

# Phosphatidylglycerophosphate synthases from *Arabidopsis thaliana*

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**Abstract** Two *Arabidopsis thaliana* genes were shown to encode phosphatidylglycerophosphate synthases (PGPS) of 25.4 and 32.2 kDa, respectively. Apart from their N-terminal regions, the two proteins exhibit high sequence similarity. Functional expression studies in yeast provided evidence that the 25.4 kDa protein is a microsomal PGPS while the 32.2 kDa protein represents a preprotein which can be imported into yeast mitochondria and processed to a mature PGPS. The two isozymes were solubilized and purified as fusion proteins carrying a His tag at their C-terminus. Enzyme assays with both membrane fractions and purified enzyme fractions revealed that the two *A. thaliana* isozymes have similar properties but differ in their CDP-diacylglycerol species specificity. © 2001 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

**Key words:** CDP-diacylglycerol; Functional expression; Membrane lipid synthesis; Phosphatidyltransferase; Phosphatidylglycerophosphate synthase; *Arabidopsis thaliana*

## 1. Introduction

Phosphatidylglycerol (PG) is an anionic phospholipid found in almost all bacterial membranes as well as in the different membrane systems of eukaryotic organisms. Biochemical and genetic studies provided evidence that anionic phospholipids have important functional roles. Although they can substitute for one another in certain cellular functions, PG appears to be indispensable for essential functions such as photosynthesis and oxidative phosphorylation [1–6].

In all organisms PG is synthesized from CDP-diacylglycerol (CDP-DAG) and glycerol-3-phosphate by the action of a phosphatidylglycerophosphate synthase (PGPS) which transfers the phosphatidyl group from CDP-DAG to glycerol-3-phosphate. Subsequently, a PGP phosphatase rapidly converts PGP to PG. PGPSs from both prokaryotes and eukaryotes are integral membrane proteins. In yeast cells PGPS is exclusively located in the inner mitochondrial membrane, which provides the substrate for the formation of cardiolipin, the typical mitochondrial membrane lipid [5,7,8]. On the other hand, in plants PG synthesis has been demonstrated to occur in different subcellular compartments [9]. Apart from the mitochondrial pathway conserved in all eukaryotes [4,8–11], plants possess a plastidial pathway for PG synthesis, which

is located in the inner envelope membranes and which is the dominant one for PG synthesis in leaves [12]. Furthermore, the endoplasmic reticulum of plants as well as of animal cells possesses all enzymic activities required for de novo PG synthesis [10,13]. In animal cells, however, this pathway appears to be of minor importance compared to the mitochondrial pathway [4].

Analysis of PGP synthases from bacteria and from mitochondria of yeast and mammals revealed that they represent two distinct groups. The bacterial PGPSs of about 20 kDa contain the typical CDP-alcohol binding motif that is also found in other phosphatidyltransferases such as the cardiolipin synthases from eukaryotes [3,14–17] whereas the mitochondrial PGPSs of about 60 kDa belong to the HKD protein family as the bacterial cardiolipin synthases [4,5,18]. In contrast to the PGPSs of bacteria, yeast and mammals, little is known about the PGPSs from plant tissues apart from their subcellular localization.

To characterize the plant PGPSs and to elucidate to which groups the isofunctional enzymes of the different subcellular compartments belong, we have functionally analyzed *Arabidopsis thaliana* genes which might encode phosphatidyltransferases. By heterologous expression in microorganisms and enzymic assays, two PGPS genes were identified. Both encoded polypeptides exhibit substantial sequence similarity to bacterial PGPSs. Here we report the functional expression of the respective cDNAs in *Escherichia coli* and yeast. In addition, we present the properties of the two plant PGPS isozymes determined with both membrane fractions and purified enzyme fractions isolated from the transgenic microorganisms.

## 2. Materials and methods

### 2.1. Cloning of cDNAs encoding CDP-diacylglycerol dependent enzymes

The following primer sequences were deduced from the four genomic *A. thaliana* sequences (A: AF118223.2, B: AC004697.2, C: AL132970.2, D: AL035540) and the *A. thaliana* cDNA sequence (E: AJ000539) encoding putative CDP-diacylglycerol dependent enzymes: A1: ATGGCGATTACAGATCTCTAAG, A2: TTACCTTAGATAAGTGATCCATG, B1: ATGCTCAGATCCGGTCTGGC, B2: CTACTTCATTAGTACTTTCCATATC, C1: ATGGGTGAAGAAGACACCGCGAC, C2: CTACTTCTTTAGCAGTACTCTCCATATC, D1: ATGGCTAAACAGAGACCG, D2: TCAAGGCTTCTTATGCTG, E1: ATGGCTAAAAGGAGAGACCTAG, E2: TCAAGGCTTCTGCTGCTTCTC. These primers were used to amplify the respective open reading frames by RT-PCR. First strand cDNA was prepared with MMLV reverse transcriptase (Promega, Madison, WI, USA) with mRNA isolated from *A. thaliana* ecotype Columbia. PCR was performed with Pfu polymerase (Stratagene, La Jolla, CA, USA). PCR products were cloned into pUC19 or pBlue-script, respectively, and sequenced. The open reading frames were

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**Abbreviations:** PG, phosphatidylglycerol; PGP, phosphatidylglycerophosphate; PGPS, phosphatidylglycerophosphate synthase

subcloned into pQE70 (Qiagen, Hilden, Germany) for expression in *E. coli* and pYES2 (Invitrogen, Groningen, The Netherlands) for expression in *Saccharomyces cerevisiae*. All restriction sites as well as the His tag for expression in yeast were added by PCR, for expression in *E. coli* the His tag provided by pQE70 was used.

## 2.2. Strains, culture conditions and preparation of membranes

*E. coli* XL1 blue was used for cloning, propagation of plasmids and expression studies. Standard cultivation methods were used for *E. coli*. For expression of heterologous proteins, this strain was cultivated at 30°C and exponentially growing cultures were induced with 1 mM isopropyl- $\beta$ -D-thiogalactopyranoside. Cells were harvested 2 h after induction and washed with 10 mM Tris-H<sub>2</sub>SO<sub>4</sub>, pH 7.4. The same buffer including 1 mM phenylmethylsulfonyl fluoride was used during membrane preparation. One volume of glass beads (0.25–0.5 mm) and two volumes of buffer were added to the cell sediment. The cells were vortexed for 5 min, cooled on ice and immediately sonicated for 5 min. The 5000 $\times$ g supernatant was used for ultracentrifugation at 150 000 $\times$ g. Membranes were resuspended in 5 mM Tris-H<sub>2</sub>SO<sub>4</sub> pH 7.4, containing 50% glycerol and stored at -20°C.

The yeast strain *S. cerevisiae* UTL7A (MAT $\alpha$ , ura3-52, leu2-3, 112, trp1) was cultivated in minimal medium containing 2% glucose as described by Warnecke et al. [19]. Protein expression was induced by exchanging the growth medium against minimal medium containing 2% galactose. Cells were harvested 12 h after induction and mitochondria and microsomal membranes were prepared as described by Zinser and Daum [20]. Membrane fractions were stored in 5 mM Tris-H<sub>2</sub>SO<sub>4</sub> pH 7.4, containing 50% glycerol at -20°C. Protein was determined using Protein Assay (Bio-Rad, Hercules, CA, USA) and bovine serum albumin as standard. Standard methods were used for SDS-PAGE analysis. For Western blotting Penta-HIS<sup>®</sup> antibody and 6 $\times$ HIS protein ladder (Qiagen) were used according to the manufacturer's protocol.

**2.2.1. Purification of PGPS1 and PGPS2.** Membrane fractions were prepared from *E. coli* cells expressing PGPS2 and yeast cells expressing PGPS1, as described above, but resuspended in 50 mM sodium phosphate, pH 7.4 at a final protein concentration of 15 mg/ml. For solubilization membrane fractions were diluted with water to a final concentration of 5 mg/ml, mixed with the same volume of 2 $\times$ column buffer (100 mM sodium phosphate, pH 7.4, 600 mM NaCl, 20 mM imidazole, 20% glycerol (w/v), and 4% Triton X-100 (w/v)), stirred on ice for 30 min and applied to ultracentrifugation at 150 000 $\times$ g for 2 h. The supernatant was applied on a nickel-NTI column (9 ml; Qiagen) equilibrated with 1 $\times$ column buffer. The column was washed with 50 ml 1 $\times$ column buffer containing 30 mM imidazole and proteins were eluted with 1 $\times$ column buffer containing 100 mM EDTA. Fractions with highest PGP synthase activities were dialyzed against 10 mM Tris-H<sub>2</sub>SO<sub>4</sub>, pH 7.4, 2% Triton X-100 (w/v) overnight, centrifuged at 150 000 $\times$ g for 2 h and the supernatants were frozen in liquid nitrogen and stored at -80°C.

## 2.3. Enzyme assays

Enzymatic activities using 5 ng to 50  $\mu$ g protein were determined at 30°C in a total volume of 100  $\mu$ l. PGPS assays were carried out as described before [11]. If not otherwise stated, the reaction mixture was composed of 100 mM Bis-Tris-propane-HCl pH 8.5, 10 mM MnCl<sub>2</sub>, 5% glycerol (w/v), 1% Triton X-100 (w/v), 100  $\mu$ M glycerol-3-phosphate, 50  $\mu$ M CDP-DAG (Sigma, Deisenhofen, Germany), 10 mM dithiothreitol and 3.2  $\mu$ M *sn*-[U-<sup>14</sup>C]glycerol-3-phosphate (154 mCi/mmol, Amersham Pharmacia, Buckinghamshire, UK). Phosphatidylinositol synthase assays were performed in 50 mM Tris-HCl pH 8, 2.5 mM MnCl<sub>2</sub>, 5% glycerol (w/v), 0.3% Triton X-100 (w/v), 0.1 mM CDP-DAG and 47 nM *myo*-[2-<sup>3</sup>H]inositol (21 Ci/mmol, NEN, Boston, MA, USA). Phosphatidylserine synthase assays were performed in 50 mM Tris-HCl pH 8.5, 0.3% Triton X-100 (w/v), 0.1 mM CDP-DAG, 10 mM MgCl<sub>2</sub>, and 7.6  $\mu$ M L-[U-<sup>14</sup>C]serine (131 mCi/mmol, Hartmann Analytic, Braunschweig, Germany). Cardiolipin synthase assays were performed as described before [21]. The reactions were stopped and lipids were extracted by addition of 0.6 ml chloroform, 0.6 ml methanol and 0.6 ml 1 M KCl, 0.2 M H<sub>3</sub>PO<sub>4</sub>. After phase separation the organic phase was used for scintillation counting or analysis of the reaction products by TLC in chloroform:acetone:methanol:acetic acid:water (9:5:2:2:1 (v/v), solvent system 1) or chloroform:methanol:25% NH<sub>3</sub> (60:30:3 (v/v), solvent system 2). Radioactively labelled lipids were visualized with a Bioimager FLA 3000

(Raytest, Straubing, Germany) and identified by cochromatography of authentic lipids.

## 3. Results and discussion

### 3.1. Identification of *A. thaliana* PGPS genes

Database searches using the sequences of known CDP-DAG dependent enzymes resulted in the identification of five *A. thaliana* genes which showed significant sequence similarities to either PGPS from bacteria or cardiolipin synthase and phosphatidylinositol synthase from eukaryotes. The open reading frames of these genes were amplified by RT-PCR, cloned into expression vectors and expressed in *E. coli* and yeast. Subsequently, enzyme assays were carried out with subcellular fractions of the transgenic microorganisms using CDP-DAG and glycerol-3-phosphate or inositol or serine or PG as substrates. The reaction products were finally analyzed by TLC and compared with those of a control fraction derived from transgenic cells harboring the respective empty vector only. In that way, the five cDNAs were found to encode three different phosphatidyltransferases. The phosphatidyltransferases encoded by the genes AC004697.2 and AL132970.2 specifically transferred a phosphatidyl group from CDP-DAG to glycerol-3-phosphate and, thus, represent PGPSs termed PGPS1 and PGPS2. As shown in Fig. 1, the transgenic yeast cells expressing the respective cDNAs formed distinctly higher levels of PGP than the control cells. PGP was partially converted to PG by the action of the yeast PGP phosphatase. In contrast to the cDNA of PGPS1, that of PGPS2 was functionally expressed not only in yeast but also in *E. coli* where the specific PGPS2 activity was 10-fold higher than in yeast. The analysis of the reaction products gave a pattern similar to that shown in Fig. 1. Both PGPS1 and

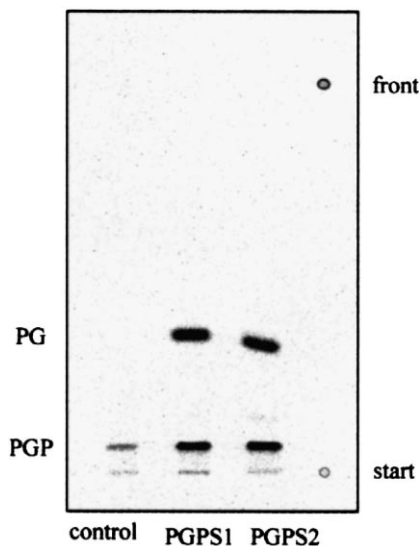


Fig. 1. Reaction products formed from CDP-DAG and [U-<sup>14</sup>C]glycerol-3-phosphate by membrane fractions from yeast harboring only the empty vector or expressing either PGPS1 or PGPS2 corresponding to 10  $\mu$ g protein in each case. Reaction products were separated by TLC in solvent system 1 and radioactively labelled lipids were visualized with a Bioimager FLA 3000. Because the assay was optimized for plant PGPS the endogenous PGPS of yeast displayed low activities of 0.02 nmol/min/mg protein corresponding to 0.25% of the activity of the yeast cells expressing either PGPS1 or PGPS2.

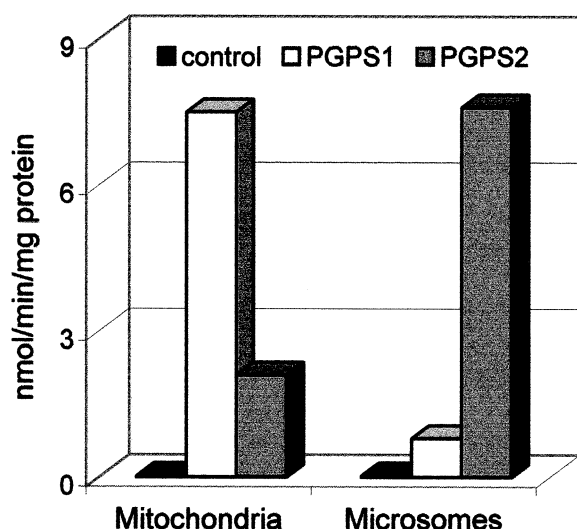


Fig. 2. Subcellular localization of PGPS1 and PGPS2 expressed in yeast cells. The incorporation rates of [U- $^{14}$ C]glycerol-3-phosphate into PG and GPG by mitochondrial and microsomal fractions of yeast control cells and of yeast cells expressing either PGPS1 or PGPS2 are given.

PGPS2 activity was recovered in the membrane fractions of the host cells while the soluble fractions displayed negligibly low activities only. This localization is consistent with the two PGPS sequences deduced from the cDNAs. The sequences have a length of 296 (PGPS1) and 233 (PGPS2) amino acid residues, respectively, and three predicted membrane spanning domains, by which the proteins are presumably anchored in the membranes. Apart from the N-terminal region, the two sequences are very similar to each other (79% identity) and exhibit substantial similarity to the bacterial PGPSs and the eukaryotic cardiolipin synthases [3,14–17]. Especially the central region of the sequences covering the typical CDP-alcohol binding motif  $\text{DX}_2\text{DGX}_2\text{ARX}_8\text{GX}_3\text{DX}_3\text{D}$  is highly conserved. Hence, the *A. thaliana* PGPS isozymes belong to the same group of phosphatidyltransferases as the bacterial PGPSs, but they differ structurally from the mitochondrial PGPSs of yeast and mammals, which are members of the HKD protein family.

Unlike the PGPS2 sequence, that of PGPS1 has an N-terminal extension which is predicted to represent a cleavable transit peptide for the import into plastids and with a lower probability for the import into mitochondria. Our experiments, which showed that the cDNA of PGPS1 unlike that of PGPS2 was functionally expressed in yeast but not in *E. coli*, support the prediction and suggest that the preprotein can be imported into yeast mitochondria and processed to a catalytically active PGPS. To support this assumption, PGPS activities were measured in mitochondrial and microsomal fractions isolated from transformed yeast cells. In yeast cells expressing the PGPS2 cDNA, highest specific PGPS activities were determined in the microsomal fraction (Fig. 2). On the other hand, in yeast cells expressing the PGPS1 cDNA, distinctly higher PGPS activities were measured in the mitochondrial than in the microsomal fractions (Fig. 2). In addition, expression studies were carried out with modified PGPS constructs inserting a His tag at the C-terminus of the PGPS proteins. The analysis of the respective fractions by Western

blotting with monoclonal antibodies against the His tag showed that the enzymatic activity of PGPS1 correlated with a processed protein with a mass even smaller than that of PGPS2 (Fig. 3) instead of the expected 32.2 kDa. Hence, these data suggest that the PGPS1 cDNA encodes a preprotein with a cleavable transit peptide at its N-terminus which can be imported into yeast mitochondria and processed to catalytically active PGPS.

As described above, in plant cells PGPS is not only located in mitochondria and endomembranes, but also in plastids. In plant cells the preprotein encoded by AC004697.2 is perhaps targeted to both mitochondria and plastids. Such a twin targeting has been demonstrated to occur in plant cells [22]. A twin targeting of the PGPS1 preprotein is in line with the predicted targeting of the polypeptide mentioned above and with the properties of the two plant enzymes which were found to be very similar, although subcellular fractions from different plant species have been used as enzyme source (see below). In addition, this is supported by the transient expression studies of Hagio et al. [23], which were carried out in parallel to our experiments and which showed that a fusion protein of PGPS1 with a green fluorescent protein was targeted to chloroplasts and to other organelles, which were tentatively identified as mitochondria. According to these data it is possible that in *A. thaliana* the plastidial and mitochondrial PGPSs are encoded by the same gene, but further experiments are required to support this assumption.

### 3.2. Properties of the *A. thaliana* PGPS isozymes

The properties of the two *A. thaliana* PGPS isozymes were determined using both membrane fractions isolated from the microorganisms, transformed with a PGPS construct and purified enzyme fractions as enzyme source. Enzyme assays with membrane fractions showed that PGPS2 displayed distinctly higher specific activities in *E. coli* than in yeast membranes but very similar properties regardless of whether it was expressed in *E. coli* or in yeast. Furthermore, the addition of a His tag at the C-terminus of the PGPS proteins was found to have no obvious effect on the enzymatic properties of the two isozymes. Hence, to facilitate protein purification, the His tagged PGPS proteins were solubilized from the host membranes, namely PGPS1 was extracted from the yeast mitochondrial membranes while PGPS2 was purified from *E. coli* membranes because of its higher specific activities in *E. coli* than in yeast. Solubilization of both isozymes was achieved by

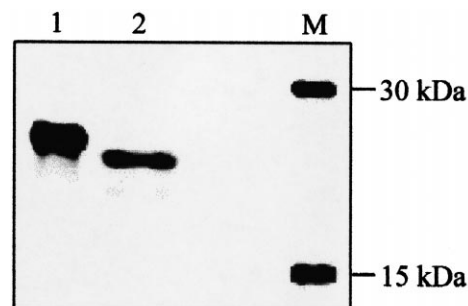


Fig. 3. Western blot analysis of PGPS1 and PGPS2 expressed as fusion proteins with C-terminal His tag (lane 1, PGPS2 fraction purified from *E. coli* membranes; lane 2, PGPS1 fraction purified from yeast mitochondria; M, marker). Unlike PGPS2, PGPS1 was barely detectable in its unpurified form.

extracting the *E. coli* membranes and the yeast mitochondrial membranes, respectively, with 2% (w/v) Triton X-100. The subsequent separation of the solubilized protein fractions on  $\text{Ni}^{2+}$  chelate affinity resin resulted in an appreciable enrichment of the PGPS activities. Both the purified PGPS1 and PGPS2 fractions showed specific activities of about 1  $\mu\text{mol}/\text{min}/\text{mg}$  protein. SDS-PAGE analysis of the protein fractions revealed that PGPS2 was purified to near homogeneity. While the fusion protein of about 25 kDa was only a minor component in the *E. coli* membranes, it was the main protein in the fractions eluted with EDTA from the column (Fig. 4). The purified PGPS1 fraction was less homogeneous than that of PGPS2, but a distinct enrichment of the mature PGPS1 fusion protein of about 25 kDa was achieved.

Unlike the results obtained with the membrane fractions (Fig. 1), PGP was formed as sole reaction product from CDP-DAG and  $[\text{U-}^{14}\text{C}]$ glycerol-3-phosphate by the purified enzyme fractions. In addition, these fractions specifically transferred the phosphatidyl group from CDP-DAG to glycerol-3-phosphate, but not to inositol, serine, choline or PG. This substrate specificity confirmed the identity of the expression products as PGPSs. A comparison of the properties of the purified enzyme with those of the respective membrane bound form gave consistent results. Moreover, these experiments revealed that the two *A. thaliana* isozymes have similar properties. Both isozymes showed a pH optimum of about 8.5 and required divalent cations, especially  $\text{Mn}^{2+}$  for activity. Highest PGPS1 activity was obtained in the presence of 2.5 mM  $\text{Mn}^{2+}$  while PGPS2 required 8 mM  $\text{Mn}^{2+}$  for maximal activity. In addition, the activities of both isozymes were stimulated by Triton X-100 but to a different extent. The PGPS1 activity was stimulated by a factor of two and gave highest activities at 0.8% Triton X-100 while PGPS2 activity showed a 10-fold stimulation at optimal Triton X-100 concentrations of 0.5%. Such properties have also been reported for mung bean mitochondria and castor bean microsomes [10,11]. In addition, these properties are also typical of the plastidial PGPS from pea leaves (Frentzen, unpublished). Hence, the PGPS isozymes from plants display properties typical of the bacterial PGPS, but they differ from the bacterial enzymes with regard to the cation specificity. While the bacterial enzymes are more active with  $\text{Mg}^{2+}$  than with  $\text{Mn}^{2+}$  [24,25], the plant enzymes such as those of *A. thaliana* are more active

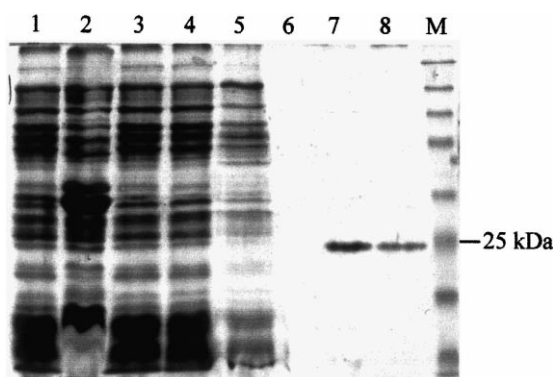


Fig. 4. SDS-PAGE analysis of proteins at different steps of PGPS2 fusion protein purification (lane 1, membrane fraction; lane 2, unsolubilized proteins; lane 3, solubilized proteins; lanes 4 and 5, proteins not retained on the column; lanes 6–8, fractions 1–3 eluted with EDTA from the column; lane M, marker).

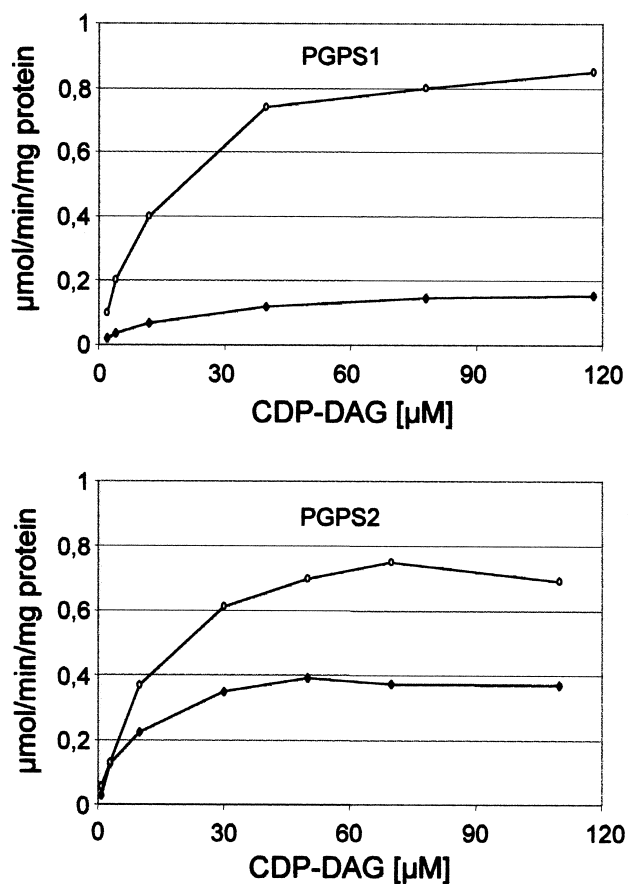


Fig. 5. Activities of the two PGPS isozymes as a function of the concentration of CDP-dipalmitoylglycerol (○) and CDP-dioleoylglycerol (◆).

with  $\text{Mn}^{2+}$  than with  $\text{Mg}^{2+}$ . Even at optimal  $\text{Mg}^{2+}$  concentrations of 130 and 50 mM for PGPS1 and PGPS2, respectively, the activities of the two isozymes only comprised about 10% of their  $\text{Mn}^{2+}$  dependent activities. Unlike  $\text{Mg}^{2+}$ ,  $\text{Ca}^{2+}$  did not stimulate the two *A. thaliana* isozymes, but it inhibited the  $\text{Mn}^{2+}$  dependent activities at low concentrations of 0.1–0.5 mM which gave a 30–70% inhibition. Such an inhibition by  $\text{Ca}^{2+}$  has also been reported for the PGPS of *E. coli* [24].

Both PGP isozymes showed typical saturation kinetics when their activities were determined as a function of the substrate concentration (Fig. 5). Very similar apparent  $K_M$  values were calculated for the two *A. thaliana* isozymes, namely 52 and 62  $\mu\text{M}$  for glycerol-3-phosphate and 12 and 17  $\mu\text{M}$  for CDP-dipalmitoylglycerol. Almost identical apparent  $K_M$  values similar to those of the *A. thaliana* isozymes were also reported for the mitochondrial and microsomal PGPS isozymes from castor bean endosperm [10]. Furthermore, the mitochondrial PGPS from mung bean seedlings and the plastidial enzyme from pea leaves also have apparent  $K_M$  values in the  $\mu\text{M}$  range ([11], Frentzen, unpublished).

The analysis of the CDP-DAG species specificity revealed that both *A. thaliana* PGPS isozymes were more active with CDP-dipalmitoylglycerol than with CDP-dioleoylglycerol (Fig. 5). As has been reported for the PGPS from mung bean mitochondria [11], the observed species specificity of the two *A. thaliana* isozymes was due to differences in the apparent  $V_{\text{max}}$  values. PGPS1, however, displayed a three-

fold higher difference in these values than PGPS2 and, thus, a more pronounced CDP-DAG species specificity than PGPS2 (Fig. 5). A pronounced CDP-DAG species specificity similar to that of PGPS1 is also a typical property of the PGPS from mung bean mitochondria and pea chloroplasts ([11], Frentzen, unpublished). Hence, the structural similarity of the two *A. thaliana* PGPS isozymes to the respective bacterial enzymes and their structural differences to the PGPSs of yeast and mammalian mitochondria was clearly reflected in the properties of the plant enzymes.

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