Alkyl-Dihydroxyacetonephosphate Synthase

FATE IN PEROXISOME BIOGENESIS DISORDERS AND IDENTIFICATION OF THE POINT MUTATION UNDERLYING A SINGLE ENZYME DEFICIENCY

(Received for publication, November 10, 1997, and in revised form, February 11, 1998)

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Peroxisomes play an indispensable role in ether lipid biosynthesis as evidenced by the deficiency of ether phospholipids in fibroblasts and tissues from patients suffering from a number of peroxisomal disorders. Alkyl-dihydroxyacetonephosphate synthase, a peroxisomal enzyme playing a key role in the biosynthesis of ether phospholipids, contains the peroxisomal targeting signal type 2 in a N-terminal cleavable precursor. Using a polyclonal antiserum raised against alkyl-dihydroxyacetonephosphate synthase, levels of this enzyme were examined in fibroblast cell lines from patients affected by peroxisomal disorders. Strongly reduced levels were found in fibroblasts of Zellweger syndrome and rhizomelic chondrodysplasia punctata patients, indicating that the enzyme is not stable in the cytoplasm as a result of defective import into peroxisomes. In a neonatal adrenoleukodystrophy patient with an isolated import deficiency of proteins carrying the peroxisomal targeting signal type 1, the precursor form of alkyl-dihydroxyacetonephosphate synthase was detected at a level comparable to that of the mature form in control fibroblasts, in line with an intraperoxisomal localization. A patient with an isolated deficiency in alkyl-dihydroxyacetonephosphate (DHAP) synthase activity had normal levels of this protein. Analysis at the cDNA level revealed a missense mutation leading to a R419H substitution in the enzyme of this patient. Expression of a recombinant protein carrying this mutation in Escherichia coli yielded an inactive enzyme, whereas a comparable control recombinant enzyme was active, providing further proof that this substitution is responsible for the inactivity of the enzyme and the phenotype. In line with this result is the observation that wild-type alkyl-DHAP synthase activity can be inactivated by the arginine-modifying agent phenylglyoxal. The enzyme is efficiently protected against this inactivation when the substrate palmitoyl-DHAP is present at a saturating concentration. The gene encoding human alkyl-dihydroxyacetonephosphate synthase was mapped on chromosome 2q31.

An increasing number of inherited diseases have been identified in recent years in which peroxisomal functions are impaired. The prototype of this group of disorders is the Zellweger syndrome, first described in 1964 (1). Goldfischer and co-workers (2) discovered in 1973 that morphologically distinguishable peroxisomes were absent in hepatocytes of patients affected by this disorder, and this discovery was followed a decade later by the observation that there is an accumulation of very long chain fatty acids in the plasma of these patients (3) and that plasmalogens (a special kind of ether phospholipids) were deficient in tissues and erythrocytes from these patients (4).

The peroxisomal disorders identified today can be subdivided into three groups depending upon whether there is a generalized loss (A), a multiple loss (B), or a single loss (C) of peroxisomal functions (5). The Zellweger syndrome, as well as neonatal adrenoleukodystrophy (NALD),1 are examples of disorders belonging to group A. Rhizomelic chondrodysplasia punctata (RCDP) is an example of a disorder belonging to group B, in which peroxisomes are present and only a limited number of peroxisomal functions is impaired. Four distinct biochemical abnormalities have been found in RCDP, including a deficiency in the activities of phytanoyl-CoA hydroxylase and the two peroxisomal enzymes involved in ether phospholipid synthesis, i.e. dihydroxyacetonephosphate (DHAP) acyltransferase and alkyl-dihydroxyacetonephosphate synthase (alkyl-DHAP synthase) (6). Furthermore, the peroxisomal 3-oxoacyl-CoA thiolase is not 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 peroxisomal targeting signal (PTS) 2 (7, 8), which was first identified in the cleavable precursors of mammalian peroxisomal thiolases (9). Due to the deficient activities of the enzymes mentioned above, RCDP patients are deficient in plasmalogens and accumulate phytanic acid in plasma (5).

In recent years, enormous progress has been made in identifying genes whose products are involved in peroxisome biogenesis and mutations in these genes have been shown to be present in patients affected by peroxisomal disorders (10). The first gene identified involved in a peroxisomal disorder (Zellweger syndrome) was the peroxisome assembly factor 1 (PAP1, renamed as PEX2), which encodes a 35-kDa zinc binding integral membrane protein (11). Mutations in the gene encoding the human PTS1 receptor (PEX5) define complementation group 2 of the peroxisomal biogenesis disorders (12). Whereas a point mutation leading to a N489K substitution caused an

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1 The abbreviations used are: NALD, neonatal adrenoleukodystrophy; DHAP, dihydroxyacetonephosphate; PTS, peroxisomal targeting signal; RCDP, rhizomelic chondrodysplasia punctata; PCR, polymerase chain reaction.
isolated deficiency in PTS1 import, a mutation leading to a premature stop codon resulted in a deficiency in both PTS1 and PTS2 import, suggesting that the PTS1 receptor may also be involved in PTS2 import, at least in mammals. In RCDP, mutations in the PTS2 receptor (PEx7) are observed leading to deficiencies in PTS2 protein import (13–15).

A few patients have been identified that have an isolated deficiency in one of the peroxisomal enzymes involved in ether phospholipid synthesis, i.e. DHAP acyltransferase (16–18) or alkyl-DHAP synthase (19), respectively. These patients show severe clinical abnormalities comparable to RCDP stressing the importance of ether phospholipids for human physiology.

The molecular cloning of the cDNA encoding guinea pig and human alkyl-DHAP synthase (20, 21) revealed the presence of a cleavable presequence containing a PTS2 motif, a finding in line with its deficiency in RCDP. The availability of a specific antiserum against alkyl-DHAP synthase has now enabled us to examine the level and size of this enzyme in human peroxisomal disorders. Furthermore, the isolated alkyl-DHAP synthase deficiency reported in the only patient identified so far (19) has been examined at the cDNA level and led to the identification of a critical residue for enzymatic activity.

EXPERIMENTAL PROCEDURES

Materials

An enhanced chemiluminescence (ECL) detection kit was obtained from NEN Life Science Products. Horseradish peroxidase-conjugated goat anti-rabbit IgG was a product of Bio-Rad. Oligonucleotides were manufactured by Isogen, Maarssen, The Netherlands. M-Marine leukemia virus reverse transcriptase was a product of New England Biolabs. Taq DNA polymerase was from Promega, Madison, WI. Phenylglyoxal monohydrate was from Acros Organics, Geel, Belgium.

Methods

Cell Culture—Fibroblasts were cultured and harvested by gentle trypsinization and washing by low speed centrifugation as described before (22). The NALD patient with an isolated PTS1 import deficiency (complementation group 2 of the Kennedy Krieger institute or group 4 of the Amsterdam nomenclature) has been described before (7). The alkyl-DHAP synthase deficient patient has also been described before (19). The RCDP patients studied in this paper belong both to complementation group 11 of the Kennedy Krieger Institute or group 1 of the Amsterdam nomenclature (23). The two Zellweger patients studied in this paper belong both to complementation group 1 of the Kennedy Krieger Institute or group 2 of the Amsterdam nomenclature (23).

Western Blotting and in Vitro Transcription/Translation—The antisense was used of recombinant guinea pig alkyl-DHAP synthase as described before (24). SDS-polyacrylamide gel electrophoresis on 9% acrylamide gels was performed according to Laemmli (25), and proteins were transferred to nitrocellulose membranes. Nonspecific binding sites were blocked overnight at 4 °C with 4% milk powder and 0.4% Tween-20 in phosphate-buffered saline. Subsequent manipulations were done at room temperature. The membranes were incubated with the antisera for 1 h at a dilution of 1/2000 in phosphate-buffered saline containing 0.1% milk powder and 0.01% Tween-20. After washing, the membrane was incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG for 1 h. Peroxidase activity was detected with ECL.

For the in vitro transcription/translation of alkyl-DHAP synthase, a construct was created in a pET-15B vector containing the complete open reading frame of human alkyl-DHAP synthase precursor (21) downstream of the T7 promoter. This construct was translated in vitro using TnT coupled reticulocyte lysate system (Promega) according to the manufacturer’s instructions.

RESULTS

In Fig. 1A a Western blot analysis is shown from fibroblast homogenates derived from controls and patients with the indicated peroxisomal disorders using the antiserum against recombinant guinea pig alkyl-DHAP synthase. Human alkyl-DHAP synthase in control fibroblasts runs at a slightly higher molecular weight than the guinea pig counterpart (compare lanes 1, 2, and 3), as has been observed before for liver (24). In Zellweger syndrome and RCDP, alkyl-DHAP synthase is present at strongly reduced levels, although some residual mature protein is observed as well as protein running at a higher molecular weight, which may correspond to the precursor form of alkyl-DHAP synthase. In a NALD patient having an isolated PTS1 import deficiency, alkyl-DHAP synthase is present at a

| Code     | Nucleotides | Sequence                  |
|----------|-------------|---------------------------|
| DF1−21M13| 160−179a    | TGTTAAAACGCCGAGCCTTTGAACTGAGGAAGCC |
| DF2 M13rev| 1078−1097b  | CAGGAAAACGCTGAGCTACCCGTATGGATGAGGATG |
| DF3−21M13| 1034−1054a  | TGTTAAAACGCCGAGCCTTTGAACTGAGGAAGCC |
| DF4 M13rev| 1978−1997b  | CAGGAAAACGCTGAGCTACCCGTATGGATGAGGATG |

a Forward.  
b Reverse.
acid sequence and the human wild-type sequence (21).

Only one difference was observed between the deduced amino acid sequence of this patient and the wild-type sequence, a G to A transition at nucleotide 1256 (Fig. 3A). This transition creates a HphI site in the mutant cDNA, and the sensitivity of the mutant cDNA to this restriction enzyme confirms that the G to A transition is not a sequencing artifact (Fig. 3B). To obtain further proof that this substitution is responsible for the inactivity of the enzyme in this patient, and that it does not represent a natural polymorphism, recombinant pControl and pMutant constructs were prepared as indicated in Fig. 4A (compare legend). Both constructs were expressed in E. coli. As can be seen in Fig. 4 the recombinant mutant protein and its control counterpart were expressed at very similar levels both when assayed with Coomassie staining (Fig. 4B) or immunostaining (Fig. 4C). However, no alkyl-DHAP synthase activity was detected in E. coli homogenates expressing the recombinant mutant protein (encoded by the pMutant construct) with His-419, whereas the expression of the recombinant control protein (encoded by the pControl construct) with Arg-419 resulted in an enzymatic activity of 1.87 milliunit/mg protein. These results provide convincing proof that this R419H mutation is responsible for the inactivity of the enzyme in this patient and indicates that this arginine is essential for alkyl-DHAP synthase activity.

Fig. 5A shows that alkyl-DHAP synthase activity is hardly affected by the serine-modifying agent phenylmethanesulfonyl fluoride at concentrations of 0.5 or 1 mM. Only at higher concentrations some inhibition of enzyme activity is observed. By contrast, alkyl-DHAP synthase activity is efficiently inhibited by concentrations of 0.5 and 1 mM of the arginine-modifying agent phenylglyoxal. This inhibition appears to be most efficient in incubations in bicarbonate buffer. Subsequent experiments (Fig. 5B) indicate that the inhibition by 2 mM phenylglyoxal follows pseudo-first order kinetics with \( k = 0.067 \) min\(^{-1}\). The presence of the substrate palmitoyl-DHAP protects...
the enzyme against phenylglyoxal-mediated inactivation resulting in an inactivation constant ($k = 0.006 \text{ min}^{-1}$) that is even somewhat lower than the one observed in the absence of inhibitor ($k = 0.009 \text{ min}^{-1}$).

**FISH** mapping of a 1850-base pair human alkyl-DHAP synthase cDNA probe resulted in signals on one pair of chromosomes, i.e., the long arm of chromosome 2, region q31 (Fig. 6). No additional locus is revealed by FISH detection under the conditions used. Therefore, we conclude that the gene encoding human alkyl-DHAP synthase is located on chromosome 2q31.

**DISCUSSION**

The discovery that plasmalogens are deficient in the tissues from patients suffering from Zellweger syndrome stressed unambiguously the indispensable role of peroxisomes for ether phospholipid biosynthesis (4). The activities of the first two (peroxisomal) enzymes in this biosynthetic route, DHAP acyltransferase and alkyl-DHAP synthase, are deficient in Zellweger syndrome, as well as in several other peroxisomal disorders including RCDP (5, 6). Recently, we succeeded in cloning of the cDNA encoding alkyl-DHAP synthase, and this led to the discovery that this enzyme is synthesized as a precursor protein containing a PTS2 motif in a cleavable presequence (20, 21). The availability of an antiserum against alkyl-DHAP synthase enabled us to study the molecular basis of the deficiency of this enzyme in more detail. The strongly reduced levels in fibroblasts derived from patients affected by Zellweger syndrome and RCDP suggest that alkyl-DHAP synthase is not stable in the cytoplasm when it is not imported into the peroxisome due to the absence of peroxisomes (Zellweger syndrome) or defective import (RCDP) (Fig. 1). Instability of peroxisomal proteins in Zellweger syndrome is also observed for the enzymes involved in $\beta$-oxidation that become rapidly degraded in the absence of functional peroxisomes (30). However, fibroblasts derived from patients suffering from Zellweger syn-

**FIG. 5. Effect of phenylglyoxal on alkyl-DHAP synthase activity.** A, effect of phenylglyoxal concentration and incubation buffer. Enzyme was incubated for 45 min at 25 °C without or with the indicated concentrations of phenylmethylsulfonyl fluoride or phenylglyoxal as described under "Experimental Procedures" prior to activity assay. Symbols: □, phenylmethylsulfonyl fluoride, Tris buffer; ○, phenylglyoxal, Tris buffer; ●, phenylglyoxal, borate buffer; △, phenylglyoxal, bicarbonate buffer. Enzyme activity is expressed as the percentage of activity measured in the absence of inhibitor. B, effect of incubation time and palmitoyl-DHAP. Enzyme was incubated for the indicated time periods without inhibitor (□), 2 mM phenylglyoxal (○), or 2 mM phenylglyoxal in the presence of 0.3 mM palmitoyl-DHAP (△) in Tris buffer as described under "Experimental Procedures." Activities are expressed as the logarithm of the percentage of the activity measured at zero time.
drome have invariably been shown to retain some residual alkyl-DHAP synthase activity, which generally amounts to approximately 10% of control fibroblasts (31), as well as a residual capacity to synthesize plasmalogens (32). These results indicate that alkyl-DHAP synthase can nevertheless fold into an active enzyme in this disorder. In this respect, it is remarkable that in Zellweger syndrome all residual signals on Western blots correspond to the precursor form rather than the mature form, already suggesting that the precursor may be active and responsible for the residual activities observed. The reduced levels of alkyl-DHAP synthase in Zellweger syndrome and RCDP fibroblasts are in line with the observations made in liver samples from patients suffering from these disorders, although in these samples actually no residual enzyme protein could be observed (24).

The residual alkyl-DHAP synthase content of RCDP fibroblasts consists of both the precursor and the mature form of the enzyme (Fig. 1). This finding is different from the observations made with peroxisomal 3-oxoacyl-CoA thiolase (the other known PTS2 protein in humans), which is only present as the 44-kDa precursor form (6). A clear explanation for this discrepancy is lacking.

Alkyl-DHAP synthase is present in the NALD patient with a specific PTS1 import defect (described in Ref. 7) at a level comparable to control fibroblasts, which is in line with the expectation that alkyl-DHAP synthase, being a PTS2 protein, is imported into the peroxisome in this patient. However, alkyl-DHAP synthase is present as its precursor form rather than as its mature form in this disorder. An analogous observation has been made with peroxisomal 3-oxoacyl-CoA thiolase, which, although imported, is also not processed in this patient (7). These findings can be explained by assuming that the protease responsible for processing of both alkyl-DHAP synthase and 3-oxoacyl-CoA thiolase is a PTS1 protein, and is thus not present in the peroxisome in this disorder. Indeed, evidence has been reported which suggests that pp110, a protein with a consensus PTS1 at its C terminus, is the protease that cleaves the peroxisomal thiolase (33). Since alkyl-DHAP synthase and peroxisomal thiolase contain sequence homology in the cleavage site (20), pp110 may as well be responsible for cleavage of alkyl-DHAP synthase. The alkyl-DHAP synthase activity in the homogenate of this patient is not impaired (even somewhat higher than the two controls) indicating that processing of the precursor is not needed for the enzyme to become active. Nevertheless, this patient still exhibits a relatively mild impairment in de novo plasmalogen synthesis when compared with controls (about a factor two lower than controls, see patient PBD018 in Ref. 34), which may point to an involvement of a PTS1 protein in ether lipid synthesis. Unfortunately, the DHAP acyltransferase activity in this patient has not been reported.

The discovery of a patient with an isolated deficiency in alkyl-DHAP synthase activity with the clinical symptoms of RCDP indicates that ether phospholipids are extremely important for human physiology and that the deficiency in ether phospholipids is actually responsible for the RCDP phenotype (5, 6). The observation that fibroblasts of this patient contain normal levels of mature alkyl-DHAP synthase protein suggests that the protein is properly incorporated into the peroxisome and that an amino acid residue essential for enzymatic activity has been mutated. The subsequent identification of this mutation responsible for the inactivity (R419H) gives us a first example of an essential residue for enzymatic activity. In agreement with this result is the observation that the enzyme can be inactivated by the arginine-modifying agent phenylglyoxal (Fig. 5). The finding that the enzyme is efficiently protected against this inactivation when substrate (palmitoyl-DHAP) is present at a saturating concentration provides a strong indication that this inactivation results from modification of an arginine residue(s) in the active site. However, experimental proof is lacking that the inhibition of enzymatic activity by phenylglyoxal is indeed a result of a modification of arginine 419. Arginines are known to be ideally suited for interaction with negatively charged phosphate groups due to their ability to form multiple hydrogen bonds with this moiety (35). Because of resonance stabilization of the guanidinium group in the side chains of these residues, arginines are poor proton donors and would probably not function as general acid catalysts (36). In this respect it is noteworthy that acetyl-dihydroxyacetone lacking the phosphate group appears not to be a substrate for alkyl-DHAP synthase, stressing the importance of the phosphate group (37). However, from the data presented in this paper it is not strictly proven that this arginine residue is actually an active site residue involved in substrate binding or catalysis, since such a mutation may as well disturb the secondary or tertiary structure of a protein to the extent that it becomes inactive. The conclusion that the R419H substitution does not reflect a natural occurring polymorphism is also stressed by the observation that this arginine residue is also present in at least two independent human expressed sequence tags clones (21) and guinea pig alkyl-DHAP synthase (20).

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