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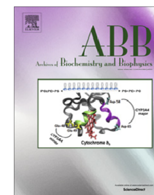

Possible allostery and oligomerization of recombinant plastidial *sn*-glycerol-3-phosphate acyltransferase

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ABSTRACT

Plastidial acyl-acyl carrier protein:*sn*-glycerol-3-phosphate acyltransferase (GPAT; EC 2.3.1.15) catalyzes the acyl-acyl carrier protein-dependent *sn*-1 acylation of *sn*-glycerol 3-phosphate (G3P) to produce lysophosphatic acid. Functional recombinant *Erysimum asperum* GPAT (EaGPAT), devoid of transit peptide, was produced in yeast. Analysis of the dependence of EaGPAT activity on increasing G3P concentration resulted in a hyperbolic response. EaGPAT exhibited a preference for 18-carbon unsaturated acyl-CoAs. Assays with concentrations of oleoyl-CoA up to 90 μM revealed an exponential response to increasing concentrations of acyl donor, and the introduction of increasing concentrations of unlabeled linoleoyl-CoA into the standard reaction mixture resulted in increased incorporation of radiolabeled oleoyl moieties into lysophosphatidic acid. Collectively, the kinetic results suggest that acyl-CoA may act as both substrate and allosteric effector. EaGPAT was also shown to oligomerize to form higher molecular mass multimers, with the monomer and trimer being the predominant forms of the enzyme. Since most allosteric enzyme exhibit quaternary structure, the self-associating properties of EaGPAT are consistent with those of an allosteric enzyme. These results could have important regulatory implications when plastidial GPAT is introduced into a cytoplasmic environment where acyl-CoA is the acyl donor supporting cytoplasmic glycerolipid assembly.

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Introduction

Plant plastidial *sn*-glycerol-3-phosphate acyltransferase (GPAT[3]; EC 2.3.1.15) catalyzes the in the *sn*-1 acylation of *sn*-glycerol 3-phosphate (G3P) to generate lysophosphatidic acid (LPA) [1]. Three types of GPAT exist in higher plant cells, localized in the plastid stroma (as a soluble protein), endoplasmic reticulum (ER) membrane, and mitochondrial membrane [2,3]. In different cell compartments, the natural acyl substrates for GPAT are distinct: in the ER and mitochondria, the endogenous acyl-donor is acyl-CoA, while in the plastid, it is acyl-acyl carrier protein (acyl-ACP) [1]. In the plastid, GPAT catalyzes the initial reaction in the Kornberg–Pricer pathway for glycerolipid biosynthesis leading to phosphatidic acid (PA), which is the precursor of various types of glycerolipids for building the subcellular membranes within the plastid (Fig. 1) [4,5]. Plastidial lysophosphatidic acid acyltransferase (LPAAT) catalyzes the acyl-ACP-dependent synthesis of PA from LPA [5]. In *Arabidopsis thaliana* (Arabidopsis), plastidial GPAT and LPAAT are often referred to as ATS1 and ATS2, respectively [5,6]. Plastidial GPAT is a soluble enzyme whereas LPAAT is membrane-bound [5]. The typical membrane lipids found in plastid are monogalactosyldiacylglycerol, digalactosyldiacylglycerol, phosphatidylglycerol (PG) and sulfoquinovosyldiacylglycerol [7]. Among these lipids, PG is predominantly synthesized within the plastid, while the others can be synthesized either by the plastidial or ER glycerolipid synthetic pathways depending on the plant species [7].

As the major phospholipid in the thylakoid membrane, PG plays a critical role in mediating the chilling sensitivity of plants [8]. Previous studies discovered that chilling tolerant plants normally have

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[3] *Abbreviations used:* ACAT, acyl-CoA:cholesterol acyltransferase; ACP, acyl-carrier protein; BN, blue native; BS3, *bis*[sulfosuccinimidyl]suberate; ER, endoplasmic reticulum; EST, expressed sequence tag; GPAT, *sn*-glycerol-3-phosphate acyltransferase; G3P, *sn*-glycerol 3-phosphate; LPA, lysophosphatidic acid; LPPAT lysophosphatidic acid acyltransferase; PA, phosphatidic acid; PG, phosphatidylglycerol; RACE, rapid amplification of cDNA ends.

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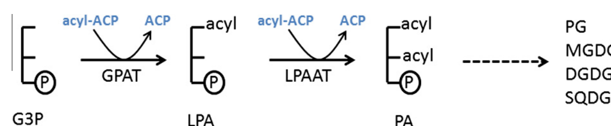

Fig. 1. Glycerolipid biosynthesis in plant plastid. Plant plastidial sn-glycerol-3-phoshate acyltransferase (GPAT; ATS1) catalyzes the *sn*-1 acylation of *sn*-glycerol 3-phosphate (G3P) to generate lysophosphatidic acid (LPA) with the endogenous acyl-donor, acyl-acyl carrier protein (ACP). Plastidial lysophosphatidic acid acyltransferase (LPAAT; ATS2) catalyzes the acyl-ACP-dependent synthesis of phosphatidic acid (PA) from LPA. In the plastid, PA is the precursor of various types of glycerolipids for building the subcellular membranes within this organelle. MGDG, monogalactosyldiacylglycerol; DGDG, digalactosyldiacylglycerol; SQDG, sulfoquinovosyldiacylglycerol; PG, phosphatidylglycerol.

a higher proportion of unsaturated fatty acids in PG in comparison to chilling sensitive plants [1]. Additionally, the fatty acid composition of PG is largely determined by the substrate selectivity of plastidial GPAT. For example, plastidial GPATs from chilling-tolerant plants such as spinach (*Spinacia oleracea*) and pea (*Pisum sativum*) exhibit increased selectivity for acyl-donors containing oleic acid ($18{:}1\Delta^{9cis}$; hereafter referred to as 18:1) compared to acyl donors containing palmitic acid (16:0) [9,10]. In contrast, GPATs from chilling-sensitive plants such *Amaranthus lividus* and squash (*Cucurbita moschata*) generally did not discriminate between acyl donors containing 18:1 versus 16:0 acyl chains [11–13]. In proof-of-concept genetic engineering studies, the introduction of plastidial GPAT from cold-tolerant Arabidopsis was found to increase chilling tolerance in tobacco (*Nicotiana tabacum*) [14] and rice (*Oryza sativa*) [15]. Conversely, the introduction of squash plastidial GPAT into tobacco resulted in a decrease in chilling sensitivity compared to the wild type [14]. In addition, introduction of *Escherichia coli* GPAT, which prefers 16:0-ACP, into Arabidopsis resulted in a decrease in chilling sensitivity presumably due to a decrease in the fluidity of the thylakoid membranes [8]. Xu et al. [16] characterized the biochemical and molecular defect of several *Arabidopsis* plastidial *gpat (ats1)* mutant lines and discovered that GPAT (ATS1) plays a coordinated regulatory role with the plastidial LPAAT (ATS2) in the biosynthesis of plastidial PG. Their results further revealed the important role of plastidial GPAT in regulating plastidial lipid metabolism and plant development.

Attempts to understand the mechanistic basis of the substrate selectivity of plastidial GPAT using a site-directed mutagenesis strategy is complicated by the fact that the amino acid sequences of chilling-resistant and chilling-sensitive plants are highly conserved [17,18]. Considerable progress in understanding structure/function in plastidial GPAT, however, was made possible through analysis of the crystal structure of the recombinant squash enzyme [17]. Modeling studies combined with site-directed mutagenesis showed that substituting Leucine 261 to a phenylalanine residue changed the specificity of squash GPAT from the native form which is non-selective to a mutant form which is selective for 18:1-ACP [13]. Crystallographic analysis did not reveal any changes in enzyme structure which suggested that the substitution to a phenylalanine residue altered the shape of the binding pocket. Homology modeling and protein structure-based sequence alignment of plastidial GPATs from chilling-resistant and chilling-sensitive plants has shown, however, that enzymes from both types of plants share a highly conserved domain containing the proposed substrate-binding cavity [19]. In contrast, the amino acid residues surrounding the substrate-binding pocket were found to be highly heterogeneous and may influence the size of substrate-binding pockets of plastidial GPATs. Using a combination of kinetic analysis and binding experiments, it was determined that recombinant squash GPAT binds acyl-ACP prior to and in the absence of G3P [19].

Erysimum asperum (western wallflower), a species within the Brassicaceae, is recognized as a cold tolerant plant in Alberta, Canada. We therefore hypothesized that this species could be a potential source of plastidial GPAT with increased selectivity for substrates containing unsaturated fatty acyl chains. The recombinant EaGPAT with deletion of the putative transit peptide was functionally produced in yeast. The recombinant EaGPAT exhibited a substrate preference for 18-carbon unsaturated acyl-CoAs. Additionally, recombinant EaGPAT exhibited an exponential response in enzyme activity to increasing concentrations of acyl-CoA suggesting that acyl-CoA could act as both substrate and positive allosteric effector. Enzyme assays with a fixed amount of [1-^{14}C]18:1-CoA and increasing concentrations of unlabeled linoleoyl ($18{:}2\Delta^{9cis,12cis}$; hereafter referred to as 18:2)-CoA resulted in a significant increase in the incorporation of radiolabel into LPA suggesting that 18:2-CoA, in addition to acting as a substrate, was acting at a site other than active site to stimulate the activity of EaGPAT. Blue native (BN)-PAGE and cross-linking experiments indicated that recombinant EaGPAT could oligomerize to form multimers with the monomer and trimer being the predominant forms of the enzyme observed following gel electrophoresis. Thus, for the first time, we present evidence for a self-associating plastidial GPAT that appears to be allosterically regulated by acyl-CoA.

Experimental

Plant materials

E. asperum plants were grown in a greenhouse under 16 h day/8 h night at 25 °C.

Cloning of plastidial GPAT full-length cDNA from E. asperum

Total RNA was extracted from the leaf tissues of the plant using RNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA). First-strand cDNA was synthesized using total RNA with QuantiTect reverse transcription kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. Based on the sequences of the conserved regions of several known plant plastidial *GPAT* genes, a cDNA fragment of a plastidial *GPAT* gene was amplified with the following primer pair: WWF1F (5′-TCCTCTTTGTAAGCCGTTCAGTATGGG-3′) and WWF1R (5′- TCTTTCTCAACCTGGGGTGGTGGTG-3′). The 5′ and 3′ ends of the full-length cDNA were amplified by rapid amplification of cDNA ends (RACE) using the GeneRacer Kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The primers used for 5′ and 3′ ends amplification were: WWF5′RACE (5′-GAAGGATTAGGGCGGTCCCTTCCAC-3′) and WWF3′RACE (5′-CGGAGAGAAAAG ATTAGTTGGGTTTCACGG-3′). For amplification of the full-length cDNA sequence of the *EaGPAT*, the following primer pair was used: WWF5′ (5′-GTA**ATG**GCTCTCACATTTTCCTCCTCCGCC-3′) and WWF3′ (5′-ATTCCAGGGTTGTGACAAGGAGACCGTTG-3′). Multiple sequence alignment was performed using ClustalW software [20] and Geneious software (http://www.geneious.com)./.

Heterologous expression of EaGPAT in yeast

Full-length and truncated *EaGPAT* cDNAs were over-expressed in yeast. The truncated cDNA fragment of *EaGPAT* was amplified without the first 291 bp (encoding 97 amino acids at the N-terminus) at the 5′ end. The full-length and the truncated cDNA fragments of *EaGPAT* were generated by PCR using the following primers: WWF5′ (5′-GTA**ATG**GCTCTCACATTTTCCTCCTCCGCC-3′) (for full-length cDNA), TWWF5′ (5′-GCC**ATG**TCCCGTACCTTCTTGGATGCGAG-3′) (for truncated cDNA fragment) and WWF3′ (5′-ATTCCAGGGTTGT-

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