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Biochemical characterization of the very long-chain fatty acid elongase ELOVL7

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ABSTRACT

Very long-chain fatty acids (VLCFAs) have a variety of physiological functions and are related to numerous disorders. The key step of VLCFA elongation is catalyzed by members of the elongase family, ELOVLs. Mammals have seven ELOVLs (ELOVL1-7), yet none of them has been purified and analyzed. In the presented study we purified ELOVL7 and measured its activity by reconstituting it into proteoliposomes. Purified ELOVL7 exhibited high activity toward acyl-CoAs with C18 carbon chain length. The calculated K_m values toward C18:3(n-3)-CoA and malonyl-CoA were both in the μM range. We also found that progression of the VLCFA cycle enhances ELOVL7 activity.

1. Introduction

Most cellular lipid molecules include as a major constituent at least one fatty acid (FA). FAs are diverse in carbon chain length and degree of saturation, which contributes greatly to the variety of functions exhibited by the lipids. The most abundant cellular FAs are long-chain fatty acids (LCFAs) with 16–18 carbons. Very long-chain fatty acids (VLCFAs), those having a chain length >20 carbons, are less abundant than LCFAs but possess unique functions that cannot be substituted for by LCFAs. LCFAs such as palmitic acid (C16:0), oleic acid (C18:1), and linoleic acid (C18:2) are mainly utilized as components of glycerophospholipids and triacylglycerol [1], the latter of which is relevant to obesity and hyperlipidemia. In contrast, most saturated or monounsaturated VLCFAs are incorporated into sphingolipids and play important functions in physiological processes such as skin barrier and myelin formation [2,3]. Moreover, polyunsaturated VLCFAs such as docosahexaenoic acid (C22:6) exhibit anti-inflammatory and anti-atherogenic effects [4].

VLCFAs are synthesized in the endoplasmic reticulum from LCFAs, which are food-derived or synthesized in the cytosol de novo by FA synthase. VLCFA elongation occurs by cycling through a 4-step reaction (condensation, reduction, dehydration, and reduction), with 2 carbons being added through each cycle [5]. In the first reaction acyl-CoA and malonyl-CoA are condensed to 3-ketoacyl-CoA by one of the FA elongases named the ELOVLs [5]. The second step is an NADPH-dependent reduction catalyzed by the 3-ketoacyl-CoA reductase KAR to produce 3-hydroxyacyl-CoA [6], and the third, a dehydration step by one of the 3-hydroxyacyl-CoA dehydratases HACD1-4 [7]. The resulting 2,3-*trans*-enoyl-CoA is finally reduced to acyl-CoA by the *trans*-enoyl-CoA reductase TER, which is NADPH-dependent [6].

The ELOVLs catalyze the first, rate-limiting step of the VLCFA elongation cycle. In mammals, seven ELOVL isozymes (ELOVL1-7) exist, and each has a specific substrate specificity and physiological functions [5]. ELOVLs are also associated with known pathologies. Dominant *ELOVL4* mutations cause a juvenile macular dystrophy (Stargardt disease-3) [8], and *Elovl4* knockout mice die soon after birth due to a deficiency in skin barrier formation [9]. Studies in *Elovl6*-null mice have revealed that ELOVL6 is involved in an obesity-induced insulin resistance [10], and ELOVL7 is reportedly involved in prostate cancer growth [11].

We recently investigated the enzyme activities of all ELOVLs under identical conditions, and determined the substrate specificity of each [12]. We demonstrated that ELOVL1 exhibits high activity toward C20–C22 saturated and monounsaturated acyl-CoAs, and ELOVL7 elongates C16–C20 acyl-CoAs, with its highest activity toward C18-CoAs. In that study we determined the activities only at a fixed concentration (50 μM) of each substrate acyl-CoA, and

Abbreviations: VLCFA, very long-chain fatty acid; FA, fatty acid; LCFA, long-chain fatty acid; SC, synthetic complete; HMF, His_6, Myc, and 3xFLAG

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used as the enzyme source total membranes from cells overproducing each ELOVL. It is also important, though, to determine both the K_m and V_{max} values of purified enzymes, so as to evaluate the contribution of each ELOVL to the VLCFA elongation reaction. Although there have been reports on the enzymatic characterization of yeast ELOVL homologs using purified enzymes [13], there have been no such reports regarding the mammalian ELOVLs. In the presented study, we subjected ELOVL7, the least studied of the mammalian ELOVLs, to purification and determination of its K_m and V_{max} values. Although ELOVL7 lost its activity when solubilized in Triton X-100, we successfully measured its activity by reconstituting it into proteoliposomes. Moreover, by comparing the activity of purified ELOVL7 with activity in a membrane fraction, we determined that progression of the VLCFA cycle is required for maximal activity of ELOVL7.

2. Materials and methods

2.1. Cell culture and transfection

HEK 293T cells were grown in 0.3% collagen-coated dishes, in Dulbecco's modified Eagle's medium (Sigma, St. Louis, MO) containing 10% fetal bovine serum and supplemented with 100 units/ml penicillin and 100 μg/ml streptomycin. Transfections were performed using Lipofectamine Plus™ Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions.

The *Saccharomyces cerevisiae* strain DEY113 (*Δprb1::KanMX4 Δpep4::MET15*) is a derivative of BY4741 (*MAT**a** his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*) [14]. DEY113 cells carrying the pYS15 or pYS19 plasmid were grown in synthetic complete (SC) medium (0.67% yeast nitrogen base and 2% D-glucose) containing nutritional supplements.

2.2. Plasmids

The pCE-puro 3xFLAG-1 plasmid is a mammalian expression vector designed to produce an N-terminal 3xFLAG-tagged protein. The pCE-puro 3xFLAG-ELOVL7 plasmid is a derivative of the pCE-puro 3xFLAG-1 plasmid and encodes 3xFLAG-ELOVL7, as described previously [12]. The pAK1018 plasmid (*URA3* marker) is a yeast expression vector designed to produce an N-terminal, tandemly oriented His_6, Myc, and 3xFLAG (HMF)-tagged protein under the control of the *TDH3* (*glyceraldehyde 3-phosphate dehydrogenase*) promoter. The pYS15 plasmid encoding *HMF-ELOVL7* was constructed by cloning *ELOVL7* cDNA from the pCE-puro 3xFLAG-ELOVL7 plasmid into the pAK1018 vector. The pYS19 plasmid encoding *HMF-ELOVL7 (AA)* was created by site-directed mutagenesis using a QuikChange kit (Stratagene, Agilent Technologies, La Jolla, CA) and primers 5′-TTCCTTCATGTATTCGCTGCTACCATCATGCCGTG-3′ and 5′-CACGGCATGATGGTAGCAGCGAATACATGAAGGAA-3′.

2.3. Purification of HMF-ELOVL7

Yeast cells expressing HMF-ELOVL7 or HMF-ELOVL7 (AA) were suspended in buffer A (50 mM HEPES-NaOH (pH 6.8), 500 mM NaCl, 10% glycerol, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and a 1× protease inhibitor mixture (Complete™ EDTA free; Roche Diagnostics, Indianapolis, IN)) and lysed by vigorously mixing with glass beads using a BeadBeater (Biospec Products, Bartlesville, OK). After removal of cell debris by centrifugation (2000×*g*, 3 min, 4 °C), the supernatant was centrifuged at high speed (100000×*g*, 60 min, 4 °C). The resulting pellet (membrane fraction) was suspended in buffer A containing 2% Triton X-100. After an additional high speed centrifugation (100000×*g*, 60 min, 4 °C), the supernatant (solubilized fraction) was incubated overnight at 4 °C with anti-FLAG M2 agarose (Sigma) while rotating. The beads were washed six times in decreasing concentrations of Triton X-100 in buffer A. The bound proteins were then eluted with buffer A containing 0.1% Triton X-100 and 100 μg/ml 3xFLAG peptide.

2.4. In vitro FA elongase assay

Membrane fractions used for in vitro elongase assays were prepared as described previously [12]. Reconstitution of proteins into proteoliposomes was performed essentially as described elsewhere [13], using phosphatidylcholine (Avanti Polar Lipids, Alabaster, AL) as the liposomal lipid and Bio-beads SM-2 adsorbents (Bio-Rad, Hercules, CA) for adsorption of Triton X-100. In vitro FA elongation assays were performed as described previously [12].

2.5. Immunoblotting

Immunoblotting was performed as described previously [15], using the anti-FLAG antibody M2 (1 μg/ml; Stratagene, Agilent Technologies, La Jolla, CA) as the primary antibody, and HRP-conjugated anti-mouse IgG $F(ab')_2$ fragment (1:7500 dilution; GE Healthcare Bio-Sciences) as the secondary antibody. Labeling was detected using Pierce Western Blotting Substrate (Thermo Fisher Scientific, Waltham, MA).

3. Results

3.1. Progression of the VLCFA elongation cycle enhances ELOVL7 activity

In our previous report, we determined the substrate specificity of ELOVL7 using total membrane proteins prepared from HEK 293T cells overproducing 3xFLAG-ELOVL7 [12]. In that experiment we included the reducing agent NADPH, which is required for the second and fourth reactions of the VLCFA elongation cycle, but not for the ELOVL7-catalyzed first reaction. Under such conditions, the ELOVL7 product 3-ketoacyl-CoA was rapidly converted to acyl-CoA via 3-hydroxyacyl-CoA and 2,3-*trans*-enoyl-CoA [12]. Here, we compared the ELOVL7 activity in the presence or absence of NADPH. Total membrane proteins prepared from HEK 293T cells transfected with the vector or with a plasmid encoding *3xFLAG-ELOVL7*, were incubated with [^{14}C]malonyl-CoA and C18:3(n-3)-CoA. After the reaction, the acyl-CoAs produced were converted to FAs by saponification, then separated by TLC. In the presence of NADPH, the levels of the acyl-CoA generated by the ELOVL7 membrane preparations were much higher than those produced by control membranes (Fig. 1A, FA), as reported previously [12]. Low levels of 3-hydroxyacyl-CoA were also detected in the reaction products generated by the ELOVL7 membrane preparations. In contrast, when the reactions were performed in the absence of NADPH, 3-ketoacyl-CoA, but no acyl-CoA or 3-hydroxyacyl-CoA, was produced by the ELOVL7 membrane preparations (Fig. 1A). An additional band (Fig. 1A, asterisk) was also generated by the ELOVL7 membrane preparations, but this may represent a by-product of 3-ketoacyl-CoA, perhaps a decarboxylated form, formed during the saponification reaction. Little 3-ketoacyl-CoA was generated by control membranes in the absence of NADPH (Fig. 1A). Although NADPH itself is not involved in the ELOVL7-catalyzed reaction, the reaction products were more abundant in the presence of NADPH (Fig. 1A, acyl-CoA plus 3-hydroxyacyl-CoA) than in its absence (Fig. 1A, 3-ketoacyl-CoA plus 3-ketoacyl-CoA*). These results suggest that for ELOVL7 to exert its maximal activity, participation of the entire VLCFA elongation cycle is required.