Purification and characterization of thiol-reagent-sensitive glycerol-3-phosphate acyltransferase from the membrane fraction of an oleaginous fungus

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Glycerol-3-phosphate acyltransferase (GPAT), responsible for the first committed, rate-limiting, step of glycerolipid synthesis, was purified to homogeneity from the membrane fraction of an oleaginous fungus, *Mortierella ramanniana* var. *angulispota*. The enzyme was solubilized from the membrane fraction by pre-treatment with 0.05% Triton X-100 and treatment of the resulting pellet with 0.3% Triton X-100. The enzyme was subsequently purified by column chromatography on heparin–Sephacrose, Yellow 86 agarose, a second heparin–Sephacrose column, Superdex-200 and hydroxylapatite Bio-Gel. Enzyme activity was finally enriched 1308-fold over that of the starting membrane fraction. SDS/PAGE of the purified fraction revealed a single band with a molecular mass of 45 kDa. Native PAGE showed a major band that corresponded to GPAT activity. Enzyme activity was inhibited by thiol reagents, suggesting that it originated from microsomes rather than mitochondria. Purified GPAT depended on exogenous oleoyl-CoA and *sn*-glycerol-3-

phosphate, with the highest activity at approx. 50 and 250 μ M, respectively, and preferred oleoyl-CoA 5.4-fold over palmitoyl-CoA as an acyl donor. Anionic phospholipids, such as phosphatidic acid and phosphatidylserine, were absolutely required for activity of the purified enzyme, and their ability to activate GPAT was influenced by the purity of the GPAT preparation. Bivalent cations, such as Mg^{2+} and Ca^{2+} , inhibited purified GPAT activity, whereas 5 mM Mn^{2+} elevated activity approx. 2-fold. These results provide new insights into the molecular characterization of microsomal GPAT, which has not been well characterized compared with mitochondrial and plastidic GPAT.

Key words: anionic phospholipids, Mn^{2+} , *Mortierella ramanniana* var. *angulispota*, native PAGE, triacylglycerol biosynthesis.

INTRODUCTION

Glycerol-3-phosphate acyltransferase (GPAT; EC 2.3.1.15) operates the first committed, rate-limiting, step of glycerolipid synthesis [1,2]. It catalyses the esterification of glycerol-3-phosphate (G3P) at the *sn*-1 position with a fatty acyl-CoA to form 1-acylglycerol-3-phosphate (lysophosphatidic acid; LPA). GPAT purification and gene cloning have been reported in *Escherichia coli* [3,4], mammalian mitochondria [5–8] and plant chloroplasts [9,10]. Microsomal GPAT, distinguished from the mitochondrial enzyme by its sensitivity to thiol reagents [11–15], has not been well characterized at the molecular level.

GPAT may play a pivotal role in regulating phospholipid and triacylglycerol (TG) biosynthesis [1,2], although the physiological roles of GPAT isoenzymes from different organelles are not well understood. Mitochondrial GPAT has specificity towards saturated fatty acyl-CoA [6,7,16,17], whereas microsomal GPAT has broader specificity towards acyl-CoA [1,2,18]. Both mitochondrial and microsomal GPAT are regulated nutritionally and hormonally, and phosphorylation–dephosphorylation mechanisms may be involved [1,17]. The contribution of mitochondrial and microsomal enzymes to glycerolipid biosynthesis and its regulation have not been well evaluated mainly because the molecular nature of microsomal GPAT is not clear. Only a few reports have described partial purification of microsomal GPAT [19–21], and the purification and gene cloning of microsomal GPAT are awaited.

Mortierella ramanniana var. *angulispota* is an oleaginous

fungus, screened for γ -linolenic acid production [22,23]. We have been studying the mechanisms of TG biosynthesis in this fungus, which is expected to be very active and may provide a suitable system. Elucidation of these mechanisms would enable us to design TG molecular species in this fungus. The subcellular distribution of TG biosynthetic enzymes in this fungus showed that diacylglycerol acyltransferase was enriched in the lipid-body fraction, whereas GPAT activity was recovered mostly in the membrane fraction [24,25]. Recently, we purified diacylglycerol acyltransferase to homogeneity from the lipid-body fraction [26]. In the present study, we solubilized and purified GPAT from the membrane fraction of *M. ramanniana* var. *angulispota*, deriving novel information relating to substrate specificity and regulation of purified GPAT.

EXPERIMENTAL

Materials

Acyl CoAs, *sn*-G3P, *sn*-1,2-diolein, phosphatidic acid (PA) prepared from egg lecithin, phosphatidylserine (PS) from bovine brain, phosphatidylethanolamine (PE) from bovine liver, phosphatidylinositol from soya bean, phosphatidylcholine (PC) from bovine liver, dioleoyl PA, dioleoyl PS, dioleoyl PC, LPA (*sn*-oleoyl-G3P), lysophosphatidylcholine from bovine liver, lysophosphatidylserine and reactive Yellow 86 agarose were purchased from Sigma (St. Louis, MO, U.S.A.). HiLoad 26/60 Superdex 200 pg and heparin–Sephacrose CL-6B were obtained

Abbreviations used: DTT, dithiothreitol; DTNB, 5,5'-dithiobis-(2-nitrobenzoic acid); GPAT, glycerol-3-phosphate acyltransferase; G3P, glycerol-3-phosphate; LPA, lysophosphatidic acid; NEM, *N*-ethylmaleimide; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PCMB, *p*-chloromercuribenzoic acid; PS, phosphatidylserine; TG, triacylglycerol.

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from Pharmacia (Uppsala, Sweden), and hydroxylapatite Bio-Gel was purchased from Bio-Rad (Richmond, CA, U.S.A.). [$1\text{-}^{14}\text{C}$]Oleoyl-CoA (58 mCi/mmol) and *sn*-[$1\text{-}^{14}\text{C}$]G3P (148 mCi/mmol) were obtained from Du Pont (New England Nuclear, Boston, MA, U.S.A.). Triton X-100 was from Nacalai Tesque (Kyoto, Japan). Silica gel 60 TLC plates were purchased from Merck (Darmstadt, Germany). All other reagents were of analytical grade.

Preparation of the membrane fraction

M. ramanniana var. *angulispora* (IFO 8187) was obtained from the culture collection of the Institute of Fermentation (Osaka, Japan). The membrane fraction from fungal cells cultured for 4 days was obtained as described previously [24], and stored at -80°C until use.

GPAT activity assay

GPAT activity was measured based on a protocol described previously [25], with slight modification. In the case of the membrane fraction and the Triton X-100 extract, the reaction mixture contained 10 mM phosphate buffer (pH 7.0), 0.15 M KCl, 50 μM [$1\text{-}^{14}\text{C}$]oleoyl-CoA (0.02 μCi), 250 μM G3P and 50 μl of enzyme source in a final volume of 100 μl . The reaction, started by adding the enzyme preparation, was conducted at 30°C for 5 min. Product formation increased linearly with time. GPAT activity was calculated from the radioactivity incorporated into PA and/or LPA (see the Results section), separated by TLC developed with chloroform/acetone/methanol/acetic acid/water (5:2:1:1:0.5, by vol.). In the case of fractions obtained from column chromatography, 250 μM PA was added to the above assay system in order to reconstitute the activity. In the experiment to study acyl-CoA specificity, 50 μM *sn*-[$1\text{-}^{14}\text{C}$]G3P (0.1 μCi) and 250 μM acyl-CoA were used under the same conditions as described above. Concentrations of *sn*-[$1\text{-}^{14}\text{C}$]G3P and acyl-CoA were used that allowed optimal ^{14}C -incorporation into LPA. One unit of enzyme activity was defined as the amount of enzyme that catalysed the formation of 1 μmol of product/min.

Solubilization of GPAT from the membrane fraction

Prior to GPAT purification, we ran several trials for solubilization of the enzyme from the membrane fraction and ultimately used Triton X-100 to solubilize GPAT through two successive steps. First, we suspended the membrane fraction (protein concentration of 5 mg/ml) in 10 mM phosphate buffer (pH 7.0) containing 0.15 M KCl, 1 mM PMSF, 1 mM EDTA, 1 mM dithiothreitol (DTT), 10% (v/v) ethyleneglycol and 0.05% Triton X-100, followed by thorough mixing for 1 h at 4°C . The mixture was then centrifuged at 100000 *g* for 1 h, and the pellet was transferred to 10 mM phosphate buffer (pH 7.0) containing 0.15 M KCl, 1 mM PMSF, 1 mM DTT, 10% ethyleneglycol, 1 $\mu\text{g}/\text{ml}$ pepstatin A, 2 $\mu\text{g}/\text{ml}$ aprotinin, 0.5 $\mu\text{g}/\text{ml}$ leupeptin and 0.3% Triton X-100. The mixture was incubated for 1 h at 4°C , with gentle stirring, and once again centrifuged at 100000 *g* for 1 h. The supernatant was immediately transferred to -80°C and stored until use.

GPAT purification

All purification steps involving column procedures were conducted at 4°C using a Pharmacia FPLC system. The solubilized fraction was filtered through a 0.22 μm filter just prior to its application on to the column. We started the column procedure with 300 ml of the 0.3% Triton X-100 extract (protein con-

Table 1 Effect of Triton X-100 concentration on solubilization of GPAT from the membrane fraction of *M. ramanniana* var. *angulispora*

The membrane fraction (5 mg/ml protein) was used to solubilize GPAT using different Triton X-100 concentrations, as described in the Experimental and Results sections, including two-step solubilization (i.e. the enzyme was solubilized from the membrane fraction by pretreatment with 0.05% Triton X-100 and treatment of the resulting pellet with 0.3% Triton X-100). Assay conditions are described in the Experimental section. Data are presented as means $\pm$ S.D. ($n = 3$).

Fraction	GPAT recovery (%)	Protein recovery (%)	Specific activity (m-units/mg of protein)
Membrane fraction	100.0	100.0	1.11 $\pm$ 0.03
0.3% Triton X-100 supernatant	48.3 $\pm$ 0.6	28.4 $\pm$ 0.8	2.17 $\pm$ 0.03
0.05% Triton X-100 pellet	118.0 $\pm$ 8.4	32.8 $\pm$ 0.9	4.86 $\pm$ 0.09
Supernatant obtained by 0.03% Triton X-100 solubilization of 0.05% Triton X-100 pellet	60.0 $\pm$ 1.8	17.0 $\pm$ 0.1	3.94 $\pm$ 0.05

Table 2 Effect of phospholipids on GPAT activity in the peak fraction of the first heparin-Sepharose column

Phospholipids were added to the GPAT assay mixture containing 50 μM [$1\text{-}^{14}\text{C}$]oleoyl-CoA, 250 μM G3P and 3 μg of the enzyme fraction. Assay conditions were as described in the Experimental section. Data are presented as means $\pm$ S.D. ($n = 3$).

Phospholipids	GPAT activity (m-units/mg of protein)
No addition	1.82 $\pm$ 0.35
PA (250 μM)	17.3 $\pm$ 1.1
PA (500 μM)	4.01 $\pm$ 0.38
PC (250 μM)	13.4 $\pm$ 1.0
PC (500 μM)	12.6 $\pm$ 1.1
PE (250 μM)	6.60 $\pm$ 0.90
PE (500 μM)	2.92 $\pm$ 0.50
PS (250 μM)	9.16 $\pm$ 1.05
PS (500 μM)	5.70 $\pm$ 1.06
LPA (250 μM)	5.78 $\pm$ 1.02
LPA (500 μM)	4.05 $\pm$ 1.01
Lysophosphatidylcholine (250 μM)	2.38 $\pm$ 0.89
Lysophosphatidylcholine (500 μM)	2.30 $\pm$ 0.65
Lysophosphatidylserine (250 μM)	2.98 $\pm$ 0.59
Lysophosphatidylserine (500 μM)	1.24 $\pm$ 0.60

centration of 170 $\mu\text{g}/\text{ml}$). To prevent activity loss, the Triton X-100 extract was divided into six equal parts (50 ml each). The extract was applied to a heparin-Sepharose CL-6B column (0.7 cm $\times$ 5 cm) pre-equilibrated with buffer A [10 mM phosphate buffer (pH 7.0) containing 0.15 M KCl, 1 mM PMSF, 1 mM DTT, 10% ethyleneglycol, 1 $\mu\text{g}/\text{ml}$ pepstatin A, 2 $\mu\text{g}/\text{ml}$ aprotinin, 0.5 $\mu\text{g}/\text{ml}$ leupeptin and 0.1% Triton X-100] in six successive batches. The column was washed with buffer A at 0.3 ml/min, then eluted with a linear gradient of 0.15–0.5 M KCl in buffer A. Fractions with enzyme activity in all six batches were combined, and made two sets (45 ml each). Concentration in a Centriprep-10 (Millipore, Bedford, MA, U.S.A.) and dilution by buffer A yielded 20 ml of desalted enzyme preparation from each set, which was subsequently applied to a Yellow 86 agarose column (1 cm $\times$ 15 cm) pre-equilibrated with buffer A in two successive batches. The column was washed at 0.3 ml/min with buffer A, followed by elution with a linear gradient of 0.15–0.5 M KCl in buffer A. Fractions possessing enzyme activity from each set were pooled, and the pooled fractions were subsequently applied to a second heparin-Sepharose CL-6B column

Figure 1 Purification of GPAT by column chromatography

(A) First heparin-Sepharose column; (B) Yellow 86 agarose column; (C) second heparin-Sepharose column; (D) Superdex 200 column; (E) hydroxylapatite column. Experimental details are given in the Experimental section. GPAT activity (■) and protein concentration (○) were

(0.7 cm × 5 cm) pre-equilibrated with buffer A. The column was washed with buffer A at 0.3 ml/min, followed by elution with a linear gradient of 0.15–0.5 M KCl in buffer A. Fractions with enzyme activity from both sets were combined, and concentrated in a Centriprep-10. The concentrate (2 ml) was applied to a HiLoad 26/60 Superdex 200 pg gel-filtration column pre-equilibrated with buffer A and eluted with the same buffer at 2 ml/min. Fractions with enzyme activity obtained from the gel-filtration column were pooled and concentrated to 2 ml in a Centriprep-10. The concentrate was then loaded on to a hydroxylapatite Bio-Gel column (1 cm × 7.5 cm) pre-equilibrated with buffer A. The column was washed with buffer A at 0.3 ml/min, washed with 0.2 M phosphate in buffer A at 0.3 ml/min, and eluted with a linear gradient of 0.2–0.4 M phosphate in buffer A. All fractions with enzyme activity were stored at -80°C between column procedures. Freeze-thawing of enzyme preparations appeared to have little effect on activity. The final enzyme preparation was also stored at -80°C until use. At this temperature, the enzyme retained approx. 95% of its activity for 3 months.

Other analytical methods

Total protein was measured with Coomassie Brilliant Blue dye reagent (Bio-Rad) as described previously [27], in the presence of 0.05 M NaOH. SDS/PAGE was conducted using a 12.5% (w/v) polyacrylamide gel (Bio-Rad) based on the protocol of Laemmli [28]. Native PAGE was conducted as described previously [29], with slight modifications. A gel (7.5%) containing 7.5% (w/v) acrylamide, 0.15% bisacrylamide, 5% (v/v) glycerol and 90 mM Tris/HCl (pH 7.5) was prepared and run at 4°C in 36 mM Tris/HCl buffer (pH 7.5) containing 0.1% Triton X-100 at 100 V for 2 h. Protein bands were detected with a silver staining kit (Pharmacia) in both cases, while the running gel was treated with 20% (w/v) trichloroacetic acid to fix proteins on native PAGE.

RESULTS

Solubilization and purification of GPAT from the membrane fraction

We tested several detergents for their ability to solubilize GPAT from the membrane fraction of *M. ramanniana* var. *angulispora*. Among these, Triton X-100 provided the highest yield of solubilized GPAT activity and was used to solubilize GPAT in the present study. Varying Triton X-100 concentration, we found 0.3% Triton X-100 was the most suitable for GPAT solubilization. On the other hand, 0.05% Triton X-100 treatment increased GPAT recovery in the resultant pellet, which may have been due to removal of inhibitory factor(s). These observations prompted us to conduct a two-step solubilization in which the membrane fraction was initially solubilized with 0.05% Triton X-100 and the resultant pellet was solubilized with 0.3% Triton X-100 (see the Experimental section for more details). GPAT specific activity after the two-step solubilization was approx. 2-fold higher than that in the 0.3% Triton X-100 extract (Table 1). After solubilization from the membrane fraction, LPA was the only reaction product from $[1-^{14}\text{C}]$ oleoyl-CoA, whereas $[^{14}\text{C}]$ PA, but almost no $[^{14}\text{C}]$ LPA, appeared as a reaction

determined as described in the Experimental section. Solid lines with no symbols represent the concentration of KCl (A–C) or phosphate (E). Fractions indicated by bars were used for further purification (A–D) or as the final preparation (E). mU, m-units.

Table 3 GPAT purification from the membrane fraction of *M. ramanniana* var. *angulispora*

Total GPAT activity in fractions obtained after column procedures was measured in the presence of 250 μ M PA, while that in the membrane fraction and the Triton X-100 extract was measured in the absence of PA. Experimental details are given in the Experimental section.

Purification step	Protein (mg)	Total activity (m-units)	Specific activity (m-units/mg of protein)	Recovery (%)	Purification (fold)
Membrane fraction	300	333	1.11	100	1
Triton X-100 extract	51	201	3.94	60	4
Heparin–Sepharose	7.2	80.4	11.2	24	10
Yellow 86 agarose	0.86	54.0	63.0	16	57
Second heparin–Sepharose	0.20	30.0	150	9	135
Superdex 200	0.026	16.2	623	4.9	561
Hydroxylapatite	0.0021	3.05	1452	0.92	1308

product in the membrane fraction. The coupling of GPAT and LPA acyltransferase was destroyed after Triton X-100 solubilization. We monitored the stability of Triton X-100-solubilized GPAT at -80°C , -30°C and 4°C , because enzyme stability is an important factor in successful enzyme purification. GPAT activity in the Triton X-100 extract was stable at -80°C for 3 months, while 50 % activity remained at -30°C after 15 days. Following storage at 4°C , the enzyme was stable for approx. 12 h, but, thereafter, its activity declined very rapidly. This observation directed us to complete every column procedure within 12 h at 4°C to avoid loss of enzyme activity during column chromatography.

Preliminary experiments showed that approx. 90 % of GPAT activity was lost after the first heparin–Sepharose column, which we suspected was due to elimination of certain phospholipids during column procedures, and we tested this by incorporating phospholipids into the GPAT assay mixture (Table 2). Most phospholipids increased GPAT activity; PA, PC and PS at 250 μ M (24 mol % in Triton X-100 micelle) were highly active. We therefore added 250 μ M PA to the assay mixture to reconstitute GPAT activity after column procedures.

Figure 2 SDS/PAGE of purified GPAT

Purified GPAT fraction (30 ng of protein) from the hydroxylapatite column was subjected to SDS/PAGE under reducing conditions using a 12.5% gel, followed by silver staining. Molecular mass standards (Sigma) are indicated by arrows on the left.

Figure 3 Native PAGE of purified GPAT

Purified GPAT fraction (30 ng of protein) from the hydroxylapatite column was subjected to native PAGE using a 7.5% gel. Preparation of the gel and running conditions are described in the Experimental section. After running the gel, one lane was cut into 0.5 cm slices. Each slice was crushed with a spatula in buffer A and assayed for GPAT activity. GPAT activity was measured based on the protocol in the Experimental section, except for a final volume of 200 μ l, and the radioactivity in LPA was counted (A). A second lane was silver stained (B).

Column chromatography profiles for GPAT purification are shown in Figures 1(A)–1(E). Heparin–Sepharose was used twice, since most proteins that eluted with the GPAT activity peak from the first heparin–Sepharose column did not bind to the second heparin–Sepharose column, while GPAT activity bound in both cases (Figures 1A and 1C). This was reproducible and may have been due to differences in sample constituents. Two major GPAT peaks appeared in heparin–Sepharose, which were combined, probably due to heterogeneity in charges since Superdex-200 gel-filtration chromatography eluted GPAT activity as a single peak (Figure 1D). Although Yellow 86 agarose (Figure 1B) and hydroxylapatite Bio-Gel (Figure 1E) also separated minor GPAT peaks, the final GPAT preparation contained the majority of the GPAT activity.

GPAT purification is summarized in Table 3. The final purified GPAT preparation was enriched 1308-fold from the membrane fraction with a yield of 0.92 %. The polypeptide profile of the purified GPAT fraction was analysed by SDS/PAGE (Figure 2).

Figure 4 Substrate dependence of purified GPAT

Purified GPAT (30 ng of protein) was assayed with 250 μM *sn*-G3P at different [$1\text{-}^{14}\text{C}$]oleoyl-CoA concentrations (**A**) or with 50 μM [$1\text{-}^{14}\text{C}$]oleoyl-CoA at different *sn*-G3P concentrations (**B**). Assay conditions are described in the Experimental section. Data are presented as means of duplicate experiments. U, units.

Silver staining showed that the final preparation contained a single band of 45 kDa. Native PAGE of the final preparation revealed a major band that corresponded to GPAT activity with basic properties (Figure 3).

Characterization of purified GPAT

The optimum pH of purified GPAT was 7.0, similar to GPAT in the Triton X-100 extract. Purified GPAT was thermolabile; preincubation of the enzyme preparation at 40 °C for 40 min completely abolished activity. Purified GPAT activity depended on exogenous oleoyl-CoA and G3P, with the highest activity at 50 and 250 μM respectively (Figure 4). Double-reciprocal plots of the substrate dependence fitted Michaelis–Menten kinetics at lower concentrations of substrates (up to 50 μM for oleoyl-CoA and 150 μM for G3P). The apparent K_m for oleoyl-CoA was 15 μM and the apparent K_m for G3P was 25 μM . V_{max} for oleoyl-CoA was 1.5 units/mg of protein and the V_{max} for G3P was 1.1 units/mg of protein. Higher concentrations of both substrates inhibited GPAT activity.

The acyl-acceptor specificity experiment indicated that purified GPAT did not use *sn*-1,2-diolein, LPA or lysophosphatidylcholine as a substrate, suggesting that the final enzyme preparation does not contain diacylglycerol acyltransferase,

Table 4 Acyl-CoA specificity of purified GPAT

Different acyl-CoAs (250 μM) were added to the GPAT assay mixture containing 50 μM *sn*-[$1\text{-}^{14}\text{C}$]G3P and purified GPAT (30 ng of protein). Assay conditions are described in the Experimental section. Data are presented as means $\pm$ S.D. ($n = 3$).

Acyl-CoA (250 μM)	GPAT activity (m-units/mg of protein)
Octanoyl-CoA	44 $\pm$ 6
Lauroyl-CoA	85 $\pm$ 11
Myristoyl-CoA	158 $\pm$ 21
Palmitoyl-CoA	155 $\pm$ 18
Stearoyl-CoA	196 $\pm$ 13
Oleoyl-CoA	835 $\pm$ 45

Table 5 Effect of reagents on purified GPAT

Purified GPAT (30 ng of protein) was assayed with 50 μM [$1\text{-}^{14}\text{C}$]oleoyl-CoA and 250 μM *sn*-G3P in the presence of different reagents. Assay conditions are described in the Experimental section. Data are presented as means $\pm$ S.D. ($n = 3$).

Reagent	GPAT activity (% of control)
DTT (1 mM)	182 $\pm$ 7
EDTA (1 mM)	19 $\pm$ 3
DTNB (1 mM)	23 $\pm$ 6
PCMB (1 mM)	36 $\pm$ 7
NEM (5 mM)	21 $\pm$ 3
Mg ²⁺ (5 mM)	25 $\pm$ 4
Ca ²⁺ (5 mM)	30 $\pm$ 5
Mn ²⁺ (2.5 mM)	153 $\pm$ 8
Mn ²⁺ (5 mM)	210 $\pm$ 18
Mn ²⁺ (10 mM)	207 $\pm$ 23

Table 6 Effect of phospholipids on purified GPAT

Purified GPAT (30 ng of protein) was assayed with 50 μM [$1\text{-}^{14}\text{C}$]oleoyl-CoA and 250 μM *sn*-G3P in the presence of different phospholipids (250 μM). Assay conditions are described in the Experimental section. Data are presented as means $\pm$ S.D. ($n = 3$).

Phospholipid	GPAT activity (m-units/mg of protein)
No addition	0
Dioleoyl PA	1298 $\pm$ 83
PA (from egg lethicin)	955 $\pm$ 52
Dioleoyl PS	1873 $\pm$ 92
PS (from bovine brain)	1559 $\pm$ 82
Dioleoyl PC	103 $\pm$ 27
PC (from bovine liver)	0
PE (from bovine liver)	0
Phosphatidylinositol (from soya bean)	0
Oleoyl LPA	193 $\pm$ 24
Lysophosphatidylserine	262 $\pm$ 33
Lysophosphatidylcholine (from bovine liver)	0

lysophosphatidic acid acyltransferase or lysophosphatidylcholine acyltransferase activity.

Purified GPAT preferred oleoyl-CoA 5.4-fold over palmitoyl-CoA as an acyl donor (Table 4). Saturated fatty acyl-CoAs, such as myristoyl- and stearoyl-CoA, were only approx. 20% as effective as oleoyl-CoA as a substrate, while medium chain acyl-CoAs were inefficient as acyl donors for purified GPAT.

Thiol reagents, such as 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB), *N*-ethylmaleimide (NEM) and *p*-chloromercuribenzoic acid (PCMB), inhibited purified GPAT (Table 5), suggesting that it originated from microsomes rather than mitochondria. In

Figure 5 Concentration dependence of purified GPAT activation on PA and PS

PA (□) or PS (■) at different concentrations was added, dried under N_2 , and suspended in the GPAT assay mixture containing 50 μ M [$1-^{14}C$]oleoyl-CoA, 250 μ M *sn*-G3P and purified GPAT (30 ng of protein). Detailed assay conditions are described in the Experimental section. Mol% was calculated as [(mol of phospholipid)/(mol of phospholipid and Triton X-100)] $\times$ 100. The highest GPAT activity was achieved at 24 mol% (250 μ M) for both PA and PS. Data are presented as means $\pm$ S.D. ($n = 3$). U, units.

contrast, DTT, which protects thiol groups, enhanced GPAT activity. Metal ions Mg^{2+} and Ca^{2+} , inhibited GPAT activity, whereas Mn^{2+} ions elevated activity at 5 mM (Table 5). GPAT activity was inhibited by EDTA probably due to Mn^{2+} chelation.

Purified GPAT was activated by anionic phospholipids, such as PA and PS (Table 6), as in the peak fraction of the first heparin–Sephacrose column (see Table 2). The ability to activate GPAT by phospholipids was changed after GPAT purification. PS was more effective than PA in activating purified GPAT, and PC, which significantly activated crude GPAT, did not affect GPAT activity. Note that purified GPAT could not exhibit enzyme activity without exogenous phospholipids. Concentration dependence of PA and PS to activate purified GPAT showed that 250 μ M (24 mol%) was the most effective for PA and PS, and PS was more effective than PA at all concentrations (Figure 5). These results suggest these anionic phospholipids serve as lipid boundaries rather than cofactors for GPAT activation.

DISCUSSION

In the present paper, we present solubilization, purification to homogeneity, and characterization of GPAT from the membrane fraction of an oleaginous fungus, *M. ramanniana* var. *angulispora*. To avoid enzyme loss during column procedures, we added exogenous phospholipids for its reactivation and repeated small-scale column chromatography in order to minimize exposure of the enzyme preparation at 4 °C. Since the enzyme preparation was very stable at –80 °C, the repetition of short-column procedures at 4 °C and storage at –80 °C minimized the decrease in GPAT activity.

SDS/PAGE and native PAGE of purified GPAT from *M. ramanniana* var. *angulispora* revealed a molecular mass of 45 kDa and a basic isoelectric point. This molecular size was distinct from that of GPAT in *E. coli* [4] and mammalian mitochondria [6–8], which is approx. 83–90 kDa. Plastidial GPAT isoenzymes

have molecular masses of 30–40 kDa, but these are not membrane-bound and their isoelectric points are 5.5–6.6 [9]. The apparent K_m for G3P in purified GPAT from *M. ramanniana* var. *angulispora* was much lower than the corresponding values in *E. coli* [4] and mammalian mitochondria [6,7], whereas that for oleoyl-CoA was slightly higher than the values in *E. coli* [4] and mammalian mitochondria [6], indicating that GPAT in *M. ramanniana* var. *angulispora* had higher affinity for G3P. These data indicate that purified GPAT in the present study represents an enzyme that is different from previously known GPAT.

In mammalian cells, microsomal GPAT is sensitive to thiol reagents, whereas mitochondrial GPAT is resistant [11–15]. GPAT purified from the membrane fraction of *M. ramanniana* var. *angulispora* was inhibited by thiol reagents, such as DTNB, NEM and PCMB (Table 5), suggesting that it originated from microsomes rather than mitochondria. Protection of thiol groups by DTT enhanced GPAT activity in *M. ramanniana* var. *angulispora*, which also agreed with previous observations for microsomal GPAT [11]. Another comparison between microsomal and mitochondrial GPAT in mammalian cells is the products of the enzyme reactions; the main product in microsomes is PA, whereas that in mitochondria is LPA [17,30]. When the membrane fraction of *M. ramanniana* var. *angulispora* was incubated with [$1-^{14}C$]oleoyl-CoA and G3P, only radiolabelled PA, but no radiolabelled LPA, formed [25]. This indicates that most of the GPAT activity in the membrane fraction coupled with LPA acyltransferase, and therefore that it is localized in microsomes. Taken together, it is most probable that the new type of GPAT purified from the membrane fraction of *M. ramanniana* var. *angulispora* originated from microsomes.

Substrate specificities of GPAT have been extensively studied to elucidate the mechanism behind fatty acid distribution of phospholipids and TG. Microsomal GPAT used both saturated and unsaturated fatty acyl-CoA in mammals [1,12,13,31,32] and plants [18,33,34], whereas mitochondrial GPAT preferred palmitoyl-CoA over unsaturated fatty acyl-CoA [6,7,11,13,17]. Acyl-CoA specificity of purified GPAT from *M. ramanniana* var. *angulispora* is distinct from mitochondrial GPAT, and preferred oleoyl-CoA over palmitoyl-CoA. The present study provides direct evidence, using a purified enzyme, that thiol-reagent-sensitive microsomal GPAT uses different acyl-CoAs *in vitro*. How microsomal GPAT contributes to the asymmetric distribution of saturated and unsaturated fatty acids at the *sn*-1 and *sn*-2 positions of glycerophospholipids in comparison with mitochondrial GPAT is of great interest.

It is known that detergent-solubilized GPAT requires exogenous phospholipids to exhibit activity [4,6,7,16,19,35–38]. Suitable phospholipids for activation differ with enzyme source and preparation. Phosphatidylglycerol was suitable for purified GPAT in *E. coli* [4,38] and rat liver mitochondria [6]. Crude preparations of rat liver mitochondria [16,37] and mouse recombinant GPAT expressed in insect cells [7] showed different requirements for phospholipids. Our observation that fungal GPAT activation efficiency by different phospholipids changed with enzyme purity is consistent with previous studies. The anionic phospholipid PS was also effective in activating previously characterized GPAT [4,6,7,16,37], and was the most effective in activating purified GPAT in the fungus studied in the present paper. In contrast, PA, another anionic phospholipid which inhibited mitochondrial GPAT [6,16,37], was very effective in activating fungal GPAT in both crude and purified preparations. This raises the possibility that PA regulates the contribution of microsomal and mitochondrial GPAT in glycerolipid biosynthesis. It is also known that dioleoyl species of phospholipids are much more effective than dipalmitoyl species

[6,38]. Our observation that the dioleoyl species of PA and PS showed higher activation than the respective native phospholipids (see Table 6) is consistent with previous results. The high phospholipid-to-detergent ratio in mixed micelles was required to activate purified GPAT in *E. coli* [38] and rat liver mitochondria [6], which were classified as enzymes that require lipid boundaries rather than lipid cofactors [39]. We observed that GPAT activation by PS and PA was dose dependent and 24 mol % concentration was the most effective to activate purified GPAT (Figure 5), which indicates that GPAT from the membrane fraction of *M. ramanniana* var. *angulispora* also belongs to the group of enzymes that require a lipid boundary for their activation.

Bivalent cations, such as Mg^{2+} and Ca^{2+} , increased GPAT activity from different sources [16,19,33,40] and Mg^{2+} was added to the GPAT assay for purification [4,6,7]. In contrast to previous observations, purified GPAT in *M. ramanniana* var. *angulispora* was significantly inhibited by Mg^{2+} and Ca^{2+} , but activated by Mn^{2+} (see Table 5). Mn^{2+} was also reported to elevate GPAT activity, but its effect substituted for other metal ions [16,19]. The effect of Mn^{2+} on purified fungal GPAT did not substitute for other metal ions and appeared to be specific, although its physiological importance remains unclear. The inhibitory effect of Mg^{2+} and Ca^{2+} and the stimulatory effect of Mn^{2+} were less in the Triton X-100 extract than in the purified fraction (results not shown), suggesting depletion of endogenous metal ions during purification procedures.

Mitochondrial GPAT activity and its gene were found to respond to nutrition and hormones [5,17]. Microsomal GPAT tends to be less responsive to these factors, although its enzyme activity was reported to increase during cell differentiation [1,17]. Phosphorylation–dephosphorylation of GPAT [1,17] and lipid peroxidation [41] have been suggested to be involved in its regulation. How these regulatory factors affect GPAT and hence phospholipid and TG biosynthesis, should be addressed by purifying microsomal GPAT and cloning its genes. Regulation of GPAT includes how microsomal and mitochondrial GPAT contribute to glycerolipid biosynthesis. In addition, high GPAT activity and PA synthesis were observed in lipid particles of *Saccharomyces cerevisiae* [42–44], although GPAT activity was enriched in the membrane fraction of *M. ramanniana* var. *angulispora* [25]. Findings from the present study provide new insights into the molecular characterization of microsomal GPAT, which has not been well characterized compared with mitochondrial and plastidic GPAT. Further study of the structure and function of microsomal GPAT should reveal the regulatory mechanisms behind phospholipid and TG biosynthesis.

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