Polymerase Chain Reaction-based Cloning of Alkyl-dihydroxyacetonephosphate Synthase Complementary DNA from Guinea Pig Liver*

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Peroxisomes are indispensable organelles for ether lipid biosynthesis in mammalian tissues, and the deficiency of these organelles in a number of peroxisomal disorders leads to deficiencies in ether phospholipids. We have previously purified the committed enzyme for ether lipid biosynthesis, i.e. alkyl-dihydroxyacetonephosphate synthase, to homogeneity. We have now determined the N-terminal amino acid sequence, as well as additional internal sequences obtained after cyanogen bromide cleavage of the enzyme. With primers directed against the N-terminal sequence and against a cyanogen bromide fragment sequence, a 1100-bp cDNA fragment was obtained by conventional polymerase chain reaction using first-strand cDNA from guinea pig liver as a template. The 5' and 3' ends of the cDNA were obtained by rapid amplification of cDNA ends. The open reading frame encodes a protein of 658 amino acids, containing the N-terminal amino acid sequence as well as the cyanogen bromide cleavage fragment sequences. The derived amino acid sequence includes a mature protein 600 amino acids long and a precursor sequence 58 amino acids long. The latter contains a stretch of amino acids known as peroxisomal targeting signal 2. The size of the mRNA was estimated to be around 4200 nucleotides. Recombinant His-tagged alkyl-dihydroxyacetonephosphate synthase expressed in Escherichia coli was enzymatically active.

The biosynthesis of ether phospholipids requires the concerted action of two enzymes and starts with the acylation of dihydroxyacetonephosphate (DHAP)1 by the enzyme DHAP acyltransferase (EC 2.3.1.42). The ether linkage is then introduced by a second 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. Both enzymes are mainly, if not exclusively, located in peroxisomes (4).

The importance of peroxisomes for human physiology was emphasized by the discovery of inherited diseases in humans caused by the loss of one or more peroxisomal functions (5). The prototypic Zellweger syndrome is a severe disorder characterized by a general loss of peroxisomal functioning, including impaired ether lipid synthesis, defective peroxisomal β-oxidation, and defective phytic acid oxidation. A disorder with only a limited loss of peroxisomal functions is rhizomelic chondrodysplasia punctata, in which phytic acid oxidase, DHAP acyltransferase, and alkyl-DHAP synthase are impaired. Furthermore, in this disorder the peroxisomal 3-oxoacyl-CoA thiolase is present as a 44-kDa precursor rather than as the 41-kDa mature enzyme (6).

The deficiency of ether phospholipids in a number of peroxisomal disorders has clearly emphasized the indispensable role of peroxisomes for ether lipid synthesis in humans by indicating that this process cannot be taken over anywhere else in the cell. Studies on the peroxisomal enzymes required for glycerol-ether bond formation have mainly been confined to experiments with crude subcellular or purified peroxisomal fractions. Although these studies have yielded highly relevant and valuable information on the intraperoxisomal localization and enzymological properties of the enzymes, including their mechanism of action (for recent review, see Ref. 7), they have provided little information on their structure. DHAP acyltransferase has only recently been purified to near homogeneity from guinea pig liver (8) and from human placenta (9), and enzymatic activity for guinea pig liver and human placenta was shown to reside in proteins of 69 and 65 kDa, respectively. Partial purifications of alkyl-DHAP synthase from Ehrlich ascites cells (10) and from guinea pig liver (11) have been described, but no information on molecular weight was reported. Recently, we succeeded in the purification of alkyl-DHAP synthase from guinea pig liver to homogeneity and provided evidence that the enzyme consisted of a single polypeptide chain with a molecular mass of 65 kDa (12). Both N-terminal and internal amino acid sequences have now been obtained from this protein, and we here report on the cloning and expression of the cDNA.

Ether phospholipids constitute a special class of natural phospholipids. In mammals, these have either an alkyl or an alkenyl ether linkage at the sn-1 position and an acyl ester linkage at the sn-2 position of the glycerol backbone. Little is known about the specific functions of ether phospholipids. The only representative for which biological roles have clearly been established is 1-alkyl-2-acetyl-sn-glycero-3-phosphocholine, better known by its trivial name, platelet activating factor (1).

Other possible functions of ether phospholipids may include the protection of cells against certain types of oxidative stress by plasmalogens (2, 3).

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) Y08826.

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1 The abbreviations used are: DHAP, dihydroxyacetonephosphate; IPTG, isopropyl-β-D-thiogalactopyranoside; PCR, polymerase chain reaction; bp, base pair; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid; PTS2, peroxisomal targeting signal 2.
sequence coding for alkyl-DHAP synthase. The deduced amino acid sequence indicates that the mature enzyme is preceded by a 58-amino acid N-terminal extension containing a targeting signal to direct the protein to peroxisomes.

EXPERIMENTAL PROCEDURES

Materials

ProBlott was from Applied Biosystems, Foster City, CA. Cyanogen bromide was from Aldrich. Triton X-100 was from Serva, Heidelberg, Germany. Moloney murine leukemia virus reverse transcriptase was bought from New England Biolabs. DNA polymerase and pGEM-T vector systems were from Promega, Madison, WI. Oligonucleotides were from New England Biolabs.

Methods

Enzyme Purification—Purification of alkyl-DHAP synthase from guinea pig liver was done as described before, with some changes (12). Rather than with a Triton X-100 gradient from 0.075 to 0.4% (w/v) in buffer C from Stratagene, La Jolla, CA, and pET-15b vector was from Novagen, Madison, WI.

Cyanogen Bromide Cleavage—Cyanogen bromide cleavage was based on the methods described by Gross (13). Briefly, alkyl-DHAP synthase (30 μg) was treated in 100 μl of 70% aqueous formic acid with 10 mg/ml cyanogen bromide at room temperature in the dark under nitrogen. After 24 h, 0.5 ml of H2O was added and the material was lyophilized. This was repeated once for total removal of cyanogen bromide.

Electrophoresis and Amino Acid Sequencing—Samples (10 μg of alkyl-DHAP synthase in case of N-terminal analysis, 30 μg of alkyl-DHAP synthase in case of internal analysis) were separated electrophoretically on a 10% T/4% C tricine SDS polyacrylamide gel as described by Schägger and von Jagow (14). The proteins or peptides were then eluted batchwise with 0.4% (w/v) Triton X-100 in buffer C from the concanavalin A column. Peak fractions were concentrated via a trichloroacetic acid precipitation followed by an acetone/ammonia precipitation. When protein was isolated for cyanogen bromide cleavage, a final purification step with the Bio-Rad PrepCell was used to obtain completely pure alkyl-DHAP synthase.

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Reverse Transcription—Total RNA was isolated from 1.5 g of guinea pig liver using a LiCl/urea method (15). Poly(A)+ RNA was isolated with the Oligotex-dt kit (Qiagen) according to the manufacturer’s instructions. The reverse transcriptase reaction (total volume, 30 μl) contained approximately 5 μg of heat-denatured (5 min at 95°C) mRNA, 75 units of Moloney murine leukemia virus reverse transcriptase, 1 μm of each dNTP, 40 units of RNAGuard, 50 mM Tris-HCl (pH 8.3), 8 mM MgCl2, 30 mM KCl, 10 mM dithiothreitol, 4 μM dT-17 primer, and 17 μg/ml random hexamers. The dT-17 primer contained a Sall site at the 5’ end (GACGTCGACTTTTTTTTTTTTTTT). The reaction was first incubated for 10 min at 23°C and then for 60 min at 37°C in the case of the hexamer primed reaction and just 60 min at 37°C in the case of the dT-17 primed reaction. PCR was performed in a volume of 50 μl with sense primer 1 against amino acids 8–16 of the N-terminal sequence (CCIGCCACGCGCICCGAC/G/C/G/T/G/CG/C/G/T/AC) and antisense primer 2 against amino acids 2–7 of fragment IV/VII (compare Fig. 1) (CC(A/G)AA(T/C)TG(A/G)AA(T/C)TG(T/C)TG(A/G) TT), both at a concentration of 1 μM, 200 μM of each dNTP, 2 units of Taq DNA polymerase, 10 mM Tris-HCl (pH 9 at 25°C), 50 mM KCl, 0.1% Triton X-100, and 1.5 mM MgCl2. The PCR temperature profile consisted of 2 min at 95°C and 35 cycles of 45 s at 95°C, 30 s at 57°C, 3 min at 72°C, and, finally, 7 min at 72°C. An 8-μl aliquot was analyzed on an ethidium bromide-stained 1% agarose gel. PCR fragments were cloned into a pGEM-T vector using standard techniques (16). Nucleotide sequences were determined with a T7 sequencing kit using deaza-G/A mixes to minimize the risk of sequence errors. Several independent clones were analyzed to guard against errors made by Taq DNA polymerase. Both strands were sequenced.

Amplification of cDNA Ends—The 3’ end of the alkyl-DHAP synthase cDNA was obtained by PCR using sense primer 3, corresponding to nucleotides 1072–1092 (compare Fig. 2) and a dT-17 primer containing a Sall site. dT-17 primer first-strand cDNA was used as template. The temperature profile was essentially the same as above. Reactions were analyzed on ethidium bromide-stained agarose gels and with Southern blot techniques (16). Products were cloned into the pGEM-T vector (16). Products were cloned into the pGEM-T vector.

To obtain the 5’ end of the cDNA sequence, hexamer-primed cDNA was tagged with dATP using terminal deoxynucleotidyltransferase. Excess hexamers and dNTPs from the reverse transcriptase reaction were removed using Microcon-30 microcentrators (Amicon). The recovered cDNA was then tailed in a total volume of 10 μl containing 140 mM K-acetate, pH 7.2, 1 mM CoCl2, 1 mg/ml bovine serum albumin, 0.33 mM ZnSO4, 0.25 mM dATP, and 15 units of terminal deoxynucleotidyltransferase. The reaction was incubated at 37°C for 10 min and at 65°C for 15 min. PCR was performed on this preparation using antisense primer 4, corresponding to nucleotides 283–303, and the dT-17 primer for hybridization with the introduced dA tail. PCR products were cloned and sequenced as described above. Because this method failed to obtain clones long enough to include the start codon, the procedure was repeated with an antisense primer corresponding to nucleotides 106–123.

Northern Blot Analysis—The 1100-bp fragment obtained by PCR was labeled with 40 μCi of [32P]-dCTP using a deacrylase DNA labeling kit from Ambion according to the manufacturer’s instructions. An aliquot of 20 μg total RNA was separated on a 0.6% (w/v) agarose/formaldehyde gel and transferred to a nylon sheet by capillary blotting. Blots were baked at 80°C for 2 h and prehybridized at 42°C in 1× NaCl, 50% formamide, 10% dextrose sulfate, 0.5% SDS, and 100 μg/ml herring sperm DNA. Hybridization was performed with the [32P]-labeled probe.
overnight. The nylon sheet was washed twice with 2x SSC, 0.1% SDS at room temperature for 20 min and for 10 min with 0.2x SSC, 0.5% SDS at 55°C. Autoradiograms were obtained by exposing the blot to x-ray film with an intensifier screen at 280°C for 1 week.

Expression of His-tagged Alkyl-DHAP Synthase in Escherichia coli—Guinea pig liver alkyl-DHAP synthase cDNA was obtained by PCR with sense primer ATTCTCGAGCCCGCGGCACCGGAGTCTG (nucleotides 223–241) containing an XhoI site (underlined) and antisense primer TTACCATTGTTGAAGTCT (nucleotides 2020–2037) using guinea pig liver cDNA as a template. To minimize the risk of introducing errors, Pfu DNA polymerase (Stratagene) was used. The PCR fragment was cloned after digestion with XhoI into a pET-15b vector digested with BamHI (filled in) and XhoI. The construct was transformed in E. coli strain BL21(DE3). A 100-ml culture was grown overnight in Luria-Bertani medium containing 50 μg/ml ampicillin and used to inoculate 1 liter of Luria-Bertani medium containing 50 μg/ml ampicillin. When the OD had reached 0.5, the culture was induced with 0.5 mM IPTG and grown for an additional 3 hours. Cells were harvested by centrifugation at 5000 rpm for 10 min in a Sorvall RC-2B centrifuge and resuspended in 50 ml of ice-cold 50 mM Tris, pH 8.0, 40 mM EDTA, 0.2 mg/ml lysozyme. After 30 min, an osmotic shock was given by addition of 50 ml of 50 mM Tris, pH 8.0, and 40 mM EDTA. After 30 min, the cell suspension was sonicated three times for 90 s. Thereafter, 1.9 ml of 10% Triton X-100 was added and the suspension was again sonicated for 2 min. The inclusion bodies were pelleted by centrifugation at 40,000 rpm to remove all insoluble material.

Analytical Procedures—SDS-polyacrylamide gel electrophoresis was done as described by Laemmli (17). The alkyl-DHAP synthase activity assay was performed as described previously (12). Protein was determined according to Bradford (18) with bovine serum albumin as the standard.

RESULTS AND DISCUSSION

Protein Sequences—Purified alkyl-DHAP synthase was subjected to SDS-polyacrylamide gel electrophoresis and stained with Coomassie blue R-250 and silver stain. The alkyl-DHAP synthase was visualized by staining with Coomassie blue R-250 and silver stain. A 65-kDa protein band was observed. The N-terminal amino acid sequence of alkyl-DHAP synthase, as deduced from three independent determinations, was found to be...
be Lys-Ala-Arg-Arg-Ala-Ser-Ala-ALA-Thr-Ala-Ala-Pro-Thr-Ala-Thr-Pro-Ala-Ala-Pro-Glu-Ser-Gly-Ile-Ile.

In order to get additional information about the amino acid sequence of guinea pig liver alkyl-DHAP synthase, peptides were prepared by cyanogen bromide treatment of the enzyme. The cyanogen bromide treatment resulted in the complete disappearance of the mature enzyme and yielded several fragments upon tricine SDS-polyacrylamide gel electrophoresis (Fig. 1). Only the determinations that yielded unequivocal sequences upon tricine SDS-polyacrylamide gel electrophoresis (Fig. 1). Only the determinations that yielded unequivocal sequences are depicted. Because fragment I and VII turned out to be identical, we conclude that the cyanogen bromide cleavage was not complete at all methionines.

PCR-based Cloning of cDNA—PCR performed with sense primer 1 and antisense primer 2 as described under “Experimental Procedures” yielded a 1100-base pair fragment on an ethidium bromide-stained agarose gel (results not shown). Sequencing confirmed that this band was indeed a fragment of alkyl-DHAP synthase cDNA, because this DNA fragment also coded for amino acids 16–25 of the N terminus. Furthermore, the derived amino acid sequence also included the sequence of cyanogen bromide fragment VI.

The PCR reaction products described under “Experimental Procedures” to obtain the 3′ end of the cDNA were analyzed with a Southern blot using a 32P-labeled DNA probe corresponding to the region between antisense primer 2 and sense primer 3 (data not shown). Discrete bands were observed at 500 and 1000 bp and some faint bands at 1800 and 2200 bp. Two clones of the 1000-bp fragment were obtained in the pGEM-T vector. The derived amino acid sequence of these clones included the sequence of cyanogen bromide fragment III and an in-frame stop codon. No canonical polyadenylation signal (AATAAA) was found. Therefore, we assume that the T7 primer was able to anneal internally to an A-rich sequence, rather than to the poly(A) tail.

To further validate the obtained sequence, a PCR reaction was performed with primer 3 and an antisense primer corresponding to nucleotides 2030–2057 using hexamer-primed cDNA as a template. A single band with a length of about 1000 bp was obtained; it was cloned and sequenced. The obtained clones completely confirmed the previously determined sequence.

In order to obtain the sequence information about the 5′ end of the cDNA, the PCR reaction products were analyzed by Southern blotting using a 32P-labeled synthase-specific probe (corresponding to the region between primers 1 and 4). A smear was observed ranging from 100 to about 400 nucleotides. These PCR products were cloned into the pGEM-T vector. The clones obtained this way were heterogeneous in size and contained DNA sequences completely consistent with the N-terminal amino acid sequence but did not yet contain a potential start codon. Therefore, the procedure was repeated using a primer upstream of primer 4 (see “Methods”). This yielded clones with an in frame ATG. This ATG represents almost certainly the start codon, because the surrounding sequence fits the Kozak sequence (19) very nicely and there is an in-frame TAG stop codon (nucleotides 12 to 10) upstream of this ATG.

Nucleotide and Deduced Amino Acid Sequence—Fig. 2 represents the composite sequence of the cDNA corresponding to guinea pig alkyl-DHAP synthase and the predicted sequence of the protein. The N-terminal sequence is included, as well as the sequences derived from cyanogen bromide cleavage fragments. The latter are, as expected, preceded by methionines. A presence 58 amino acids long, which is not present in the mature enzyme, is found on the cDNA level. The predicted size of the mature enzyme (600 amino acids with a calculated molecular mass of 67.0 kDa) is in good agreement with the size observed by SDS-polyacrylamide gel electrophoresis (65 kDa). A FastA homology search revealed an unexpected 24.9% identity with Saccharomyces cerevisiae D-lactate dehydrogenase (cytochrome C) precursor (EC.1.1.2.4) (20) in a 293-amino acid overlap. In particular, alkyl-DHAP synthase amino acids 366–380 are completely identical with D-lactate dehydrogenase amino acids 309–323. The significance of this homology is presently not clear.

A hydrophobicity plot of the mature enzyme according to Kyte and Doolittle (21) gave no clear evidence for the presence of a hydrophobic membrane-spanning domain (data not shown). We previously reported (12) that efficient solubilization of alkyl-DHAP synthase from membranes prepared from peroxisome-enriched subcellular fractions required the presence of detergent. To that end, we used Triton X-100. Similarly, Brown and Snyder (22) solubilized the enzyme from Ehrlich ascites cell particulate fractions with Triton X-100, whereas Horie et al. (11) used CHAPS to solubilize the enzyme from guinea pig liver peroxisomes. However, the requirement of detergents for solubilization of membrane associated proteins is not restricted to integral membrane proteins with hydrophobic transmembrane segments. This has recently been exemplified by studies on prostaglandin H₂ synthase 1. This enzyme was classified as an integral membrane protein because deter-
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Thus, both proteins may well be processed by the same peroxisomal protease. In this respect, it is interesting to note that in the peroxisomal disorder rhizomelic chondrodysplasia punctata, the peroxisomal thiolase is neither imported nor processed (6). It is conceivable that this may also be the case for alkyl-DHAP synthase and that these phenomena underlie the previously reported deficiency of alkyl-DHAP synthase enzymatic activity in rhizomelic chondrodysplasia punctata (31). The experimental addressing of these possibilities will have to await the development of specific antibodies.

Alkyl-DHAP Synthase mRNA and Expression of the Enzyme in E. coli—Northern blot analysis (Fig. 4) revealed one major band with an estimated size of about 4200 nucleotides. Two additional faint bands with a higher molecular weight were detected as well. Because these bands could not be removed by further, more stringent washing, we assume that these represent alkyl-DHAP synthase mRNAs that are not fully processed.

Induction of pET-15b-alkyl-DHAP synthase-transformed E. coli strain BL21(DE3) with IPTG resulted in the expression of a 65-kDa protein and, to a much lesser extent, of an approximately 55-kDa protein (Fig. 5). The latter most likely consists of a degradation product of the 65-kDa protein. Both proteins were found to be highly enriched in inclusion bodies, although the 65-kDa protein was partially recovered in the supernatant as well (Fig. 5, lane 6). The specific activities of alkyl-DHAP synthase were measured in homogenates, inclusion bodies, and supernatant and expressed as milliunits/mg protein. As expected, the homogenate of control cells that had been transformed with an empty vector showed no detectable synthase activity. High enzymatic activities of 3.1 and 2.3 milliunits/mg were found in homogenates and supernatants, respectively, of IPTG-induced cells transformed with a vector carrying the synthase insert. For comparison, the specific activity of a guinea pig liver homogenate under these conditions amounts to 0.03 milliunits/mg protein (12). The specific synthase activity of 0.06 milliunits/mg in inclusion bodies suggests that the enzyme is not easily accessible for substrate in these precipitates or that it is present in a less active form. The latter may hold for the expressed enzyme in general. When the amount of the 65-kDa protein in homogenate and supernatant was estimated by visual inspection of staining intensities against known amounts of bovine serum albumin, the soluble alkyl-DHAP synthase was estimated to have a specific activity amounting to about 15% of that previously determined for the enzyme purified from guinea pig liver (12). This lower value may be caused by the presence of the His-tag in the expressed enzyme or may result from less efficient folding in E. coli. Nevertheless, the fact that the expressed enzyme is active provides independent proof that we cloned the cDNA for alkyl-DHAP synthase.

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FIG. 5. Expression of His-tagged alkyl-DHAP synthase in E. coli as shown by SDS-polyacrylamide gel electrophoresis. The gel was stained by Coomassie Brilliant Blue. Lane 1, molecular mass markers; molecular masses in kDa given to the left of lane 1. Lane 2, homogenate of E. coli transformed with an empty pET-15b vector (alkyl-DHAP synthase activity, 0.00 milliunits/mg). Lane 3, homogenate of E. coli transformed with pET-15b alkyl-DHAP synthase before induction. Lane 4, homogenate of E. coli transformed with pET-15b alkyl-DHAP synthase after induction (activity, 3.17 milliunits/mg). Lane 5, inclusion bodies (activity, 0.06 milliunits/mg). Lane 6, supernatant (activity, 2.35 milliunits/mg). Lane 7, molecular mass markers. The amount of bacterial protein in each lane corresponds to 60 µl of culture.

Two targeting signals for peroxisomal proteins have been identified in recent years. Peroxisomal targeting signal 1 consists of a C-terminal tripeptide with the consensus sequence SKL (26–28). This signal is used exclusively for matrix proteins and is not processed, as evidenced by the fact that many matrix proteins can be detected by anti-SKL antibodies. A cleavable peroxisomal targeting signal 2 (PTS2) has initially been identified by Swinkels et al. (29) in the N-terminal extension of rat 3-ketoacyl-thiolase, a rather rare example of a peroxisomal protein that is not synthesized at its mature size but with an N-terminal presequence. Similar PTS2 signals appeared to be present in mature yeast thiolases (29). Fig. 3 shows that the presequence of alkyl-DHAP synthase contains the peroxisomal targeting signal 2 in the amino acid sequence at positions 37–45. In particular, the homology with the PTS2 signal in mammalian 3-ketoacyl-thiolases is excellent. It is reasonable to assume that the presence of this signal is responsible for directing alkyl-DHAP synthase to peroxisomes. It is noteworthy that the presequence of alkyl-DHAP synthase, with its 58 amino acids, is considerably longer than the N-terminal extensions of either the A- or the B-type precursors for rat thiolases, which consist of 36 and 26 amino acids, respectively (29). The reason for this and the function of the extra amino acids, which have a remarkable poly-alanine segment, is currently unknown. It has recently been reported, however, that the N terminus of PTS2 carrying proteins can be extended by a large number of additional residues and still be recognized by Pas7p, the import receptor for PTS2 proteins in S. cerevisiae (30).

Apart from the PTS2 signal, alkyl-DHAP synthase and mammalian thiolases contain additional sequence homology in their presequences, including a cysteine at the cleavage site (Fig. 3).
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