mutation analysis and expression studies (D-BP deficiency type I, five patients; D-BP deficiency type II, two patients; D-BP deficiency type III, four patients) (3–6, 10, 11). None of the patients with D-BP deficiency type I and type III showed any residual D-BP DH activity in fibroblast homogenates. In contrast, all patients with D-BP deficiency type II did display D-BP DH activity in fibroblast homogenates.

With our newly developed assay, we subsequently measured D-BP DH activity in 18 patients with deficient D-BP HY who had not yet been assigned to subgroup D-BP deficiency type I or type II. Based on these measurements we were able to assign seven of these patients to subgroup II, and 11 patients to subgroup I. In **Table 1**, the specific activities of D-BP DH measured in fibroblast homogenates of these patients are shown. Mutations are listed for the patients used to validate the D-BP DH activity assay.

Immunoblot analysis of D-BP in type II deficient patients

To study the relation between the measured D-BP DH activity and the D-BP protein level, immunoblot analysis was performed using antibodies against D-BP. In control fibroblasts, three major bands could be detected: the full-length protein (79 kDa), the D-BP HY component (45 kDa), and the D-BP DH component (35 kDa). Full length D-BP is proteolytically cleaved into two fragments. This process occurs after import of D-BP into peroxisomes because patients with a defect in the peroxisomal targeting signal (PTS)1 receptor (Pex5p), who cannot import D-BP into peroxisomes, only have full length D-BP. Cleavage of D-BP is not necessary for the activity of the protein, since D-BP activity is found in fibroblast homogenates of patients with a defective PTS1 receptor (5). In all fibroblast homogenates of D-BP deficiency type II patients, both the 79 kDa and the 35 kDa band were reduced in abundancy compared with controls, but they were present, whereas the 45 kDa band of D-BP HY was absent in all D-BP deficiency type II patient fibroblasts, except for patient 1 (data not shown).

To compare D-BP DH activity with the total amount of active D-BP DH protein, the intensities of the 79 kDa and 35 kDa bands were determined by densitometric analysis. These two intensities together are a measure for the total amount of D-BP DH present in the homogenate. In **Fig. 3**, both specific activities and blot intensities are plotted for all measured D-BP deficiency type II patients. In all patients, the measured D-BP DH activity was in good agreement with the amount of total D-BP DH protein. These results indicate that mutations in the D-BP HY fragment might destabilize the full length D-BP in a way that only little of the full-length protein reaches the peroxisome, where cleavage takes place. This destabilization of the D-BP is probably dependent on the type of mutation in the hydratase domain of the protein, since both the D-BP DH activity and the amount of protein was much higher in fibroblasts of a patient with a single amino acid substitution (N457Y) than in fibroblasts of a patient with a deletion of 22 amino acids (480–501 del). To substantiate this hypothesis, mutations of all assayed D-BP deficiency type II patients will be determined.

Summarizing, the D-BP DH assay described in this paper provides a quick and reliable method to differentiate between D-BP deficiency type I and II. This assay can be performed to determine the D-BP deficiency subgroup instead of time-absorbing methods like mutation analysis and expression studies of the protein in yeast. The D-BP DH assay in combination with the normal D-BP assay (6) make it possible to distinguish between all three types of D-BP deficiency on the basis of D-BP enzyme activities. This combination of assays is also very suitable for prenatal diagnosis of D-BP deficiency.

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