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Recombinant human dihydroxyacetonephosphate acyl-transferase characterization as an integral monotopic membrane protein

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ABSTRACT

Although the precise functions of ether phospholipids are still poorly understood, significant alterations in their physiological levels are associated either to inherited disorders or to aggressive metastatic cancer. The essential precursor, alkyl-dihydroxyacetone phosphate (DHAP), for all ether phospholipids species is synthetized in two consecutive reactions performed by two enzymes sitting on the inner side of the peroxisomal membrane. Here, we report the characterization of the recombinant human DHAP acyl-transferase, which performs the first step in alkyl-DHAP synthesis. By exploring several expression systems and designing a number of constructs, we were able to purify the enzyme in its active form and we found that it is tightly bound to the membrane through the N-terminal residues.

1. Introduction

Ether phospholipids are characterized by an ether bond, rather than an ester bond, on the *sn*-1 position of the glycerol backbone. Although the detailed physiological roles of intracellular and circulating ether linked phospholipids are not yet well understood, their peculiar physical properties impact on many aspects of cell signaling and membrane biology [1,2]. In addition to their importance as structural components of cell membranes, they are fundamental for membrane fusion and vesicle formation, and they are involved in free radical scavenging and second messenger lipids storage [3,4]. Their importance is further underlined by the pathological conditions associated to altered synthesis. In fact, most of the known peroxisomal disorders show deficient ether lipids synthesis [5,6]. Additionally, elevated ether lipids synthesis and uptake are a characteristic metabolic aberration of certain type of aggressive cancers, contributing to their invasiveness and survival [7].

The key enzymes involved in the ether phospholipids biosynthetic pathway, namely dihydroxyacetone phosphate acyltransferase (DHAPAT) and alkyl-dihydroxyacetone phosphate synthase (ADPS), catalyze the acylation of dihydroxyacetone phosphate (DHAP) to form acyl-DHAP followed by the replacement of the acyl moiety with a long fatty alcohol, yielding alkyl-DHAP [8]. Mutations in the genes encoding for DHAPAT and ADPS cause a rare inherited peroxisomal disorder, rhizomelic chondrodysplasia punctata (RCDP). RCDP patients show skeletal dysplasia and profound mental retardation, which in most of the cases lead to premature death [9].

Whereas the unusual reaction mechanism of ADPS flavin fueled the interest in the biochemical characterization of this enzyme [10–12], the difficulties associated to DHAPAT expression and purification slowed progress for decades. DHAPAT performs the first step of the ether phospholipid biosynthetic pathway, namely the acylation of DHAP to form acyl-DHAP, which is the substrate of the downstream ADPS reaction. The peroxisomal localization of DHAPAT is crucial for its enzymatic activity and stability, and it is achieved by the presence of the peroxisomal targeting signal-1 (PTS1) at the C-terminus of its amino acid sequence [13]. For long, the organization of the gene encoding for DHAPAT has remained undetermined [14], although the protein itself was isolated from human placenta [15] and guinea pig liver [16] as a 680 amino acid enzyme with a molecular mass of 77 kDa [17]. *In vivo* experiments suggested that DHAPAT activity was dependent on the presence of ADPS. In particular, kinetic experiments indicated that endogenously generated acyl-DHAP was used preferentially by ADPS in comparison with exogenously added substrate, suggesting a close interaction of the two enzymes within the peroxisomes [18,19]. The formation of DHAPAT/ADPS complex was initially characterized by

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isolating the cross-linked enzymes from rabbit Harderian gland peroxisomes, and this remains the only evidence of the hetero-trimeric complex formation so far [20].

The results presented in this study aim to develop a methodology to express and purify for the first time recombinant human DHAPAT using *P. pastoris* to enable the study of its interaction with ADPS.

2. Materials and methods

2.1. Full-length human DHAPAT in HEK-293E cells: cloning, expression and purification

Human DHAPAT cDNA (NM_014236.3) was supplied by Origene. N-terminal His_8-eGFP tag was fused to DHAPAT through PCR and cloned into pCDNA3 vector (Invitrogen). HEK-293E (Invitrogen) cells were grown in suspension according to Longo et al. [21]. The transfection mixture contained polyethylenimine (PEI; DNA:PEI = 1:3 w/w), OPTIMEM medium (Gibco, 1:20 v/v) and pCDNA3-His_8-eGFP-DHAPAT (1 μg of DNA/10^6 cells). Cells were collected after 24/48 h after transfection. Frozen cell pellets were resuspended in hypotonic buffer (10 mM Tris-HCl pH 7.5, 1 mM EDTA) with protease inhibitors Complete EDTA-Free (Roche), 1 mM PMSF, and 1 mg/ml DNAse, and homogenized on ice 30 times with a tight pestle. The sample was centrifuged at 70,000*g* for 1 h. Cell membranes were resuspended in 30 mM HEPES pH 7.8, 500 mM NaCl, 50% glycerol, and protease inhibitors. Solubilization was performed adding 1% of detergent (Anatrace) and stirring 2 h at 4 °C followed by centrifugation at 70,000*g* for 1 h. Fluorescence size-exclusion chromatography (F-SEC) experiment was performed according to Goering et al., 2014 [22], using a Superdex 75 5/150 column (GE Healthcare) and a Schimadzu HPLC system equilibrated with 30 mM HEPES pH 7.8, 500 mM NaCl, 5% glycerol and 0.03% DDM.

2.2. Bioinformatic analysis of DHAPAT amino acid sequence

The secondary structure prediction resulted from the comparison of the following servers: HHpred, Quick2D [23], PredictProtein [24], Proteus 2.0 [25]. The transmembrane (TM) helices/region prediction was performed by interrogating the following servers: POLYVIEW-2D (117–138) [26], TMAP (115–132, 168–196, 370–398, 565–580) [27], TMPRED (8–25, 165–194) [28], Octopus (510–560) [29].

2.3. Human DHAPAT deletion mutants: cloning, expression and purification in E. coli cells

DHAPAT shorter constructs were obtained by PCR, either lacking of N-terminal portions (DHAPATΔ120, DHAPATΔ135, DHAPATΔ144, DHAPATΔ150, DHAPATΔ156, DHAPATΔ285, DHAPATΔ530) or single domains (DHAPAT144-530, DHAPAT156-530, DHAPAT1-163), and cloned in pET28 vectors (Novagen) containing PreScission protease cleavable N-terminal tags (His_8-SUMO, GST and His_8-eGFP). Expression trials were performed in BL21, Origami, Rosetta, DH5α, Rosetta-gami and C41 *E. coli* strains (Novagen). GST-DHAPATΔ135 expressed using Origami was chosen for further expression and purification experiments. The culture was grown at 37 °C and 200 rpm in TB medium (Terrific Broth) until OD_{600} of 1 and induced by adding 500 μM isopropyl-β-D-1-thiogalactopyranoside, shifting the temperature to 17 °C for 20 h. Collected cells were resuspended in 50 mM Tris-HCl pH 8.0, 500 mM NaCl, 5% glycerol, protease inhibitors Complete EDTA-Free (Roche), 1 mM PMSF, and 1 mg/ml DNAse. Resuspended cells were disrupted by sonication and centrifuged at 70,000*g* for 40 min at 4 °C. The soluble fraction was loaded on a 5 ml GSTrap FF pre-packed column (GE Healthcare), equilibrated with lysis buffer, using the ÄKTA FPLC system (GE Healthcare), and eluted with 50 mM of reduced glutathione. The sample was incubated at 4 °C with GST-tagged PreScission protease, 1:100 v/v overnight. Tag and protease were removed in a second run with the GST-trap column. The fractions containing the protein were pooled and loaded on an ion-exchange column (SOURCE 15Q, GE Healthcare), followed by a size-exclusion chromatography step (Superdex 200 10/300, GE Healthcare).

2.4. Full-length human DHAPAT: cloning, expression and purification in P. pastoris cells

Human DHAPAT cDNA fused with a N-terminal His_8-GFP*uv* (PreScission protease cleavable) and codon-optimized for *P. pastoris* expression was purchased from DNA2.0, and sub-cloned in the pJexpress 902 (DNA2.0) vector. The resulting plasmid was linearized using *Sac*I enzyme (Thermo Scientific). The transformation was performed according to Lin-Cereghino et al. [30], using *P. pastoris* KM71H MutS strain (Invitrogen). The colonies were tested for DHAPAT expression by inoculating them in 24 deep-well plates containing 2 ml of BMGY medium (100 mM HK_2PO_4 pH 6.0, 6.34% Yeast Nitrogen Base, 1% glycerol, 4×10^{-5}% biotin), incubating at 30 °C, 280 rpm for 60 h. The medium was exchanged with BMM medium (100 mM HK_2PO_4 pH 6.0, 6.34% Yeast Nitrogen Base, 0.5% methanol, 4×10^{-5}% biotin) to induce protein expression, and after 48 h GFP*uv* fluorescence was measured with a Clariostar plate reader (BMG Labtech; excitation 395 nm, emission 509 nm). Positive clones were inoculated in 15 ml of BMGY medium. The pre-inoculum was incubated for 30 min at 30 °C (300 rpm). 2.5 ml of the pre-inoculum was poured in 2 L baffled-flask containing 200 ml of BMGY medium, and incubated for 72 h at 30 °C (280 rpm). Cells were harvested at 1500 g for 10 min and resuspended in 100 ml of BMM medium. To allow the induction to continue for 72 h, 0.5% methanol was added every 24 h. The best expressing clone was grown in a 5 L Bioflo 3000 Fermentor (Brunswick) at 30 °C and 72 h of methanol induction, according to Damasceno et al., 2004 [31]. Cells were collected by centrifugation at 1,500*g* for 10 min at 4 °C, resuspended in 50 mM Bicine pH 9.0, 500 mM NaCl, 10% glycerol, protease inhibitors Complete EDTA-Free (Roche), 1 mM PMSF, and 1 mg/ml DNAse, and lysed using a bead beater with zirconia beads (Biospec). Cell membranes were collected by centrifugation at 70,000*g* for 2 h at 4 °C. F-SEC experiment was performed as described above [22]. Solubilization was performed by adding 1% of FSC-12 (Anatrace) to the resuspended membranes (50 mM Bicine pH 8.2, 150 mM NaCl, 5% glycerol and protease inhibitors), stirring overnight at 4 °C. Solubilized membranes were centrifuged at 70,000*g* for 1.5 h. The supernatant was incubated with 5 ml of slurry Ni-Sepharose resin (GE Healthcare), stirring at 4 °C for 2 h. The buffers used for the washing steps had decreasing amounts (till 0,05%) of FSC-12. The elution step was performed using 300 mM of imidazole in the binding buffer. Tag-cleavage was performed incubating the sample with PreScission protease (GE Healthcare) in 1:10,000 v/v ratio, overnight at 4 °C. The second affinity chromatography step and the other purification steps were carried out using the ÄKTA FPLC (GE Healthcare), loading the sample on a pre-packed nickel column (His-trap, GE-Healthcare). The collected fractions were concentrated with the Amicon Ultra concentrator (30 kDa cutoff). Size-exclusion chromatography was performed using a Superdex 200 10/300 column (GE Healthcare), equilibrated with buffer containing 50 mM bicine pH 8.2, 50 mM NaCl, 5% glycerol and 0.05% FSC-12.