

Ether lipid biosynthesis: alkyl-dihydroxyacetonephosphate synthase protein deficiency leads to reduced dihydroxyacetonephosphate acyltransferase activities

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Abstract Recent studies have indicated that two peroxisomal enzymes involved in ether lipid synthesis, i.e., dihydroxyacetonephosphate acyltransferase and alkyl-dihydroxyacetonephosphate synthase, are directed to peroxisomes by different targeting signals, i.e., peroxisomal targeting signal type 1 and type 2, respectively. In this study, we describe a new human fibroblast cell line in which alkyl-dihydroxyacetonephosphate synthase was found to be deficient both at the level of enzyme activity and enzyme protein. At the cDNA level, a 128 base pair deletion was found leading to a premature stop. Remarkably, dihydroxyacetonephosphate acyltransferase activity was strongly reduced to a level comparable to the activities measured in fibroblasts from patients affected by the classical form of rhizomelic chondrodysplasia punctata (caused by a defect in peroxisomal targeting signal type 2 import). Dihydroxyacetonephosphate acyltransferase activity was completely normal in another alkyl-dihydroxyacetonephosphate synthase activity-deficient patient. Fibroblasts from this patient showed normal levels of the synthase protein and inactivity results from a point mutation leading to an amino acid substitution. These results strongly suggest that the activity of dihydroxyacetonephosphate acyltransferase is dependent on the presence of alkyl-dihydroxyacetonephosphate synthase protein. This interpretation implies that the deficiency of dihydroxyacetonephosphate acyltransferase (targeted by a peroxisomal targeting signal type 1) in the classic form of rhizomelic chondrodysplasia punctata is a consequence of the absence of the alkyl-dihydroxyacetonephosphate synthase protein (targeted by a peroxisomal targeting signal type 2).—de Vet, E. C. J. M., L. Ijlst, W. Oostheim, C. Dekker, H. W. Moser, H. van den Bosch, and R. J. A. Wanders. Ether lipid biosynthesis: alkyl-dihydroxyacetonephosphate synthase protein deficiency leads to reduced dihydroxyacetonephosphate acyltransferase activities. *J. Lipid Res.* 1999. 40: 1998–2003.

Supplementary key words peroxisome • peroxisomal disorders • peroxisomal targeting signal • rhizomelic chondrodysplasia punctata

Ether phospholipids are a special class of natural phospholipids. In mammals, these carry either an alkyl or an

alkenyl linkage at the *sn*-1 position of the glycerol backbone and an acyl linkage at the *sn*-2 position. The 1-alkenyl-2-acyl ether lipids are also known by their trivial name plasmalogens and are relatively abundant in mammalian tissues (1).

The biosynthesis of ether phospholipids starts with the acylation of dihydroxyacetonephosphate (DHAP) by the enzyme dihydroxyacetonephosphate acyltransferase (DHAPAT) (EC 2.3.1.42). The ether linkage is then introduced by the enzyme alkyl-DHAP synthase (EC 2.5.1.26), that catalyzes the exchange of the acyl-chain in acyl-DHAP for a long chain fatty alcohol (for a recent review see ref. 2). These two enzymes are located in peroxisomes (3).

The importance of peroxisomes for mammalian ether lipid biosynthesis was further emphasized by the discovery that ether phospholipids are severely deficient in tissues and erythrocytes in humans suffering from genetic diseases in which peroxisome biogenesis is disturbed (4, 5). The prototype of this group of disorders, i.e., the Zellweger syndrome, is biochemically characterized by a generalized loss of peroxisomal functions including the β -oxidation of very long chain fatty acids and ether phospholipid biosynthesis (5). Rhizomelic chondrodysplasia punctata (RCDP) is an example of a disorder in which peroxisomes are present and only a limited number of peroxisomal functions is impaired. This disorder is characterized by four distinct biochemical abnormalities, including a deficiency in the activities of phytanoyl-CoA hydroxylase and the two peroxisomal enzymes involved in ether phospholipid synthesis, i.e., DHAPAT and alkyl-DHAP synthase (6). Furthermore, the peroxisomal 3-ketoacyl-CoA thiolase is not

Abbreviations: DHAP, dihydroxyacetonephosphate; DHAPAT, dihydroxyacetonephosphate acyltransferase; PTS, peroxisomal targeting signal; RCDP, rhizomelic chondrodysplasia punctata.

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imported and processed and hence is present as the 44 kDa precursor rather than as the 41 kDa mature form.

The basic defect in RCDP is the inability to import proteins carrying the N-terminal peroxisomal targeting signal 2 (PTS2) (7, 8), which was first identified in the cleavable presequences of mammalian peroxisomal thiolases (9). In RCDP patients mutations have been found in the gene encoding the PTS2 receptor (PEX7) which are responsible for the loss of PTS2 dependent targeting of proteins to the peroxisome (10–12). Patients have been described who show severe clinical abnormalities comparable to classical RCDP but having an isolated deficiency in one of the peroxisomal enzymes involved in ether lipid biosynthesis, i.e., either DHAPAT (13) or alkyl-DHAP synthase (14).

The cDNAs of the first two enzymes of ether phospholipid biosynthesis, i.e., DHAPAT and alkyl-DHAP synthase, have recently been cloned. DHAPAT is synthesized as a 680 amino acid residue protein with a consensus peroxisomal targeting signal type 1 (PTS1) at its C-terminus (13, 15). In contrast, the nucleotide-derived amino acid sequence of alkyl-DHAP synthase contains a consensus PTS2 motif in an N-terminal cleavable presequence (16). Interestingly, Thai et al. (15) reported the isolation of a heterooligomeric complex of 210 kDa containing both DHAPAT and alkyl-DHAP synthase, highly suggestive for a direct protein–protein interaction between these two enzymes. The availability of these cDNA sequences enabled the resolution of the molecular basis of isolated DHAPAT deficiency (13) and alkyl-DHAP synthase deficiency (17), respectively. In the only case of alkyl-DHAP synthase deficiency analyzed so far, the alkyl-DHAP synthase enzyme protein was present at a level comparable to controls and inactivity of the enzyme was shown to be due to a point mutation leading to a R419H substitution (17).

The deficiencies of phytanoyl-CoA hydroxylase and alkyl-DHAP synthase in classical RCDP patients defective in PTS2 import are easily understood, as molecular cloning revealed the presence of PTS2 motifs in both enzymes (16, 18, 19). In the case of alkyl-DHAP synthase, fibroblasts derived from such RCDP patients show strongly reduced levels of enzyme protein, indicating that this enzyme is not stable outside the peroxisome (17). However, the deficiency in DHAPAT activity in RCDP is more puzzling, as this enzyme contains a PTS1 targeting signal rather than a PTS2. In this report we provide evidence that mutations in the alkyl-DHAP synthase gene causing the absence of this enzyme protein leads to reduced DHAPAT activities in fibroblasts comparable to the deficient activities measured in classical RCDP fibroblasts. These results strongly suggest that DHAPAT activity is dependent on the presence of alkyl-DHAP synthase protein and that the deficiency of DHAPAT in RCDP is a consequence of the absence of alkyl-DHAP synthase.

MATERIALS AND METHODS

Materials

[1-¹⁴C]hexadecanol was obtained from IRE-Nederland, Soesterberg, Netherlands. 1-O-[9,10-³H]octadecyl-*sn*-glycerol-3-phosphate

and 1-[U-¹⁴C]glycerol-3-phosphate are products of Amersham, Buckinghamshire, U.K. Palmitoyl-dihydroxyacetonephosphate was synthesized as described by Hajra, Saraswathi, and Das (20). Palmitoyl-CoA was from Sigma, St. Louis, MO.

Patients

Patient 1 was representative of a classic RCDP disorder characterized by a PTS2 import deficiency. Patient 2 had an isolated DHAPAT activity deficiency as described previously (13) and patient 3 was characterized by the isolated alkyl-DHAP synthase activity deficiency described previously (17). Patient 4 was a girl born of parents who were first cousins. Within 2 weeks after birth congenital cataracts and tight hips and knees were noted. At 11 months, mild to moderate rhizomelia with full joint motions except at knees and elbows was established. X-rays showed stippled epiphyses of distal humeri and proximal femurs. Upon admission at the age of 3.5 years, episodes of general stiffening, interpreted as seizures, were confirmed by EEG. Extremities showed generalized flexion contractures that were more severe in lower extremities. Fibroblasts, obtained from skin biopsies, were cultured in F10 medium containing initially 15% fetal calf serum and subsequently 15% newborn calf serum. Cells were harvested according to standard procedures (21). The experimental protocols used in this paper were approved by the Institutional Review Board at the Academic Medical Center in Amsterdam.

Methods

De novo plasmalogen synthesis. The de novo plasmalogen biosynthesis was determined as previously described (22). Briefly, fibroblasts were grown in the presence of [³H]alkylglycerol (84 mCi/mmol, 0.5 μCi/30 ml medium) and [¹⁴C]hexadecanol (55 mCi/mmol, 1 μCi/30 ml medium). Cellular phospholipids were extracted according to the method of Bligh and Dyer (23) and separated with two-dimensional thin-layer chromatography with HCl treatment in between the two chromatographic runs to cleave the alkenyl bond in plasmalogens as described before (24). The percentage of ¹⁴C radioactivity in the alkenyl chain of ethanolamine- and cholineplasmalogens was calculated by dividing the amount of ¹⁴C label in the alkenyl chain by the total amount of ¹⁴C label in the particular phospholipid class, respectively. [³H]alkyl-glycerol incorporation into plasmalogens bypasses peroxisomal enzyme deficiencies and occurs with equal efficiency in control and peroxisome disorder fibroblasts (24). The capacity for de novo ether lipid synthesis can thus be evaluated from the ³H/¹⁴C ratios in the alkenyl chains of ethanolamine- and choline plasmalogens, as previously described (22).

Enzyme assays. DHAPAT activity assay was done according to Ofman and Wanders (25). Incubations were performed in 75 mm sodium acetate, pH 5.4, 8 mm NaF, 8 mm MgCl, 3.35 mg/ml BSA, 10 mm glycerol-3-phosphate, 0.166 mm palmitoyl-coenzyme A, and 0.1 mm [U-¹⁴C]DHAP (10,000 dpm/nmol). The latter was prepared from 1-[U-¹⁴C]glycerol-3-phosphate as described previously (25). Aliquots of whole-cell homogenates equivalent to between 30 and 70 μg of protein were used. Alkyl-DHAP synthase activity was measured according to Schrakamp et al. (21) using the DEAE filter paper assay. The incubation mixtures consisted of 100 mm Tris/HCl, pH 8.5, 50 mm NaF, 0.05% (v/v) Triton X-100, 240 μm palmitoyl-DHAP, 100 μm [1-¹⁴C]hexadecanol (10,000 dpm/nmol), and 100 μg of whole-cell homogenate protein in a total volume of 100 μl.

Western blot analyses. The antiserum against alkyl-DHAP synthase and the immunoblotting procedure for this enzyme were described previously (26). The immunoblotting procedure for the peroxisomal 3-ketoacyl-CoA thiolase is described by Wanders et al. (27).

Mutation analysis. PCR amplification of the complete open reading frame of alkyl-DHAP synthase was done with primers

containing M13 extensions to allow sequencing with fluorescently labeled M13 primers with an Applied Biosystems 377A automated DNA sequencer following ABI protocols. The procedure and primer sequences are described by de Vet et al. (17). However, in our previous studies we were not able to amplify a part of the cDNA coding for the N-terminal half of the presequence, probably due to an extremely high GC content. For this reason, further optimization of the PCR protocol was done, which included the addition of DMSO to the reaction buffer to a final concentration of 2% (v/v) and raising the denaturation temperature from 95°C to 96°C. The primer used for amplification of this region (TGTAAAACGACGGCCAGTCAGCACAAAGCGGTAGCC) corresponds to nt -18 to -1 of the cDNA sequence tagged with a -21M13 sequence (underlined) at the 5'-end. This primer is used in combination with reverse primer DF2-M13rev in the PCR reaction (17). The open reading frame of DHAP acyltransferase was sequenced as described before (13).

RESULTS

Table 1 shows the de novo plasmalogen biosynthesis in fibroblasts from patients affected by one of the different forms of RCDP. The list includes a classic RCDP patient (PTS2 import deficiency) (7, 8), an isolated DHAPAT deficiency (patient number 1 in ref. 13), an isolated alkyl-DHAP synthase deficiency (14, 17) and a not yet biochemically characterized patient with a RCDP phenotype (patient 4). Both the reduced levels of the ¹⁴C label in the alkenyl-chains of ethanolamine- and cholineglycerophospholipids and the highly increased ³H/¹⁴C ratio in these alkenyl-chains clearly indicate a deficient de novo plasmalogen biosynthesis in all patients.

Table 2 shows the DHAPAT and alkyl-DHAP synthase activities in these 4 patients. In the classic RCDP patient, deficiencies were found of both DHAPAT and alkyl-DHAP synthase activities, whereas patients 2 and 3 had an isolated deficiency in DHAPAT and alkyl-DHAP synthase, respectively. In patient 4, however, a combined deficiency of both enzymes was found. To investigate whether patient 4 was also a classic RCDP patient, immunoblot experiments were performed to assay for the presence of alkyl-DHAP synthase (**Fig. 1A**) and peroxisomal 3-ketoacyl-CoA thiolase (**Fig. 1B**). As can be seen in **Fig. 1**, the classic RCDP

TABLE 2. Activities of dihydroxyacetonephosphate acyltransferase and alkyl-dihydroxyacetonephosphate synthase in fibroblast homogenates of patients with an RCDP clinical phenotype

Patient	DHAPAT	Alkyl-DHAP Synthase
	<i>nmol/mg · 2 h</i>	<i>pmol/min · mg</i>
1	2.0 ± 0.4	1.4 ± 0.4
2	0 ± 0	26.0 ± 7.0
3	9.5 ± 1.4	0.2 ± 0.3
4	1.5 ± 0.7	0 ± 0
Control	10.9 ± 2.5 (n = 110)	25.5 ± 7.2 (n = 8)

Patients as in Table 1. Results are expressed as mean ± SD of at least three determinations.

patient had a strongly reduced alkyl-DHAP synthase level as has been observed before (17) and the peroxisomal thiolase was present in its 44 kDa precursor form. The patient with an isolated DHAPAT deficiency showed levels of alkyl-DHAP synthase and peroxisomal thiolase as in control cells. In cells from the patient with the isolated alkyl-DHAP synthase deficiency, normal levels of the enzyme protein were found and we recently showed that enzyme inactivity in this patient resulted from a point mutation leading to a R419H substitution (17). Thiolase was present at normal levels of the correct molecular size (41 kDa) indicating intact PTS2 import. In fibroblasts of patient 4 no detectable alkyl-DHAP synthase protein levels were found. Because the peroxisomal 3-ketoacyl-CoA thiolase is apparently imported normally and converted into its mature 41 kDa form (**Fig. 1B**), these data virtually exclude the possibility that the absence of alkyl-DHAP synthase is due to a PTS2 import defect as in classic RCDP. From this result it was anticipated that patient 4 might have a defective alkyl-DHAP synthase gene, resulting in undetectable levels of the protein, and that the deficiency in DHAPAT activity might be the consequence of the absence of alkyl-DHAP synthase. If this were correct, it would also provide an explanation for the deficiency of the DHAPAT in classic RCDP patients, notwithstanding the facts that DHAPAT carries a PTS1 and that RCDP is characterized by a deficiency in PTS2 protein import.

TABLE 1. De novo plasmalogen synthesis in patients with an RCDP clinical phenotype

Patient	% Label in Alkenylchain of PE	% Label in Alkenylchain of PC	³ H/ ¹⁴ C Ratio in Alkenyl chain of PE	³ H/ ¹⁴ C Ratio in Alkenylchain of PC
1	2.9	0.3	171	7.1
2	0.0	0.8	7861	5.1
3	0.5	0.4	636	7.0
4	3.3	0.5	403	13.1
Control	80.9	3.9	0.7	0.6
5-95% range	64.5 ± 85.7	2.0 ± 8.0	0.3 ± 2.4	0.3 ± 2.0

Patient 1 was a representative of a classic RCDP disorder (PTS2 import deficiency), patient 2 had an isolated DHAPAT deficiency (13), patient 3 had an isolated alkyl-DHAP synthase deficiency (17) and the new patient 4 is briefly described in the Materials section. PE, total of ethanolamineglycerophospholipid; PC, total of cholineglycerophospholipid. Data represent a single determination from a comparative experiment using all patient cell lines simultaneously.

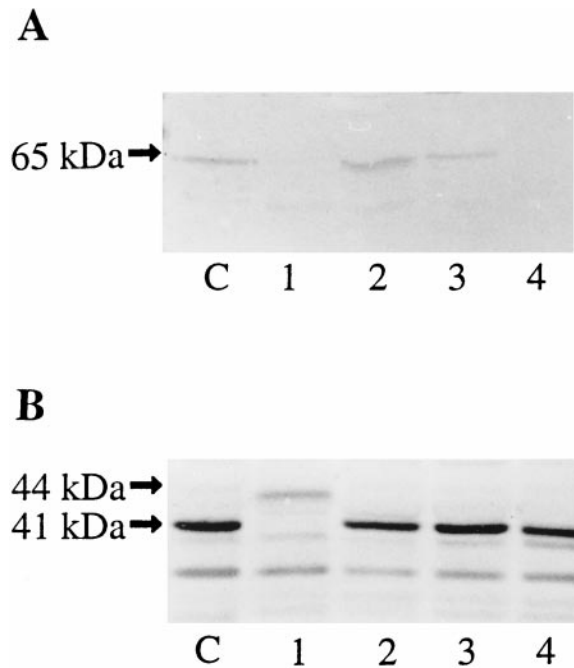


Fig. 1. Analysis of alkyl-DHAP synthase (A) and peroxisomal 3-ketoacyl-CoA thiolase (B) levels in fibroblast homogenates by Western blot immunostaining. In the case of the alkyl-DHAP synthase Western blot, 100 μ g of protein was used in each lane. In the case of the 3-ketoacyl-CoA thiolase Western blot, 50 μ g of protein was used in each lane. C, control fibroblasts; 1, patient 1 with a classical RCDP disorder; 2, patient 2 with an isolated DHAPAT deficiency; 3, patient 3 with an isolated alkyl-DHAP synthase activity deficiency; 4, a new patient with deficiencies in both DHAPAT and alkyl-DHAP synthase activities.

To investigate this possibility, the cDNA sequence encoding alkyl-DHAP synthase from this patient was analyzed by direct sequencing of reverse transcriptase-PCR products. As can be seen in **Table 3** this patient indeed had a defect in the alkyl-DHAP synthase gene. At the cDNA level a homozygous 128 bp deletion was identified. In contrast to the mutation in patient 3, which consists of an amino acid substitution leaving the open reading frame intact, the deletion in this patient 4 leads to a premature stop and explains why no 65 kDa alkyl-DHAP synthase protein was observed on immunoblot (Fig. 1A). No differences in the cDNA sequence encoding DHAPAT from patient 4 with the wild-type sequence were observed, indicating that it is unlikely that the reduced DHAPAT activity in the fibroblasts from this patient was due to a defective DHAPAT gene.

TABLE 3. Mutations in DHAPAT and alkyl-DHAP synthase as determined by direct sequencing of reverse transcriptase-PCR products

Patient	cDNA	Mutation	Consequence
2	DHAPAT	848 insertion TT	Premature stop
3	alkyl-DHAP synthase	G1256A	R419H
4	alkyl-DHAP synthase DHAPAT	Deletion 1106–1233 No mutations	Premature stop

The deficiency of plasmalogens in peroxisomal biogenesis disorders such as Zellweger syndrome and RCDP provides unambiguous evidence for the indispensable role of peroxisomes in ether phospholipid biosynthesis. Recently, the cDNAs encoding the first two peroxisomal enzymes of this pathway, i.e., DHAPAT (13, 15) and alkyl-DHAP synthase (16), have been cloned. The derived amino acid sequences revealed a consensus C-terminal PTS1 signal for DHAPAT and a PTS2 motif in a N-terminal cleavable presequence for alkyl-DHAP synthase, respectively.

The virtual absence of protein import into the peroxisomal ghosts of Zellweger syndrome and the instability of most peroxisomal enzymes outside their target organelle (5), as was recently also demonstrated for alkyl-DHAP synthase (17), provide an easy explanation for the deficient activity of both DHAPAT and alkyl-DHAP synthase in Zellweger syndrome. As it is well established that the basic defect in classical RCDP is a defect in the import of PTS2-containing proteins due to mutations in the gene encoding the PTS2 receptor (PEX7) (7, 8, 10–12), the alkyl-DHAP synthase deficiency in this disorder is also easily explained. It is rather puzzling, however, why DHAPAT activity, the enzyme being a PTS1 protein, is also strongly reduced in this disorder. As RCDP fibroblasts are still capable of PTS1 dependent protein import, there is no obvious reason to assume that DHAPAT cannot be imported in this disorder. An important clue to this question is provided in this study.

A patient with a defective alkyl-DHAP synthase gene (patient 4), resulting in the absence of the enzyme protein, exhibited a reduced DHAPAT activity comparable with the value found in classic RCDP. At present, three additional patients are under study whose fibroblasts show the same biochemical characteristics, i.e., a virtual absence of alkyl-DHAP synthase activity and protein, reduced DHAPAT activity comparable with classic RCDP, and normal thiolase processing. This indicates that the correlation between absence of alkyl-DHAP synthase protein and reduced DHAPAT activities found in patient 4 is not a coincidence.

In contrast, in another case of isolated alkyl-DHAP synthase deficiency (patient 3) in which the alkyl-DHAP synthase enzyme protein appeared normally present, albeit in an inactive form due to a point mutation (17), we found normal DHAPAT activity. These results suggest that DHAPAT activity is dependent on the presence of the alkyl-DHAP synthase protein, irrespective of whether the latter is active or not. Interestingly, alkyl-DHAP synthase activity appears not to be dependent on DHAPAT, as patients with isolated DHAPAT deficiencies have normal levels of alkyl-DHAP synthase, even if there are frameshifts in the DHAPAT cDNA sequence leading to premature stops (and thus abolishing the C-terminal PTS1 signal) (13).

By analogy, because alkyl-DHAP synthase, being a PTS2 targeted protein, is not present in the peroxisome in RCDP, it is reasonable to assume that the observed DHAPAT deficiency in this disorder is also a direct consequence of the

absence of the alkyl-DHAP synthase protein in peroxisomes. This relationship can best be understood in molecular terms by assuming that these two enzymes, DHAPAT and alkyl-DHAP synthase, are in direct physical contact with each other. Indeed, Thai et al. (15) obtained biochemical evidence that these two enzymes are present in a heteromultimeric complex in detergent extracts of rabbit Harderian gland peroxisomes. Supportive evidence for a direct interaction between these enzymes was recently also obtained using chemical cross-linking and co-immunoprecipitation (28). The advantage obtained by these two enzymes in such a complex is obvious. The product formed by DHAPAT (acyl-DHAP) can be very efficiently and directly used by alkyl-DHAP synthase to form alkyl-DHAP. In this respect it is interesting to note that a preferential use of enzymatically generated acyl-DHAP in comparison to exogenously added acyl-DHAP has been documented experimentally (29).

Multimerization of enzymes involved in a pathway is a phenomenon that is often observed. However, a few alternative possibilities are still open to explain the reduced DHAPAT activity. The alkyl-DHAP synthase protein may act as a kind of chaperone for DHAPAT, or the presence of alkyl-DHAP synthase may protect DHAPAT against proteolytic degradation. The possibility that the presence of alkyl-DHAP synthase may change the tertiary structure of DHAPAT in such a way that it becomes more active is considered less likely in that recent radiation inactivation analyses (30) have indicated that the functional unit sizes of both DHAPAT and alkyl-DHAP synthase are represented by the monomeric peptide chains. As DHAPAT contains a PTS1 signal, necessary and sufficient for targeting to the peroxisome, it is also considered unlikely that alkyl-DHAP synthase is required for targeting of DHAPAT to the peroxisome. The definitive discrimination between the possibilities described above will have to await further analysis of DHAPAT protein levels in the presence and absence of alkyl-DHAP synthase and making use of specific antibodies which are now being developed. ■■

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