# Purification of phosphatidylglycerophosphate synthase from Chinese hamster ovary cells

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Phosphatidylglycerophosphate (PGP) synthase catalyses the committed step in the biosynthesis of phosphatidylglycerol and cardiolipin in mammalian cells. Recently we isolated a Chinese hamster ovary (CHO) *PGS1* cDNA encoding PGP synthase. In the present study we purified this PGP synthase to near-homogeneity from the mitochondrial fraction of CHO-K1 cells; the final enzyme preparation gave a single 60 kDa protein on SDS/PAGE. Polyclonal antibodies raised against a recombinant CHO PGS1 protein cross-reacted with the purified 60 kDa protein and with CHO membrane proteins of 60 kDa and 62 kDa that increased after transfection with the PGS1 cDNA. The 60 and 62 kDa protein levels in a PGP synthase-defective mutant of CHO-K1 cells were markedly lower than those in CHO-K1 cells. These results indicated that the purified 60 kDa protein was PGP synthase encoded by the *PGS1* gene. In addition we found that the purified PGP synthase had no PGP phosphatase activity, indicating that phosphatidylglycerol was produced from CDP-diacylglycerol through two steps catalysed by distinct enzymes, PGP synthase and PGP phosphatase.

Key words: cardiolipin, mitochondria, phosphatidylglycerol, phospholipid.

## INTRODUCTION

The biosynthetic pathways for phosphatidylglycerol (PtdGro) and cardiolipin (CL) in mammalian cells were established by early enzymological studies, as shown in Scheme 1 (reviewed in [1]). Phosphatidylglycerophosphate (PGP) synthase catalyses the first step in the CL branch of phospholipid biosynthesis, with the displacement of the CMP moiety of CDP-diacylglycerol by L-glycerol 3-phosphate to produce PGP [2]. PGP is then rapidly dephosphorylated to generate PtdGro, which is used as a substrate together with CDP-diacylglycerol for CL synthesis. Because PGP synthase and CL synthase activity are abundant in the inner mitochondrial membrane [2–4], PtdGro and CL are thought to be synthesized in mitochondria *in vivo*. CL is located primarily in the mitochondrial inner membrane [4] and its content is especially high in cardiac muscle, ranging from 9 % to 15 % of total phospholipid, where mitochondrial energy production is thought to be important for the functioning of the heart [1].

That PGP synthase is a crucial enzyme for the biosyntheses of PtdGro and CL was indicated by the isolation of a temperature-sensitive Chinese hamster ovary (CHO) cell mutant, PGS-S, which is defective in PGP synthase activity [5]. PGP synthase activity in PGS-S is only 1 % of that in wild-type CHO-K1 cells; the biosynthetic rates and cellular contents of PtdGro and CL were also markedly decreased in the mutant [5]. We recently reported the isolation of a CHO *PGS1* cDNA [6] encoding a putative protein similar in sequence to the yeast *PGS1* gene product, PGP synthase [7]. The expression of CHO *PGS1* cDNA complements the defect in PGP synthase of PGS-S cells and corrects the decreased PtdGro and CL contents in the mutant [6,8]. These results indicate that CHO *PGS1* encodes PGP synthase. Although the cDNA of PGP synthase has been isolated, the catalytic and regulatory mechanisms of PGP synthase remain to be elucidated, partly because the complete purification of PGP synthase from a mammal has not yet been achieved [9,10].

Here we report the purification of mitochondrial PGP synthase from wild-type CHO-K1 cells. With the use of polyclonal antibodies raised against the recombinant CHO *PGS1*-encoded protein, we also demonstrate that the purified PGP synthase is encoded by CHO *PGS1*.

## EXPERIMENTAL

### Materials

All chemicals were of reagent grade or better. TLC Silica Gel 60 plates were purchased from Merck. Oligonucleotides were from Nisshinbo. L-[U-$^{14}$C]Glycerol 3-phosphate was from ICN Radiochemicals. CDP-diacylglycerol was from Serdary Research Laboratory. L-Glycerol 3-phosphate, n-octyl-$\beta$-D-glucopyranoside, Triton X-100 and metrizamide were from Sigma Aldrich. ES medium was from Nissui Pharmaceutical Co., Ltd. Ham's F-12 medium, newborn calf serum, geneticin (G418), penicillin G and streptomycin sulphate were from Life Technologies. Fetal bovine serum was from JRH Bioscience. Newborn calf serum was from ICN Flow. Blue-Sepharose, Mono Q HR 5/5, Hi Load 16/60 Superdex 200 pg, PD-10, Percoll, anti-rabbit Ig linked to horseradish peroxidase, and anti-mouse Ig linked to horseradish peroxidase were from Amersham Pharmacia Biotech. Anti-(cytochrome *c* oxidase subunit I) (anti-[COX I]) was from Molecular Probes.

### CHO cell strain and culture

Strain CHO-K1 was obtained from the American Type Culture Collection. The PGP-synthase-defective mutant, PGS-S, was described previously [5,8]. A stable transformant, CHO/cPGS1, was isolated by introducing pSVneo-cPGS1 [6] into CHO-K1, followed by selection for transformants resistant to G418

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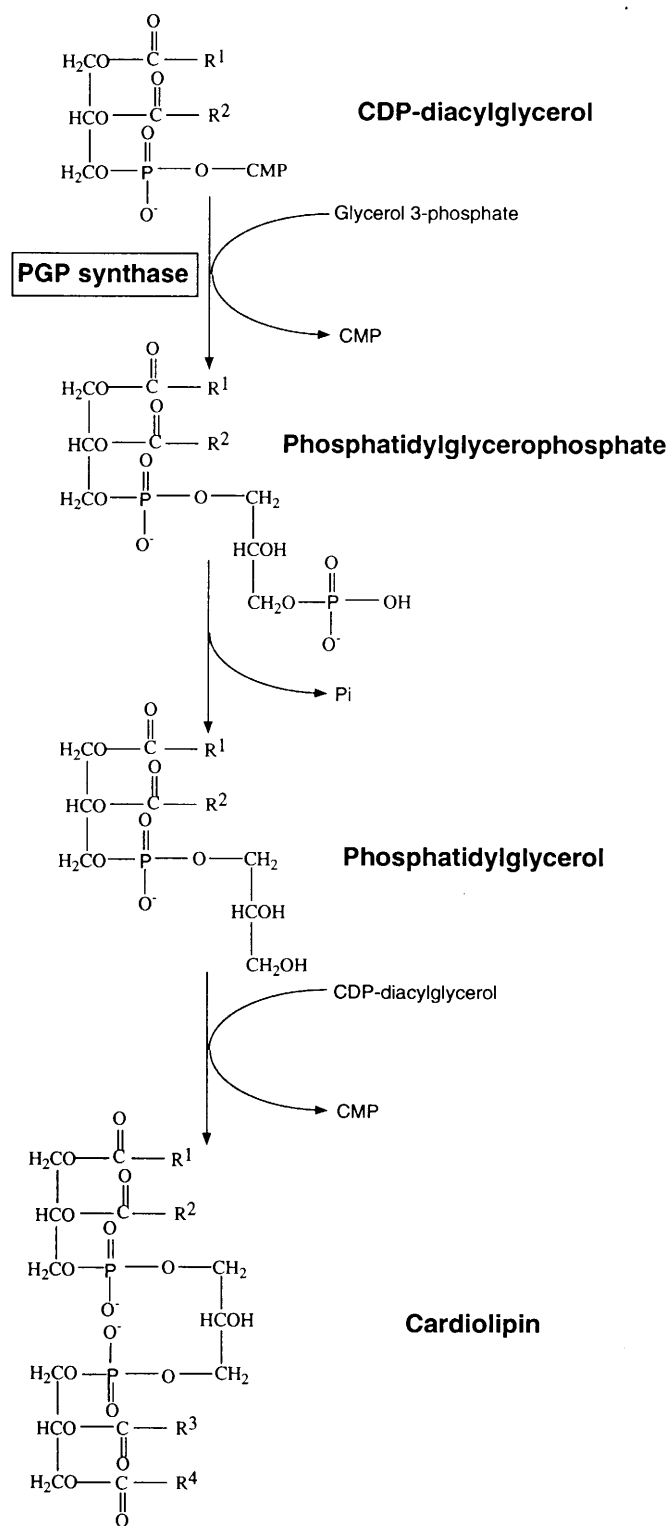
**Scheme 1  Biosynthetic pathway for PtdGro and CL in mammalian cells**

(400 μg/ml); the strain was then purified by limited dilution. Unless indicated otherwise, all strains were maintained in Ham's F-12 medium supplemented with 10 % (v/v) newborn calf serum, penicillin G (100 i.u./ml) and streptomycin sulphate (100 μg/ml), under air/CO$_2$ (19:1) at 100 % humidity and 33 °C.

## Purification of PGP synthase

Unless indicated otherwise, all procedures were performed at 4 °C or on ice. CHO-K1 cells, which were grown at 37 °C in a glass spinner-flask containing ES medium supplemented with 5 % (v/v) fetal bovine serum, L-glutamate (292 mg/l), penicillin G (100 i.u./ml), streptomycin sulphate (100 μg/ml) and NaHCO$_3$ (2.2 g/l), were collected by centrifugation at 700 *g*, then washed twice with PBS. The collected cells (6 × 10$^{10}$) were suspended in 60 ml of 0.25 M sucrose and homogenized with a motor-driven homogenizer (Iuchi). The homogenate was centrifuged at 700 *g* for 10 min to remove nuclei and debris; the resultant supernatant (post-nuclear supernatant) was centrifuged at 10000 *g* for 20 min. The resultant precipitate (crude mitochondria) was suspended in 0.25 M sucrose; the mitochondria were then purified further by hybrid Percoll/metrizamide discontinuous density gradient centrifugation as described [11]. The purified mitochondria were adjusted to 4 mg/ml protein in 5 mM Tris/HCl, pH 7.5, 0.1 M sucrose, 1 mM EDTA and 1 % (v/v) Triton X-100, then sonically disrupted. The disrupted mitochondria were centrifuged at 100000 *g* for 60 min; the supernatant (solubilized mitochondrial proteins) was stored at −80 °C for further analysis and purification of PGP synthase.

Half of the solubilized mitochondrial protein was passed at a flow rate of 4 ml/h through a Blue-Sepharose (10 ml) column equilibrated with buffer A [50 mM Tris/HCl (pH 7.5)/150 mM NaCl/0.25 M sucrose/1 mM EDTA, 0.5 % (v/v) Triton X-100]. Subsequently, the column was washed with 25 ml of buffer B [20 mM Tris/HCl (pH 7.5)/0.25 M sucrose/1 mM EDTA/1 % (w/v) n-octyl-β-D-glucopyranoside] containing 5 mM CDP and 150 mM NaCl, followed by 25 ml of buffer B containing 5 mM NADH and 150 mM NaCl. After this washing, the column was equilibrated with buffer B containing 2.5 mM CDP-diacylglycerol and incubated at 4 °C for approx. 12 h; PGP synthase activity was then eluted with buffer B containing 2.5 mM CDP-diacylglycerol. The PGP synthase fraction was diluted with buffer B to decrease the NaCl concentration to less than 20 mM. The diluted fraction was applied, at a flow rate of 0.5 ml/min, to a Mono Q HR 5/5 column equilibrated with buffer B. After the loaded column had been washed with 25 ml of buffer B containing 20 mM NaCl, PGP synthase was eluted with 30 ml of a linear NaCl gradient (20–350 mM) in buffer B. PGP synthase fractions recovered from a Mono Q column were applied, at a flow rate of 0.4 ml/min, to a Hi Load 16/60 Superdex 200 column and eluted with buffer C [150 mM sodium phosphate (pH 6.8)/1 % (w/v) n-octyl-β-D-glucopyranoside]. Solid (NH$_4$)$_2$SO$_4$ was added to the PGP synthase fraction recovered from the Superdex 200 column to 30 % satn at room temperature; the pooled active fractions were then applied, at a flow rate of 1 ml/min, to a TSK-Ether 5PW (TOSO) column. After the loaded column had been washed with 8 ml of buffer D [50 mM sodium phosphate (pH 6.8)/ 1 % (w/v) n-octyl-β-D-glucopyranoside] containing 30 %-satd (NH$_4$)$_2$SO$_4$, PGP synthase was eluted with 30 ml of buffer D, at a flow rate of 1 ml/min, containing a linear (NH$_4$)$_2$SO$_4$ gradient (30–0 % satn). The fractions eluted from the TSK-Ether column were desalted with PD-10 in buffer E [15 mM sodium phosphate (pH 6.8)/1 % (w/v) n-octyl-β-D-glucopyranoside]; the desalted fractions were used for further analysis.

## Protein determination

Unless indicated otherwise, protein concentrations were determined with bicinchoninic acid protein assay reagent (Pierce) with BSA as a standard.

**Table 1    Purification of PGP synthase from CHO cells**

PGP synthase was purified from post-nuclear supernatant prepared from CHO-K1 cells as described in the Experimental section. Protein concentrations after the TSK-Ether step were estimated by densitometric comparison of silver-stained proteins in the fractions with stained calibration bands of BSA of known concentrations after SDS/PAGE. Abbreviation: n.d., not determined.

| Purification step | Protein (mg) | Total activity (nmol/min) | Yield (%) | Specific activity (nmol/min per mg) | Purification (fold) |
|---|---|---|---|---|---|
| Post-nuclear supernatant | 12 000 | 7800 | 100 | 0.65 | 1 |
| Purified mitochondria | 250 | 1270 | 16 | 5.1 | 7.8 |
| Solubilized mitochondrial proteins | 200 | 1000 | 13 | 5.0 | 7.7 |
| Blue-Sepharose | 5.0 | 300 | 3.8 | 60 | 92 |
| Mono Q | n.d. | 26 | 0.33 | n.d. | n.d. |
| Superdex 200 | n.d. | 16 | 0.21 | n.d. | n.d. |
| TSK-Ether | 0.00032 | 3.8 | 0.049 | 12 000 | 18 000 |

## Enzyme assay

PGP synthase activity was measured as described previously [2], with some modifications. The assay was performed at 37 °C for 30 min in 50 mM Tris/HCl (pH 7.4)/0.25 mM CDP-diacyl-glycerol/0.1 mM L-[U-$^{14}$C]glycerol 3-phosphate (20 mCi/mmol)/0.25 % (v/v) Triton X-100 containing enzyme, in a total volume of 50 $\mu$l. After incubation, the lipids were extracted by the sequential addition of 600 $\mu$l of chloroform/methanol (1:2, v/v), 200 $\mu$l of chloroform and 200 $\mu$l of PBS. The lipid-containing chloroform phase was washed twice with 400 $\mu$l of chloroform/methanol/0.1 M KCl (3:47:48, by vol.); the radio-activity in the chloroform phase was then determined with a counter in a toluene-based scintillation mixture. For charac-terization of the labelled lipids, TLC on silica gel 60 plates treated with 0.5 M oxalic acid was performed with chloroform/methanol/35 % HCl (87:13:0.2, by vol.), as described previously [10].

## Preparation of an antibody against the recombinant PGS1 protein

The His$_6$-tagged partial CHO *PGS1*-encoded protein was used as an antigen with which to produce a polyclonal antibody. The recombinant protein was prepared as follows. The coding region of *PGS1* cDNA was engineered by PCR so as to add a *Bam*HI site immediately upstream of the CAG codon encoding residue 181, Gln, and a *Hin*dIII site immediately downstream of the ter-mination codon. The engineered cDNA was cloned into the *Bam*HI/*Hin*dIII site of the pQE9 vector (Qiagen), which was designed to produce a recombinant protein carrying the His$_6$ epitope at the N-terminal. The resulting construct (pQE9-cPGS1$^{181–553}$) was introduced into *Escherichia coli* strain M15 (Qiagen). The transformant grown in Luria–Bertani medium containing both ampicillin (100 $\mu$g/ml) and kanamycin (25 $\mu$g/ml) was cultivated for 4 h in the presence of 1 mM isopropyl $\beta$-D-thiogalactoside to induce the expression of the recombinant PGS1 protein. The His$_6$-tagged recombinant PGS1 protein was purified from an *E. coli* lysate with Ni$^{2+}$-nitrilo-triacetate–agarose (Qiagen) in accordance with the manu-facturer's instructions. Immunization of a rabbit and preparation of serum were performed by Charles River Japan. The antibody against the recombinant PGS1 protein was affinity-purified by adsorption on the recombinant PGS1 protein blotted to a nitrocellulose membrane [12].

## SDS/PAGE, silver staining and Western blotting

Proteins were fractionated by SDS/PAGE [10 % (w/v) gel] under reducing conditions [13]. Molecular mass standards were purchased from Bio-Rad. Proteins separated on the gel were stained with a silver staining kit (Wako). For Western blot analysis, proteins separated by SDS/PAGE were electroblotted at 22 V/cm for 60 min to an Immobilon-PSQ membrane (Millipore) in 25 mM Tris/192 mM glycine/0.02 % SDS/20 % (v/v) methanol. Then the blot was incubated with anti-(PGS1 protein) antibodies or anti-(COX I) antibodies and subsequently with anti-rabbit or anti-mouse IgG linked to horseradish peroxidase. Cross-reactive proteins were detected with en-hanced chemiluminescence Western blotting detection reagents (Amersham Pharmacia Biotech).

## Subcellular fractionation

All procedures were performed at 4 °C or on ice. Post-nuclear supernatant prepared as described above was centrifuged at 10 000 $g$ for 20 min; both the resultant precipitate (crude mito-chondria) and supernatant were collected. Mitochondria were further purified from crude mitochondria as described above. The supernatant was centrifuged at 100 000 $g$ for 1 h; the precipitate (microsomal membrane) and supernatant (cytosol) were collected.

## RESULTS AND DISCUSSION

### Purification of PGP synthase from CHO-K1 cells

Purification of PGP synthase was started with $6 \times 10^{10}$ CHO-K1 cells. Mitochondria in the crude mitochondria fraction prepared by differential centrifugation were further purified by Percoll/metrizamide discontinuous density gradient centrifugation [11]. The purified mitochondria were treated with a detergent [Triton X-100; 1 % (v/v)]; after centrifugation at 100 000 $g$ the super-natant (solubilized mitochondrial proteins) was recovered. The solubilization efficiency for PGP synthase activity was approx. 80 % (Table 1). The PGP synthase in the solubilized mito-chondrial proteins was purified by successive chromatography on Blue-Sepharose, Mono Q ion exchange, Superdex 200 gel filtration and TSK-Ether hydrophobic columns. As shown in Table 1, the overall purification of the enzyme from post-nuclear supernatant of wild-type CHO-K1 cells was 18 000-fold with a recovery yield of 0.049 %. The proteins at the different steps of the purification of PGP synthase were analysed by SDS/PAGE. The final enzyme preparation, after staining with silver, gave an apparent single band corresponding to a molecular mass of 60 kDa (Figure 1). The enzyme activity was well correlated with the amount of 60 kDa protein in the final fractions with TSK-Ether (Figure 2). The enzyme activity was also well correlated with the amount of the 60 kDa protein after Superdex 200 gel
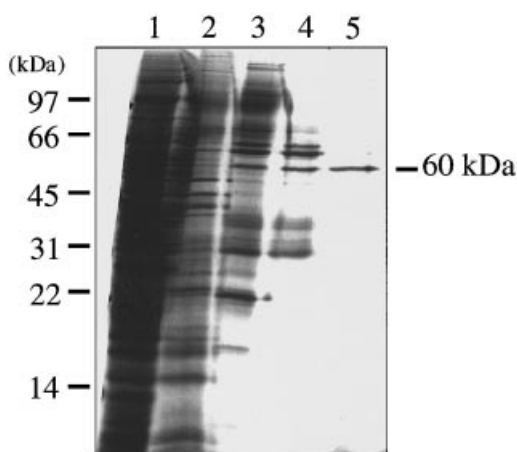
**Figure 1   SDS/PAGE analysis of proteins at the various steps of the purification of PGP synthase**

Proteins samples, each containing an equal quantity of enzyme activity (140 pmol/min), were analysed by SDS/PAGE [12.5% (w/v) gel] and detected by staining with silver. Lane 1, solubilized mitochondrial proteins; lanes 2–5, pooled active fractions from Blue-Sepharose (lane 2), Mono Q HR 5/5 (lane 3), Hi Load 16/60 Superdex 200 (lane 4) and TSK-Ether (lane 5). The molecular mass of the purified protein (60 kDa) was estimated by comparison with molecular mass standards (phosphorylase, 97 kDa; albumin, 66 kDa; ovalbumin, 45 kDa; carbonic anhydrase, 31 kDa; trypsin inhibitor, 22 kDa; lysozyme, 14 kDa), the sizes of which are indicated at the left.

filtration (results not shown). These results suggested that the purified 60 kDa protein had PGP synthase activity.

## Biochemical properties of PGP synthase

For analysis of the reaction products, purified and partly purified enzyme fractions, each containing equal amounts of enzyme activity, were incubated under the standard assay conditions; the radioactive lipids were then separated by TLC. Although radioactive PtdGro and PGP were both found in the post-nuclear supernatant, mitochondria and Blue-Sepharose purification fractions, the only radioactive lipid in the TSK-Ether fraction was PGP (Figure 3), indicating that PGP is the sole reaction product of the purified enzyme. To our knowledge this is the first demonstration that a mammalian PGP synthase can be separated from PGP phosphatase activity. These results constitute definitive evidence that PtdGro is produced from CDP-diacylglycerol through two steps catalysed by distinct enzymes, PGP synthase and PGP phosphatase.

The kinetics of the purified PGP synthase was examined. When the dependence of PGP formation on L-glycerol 3-phosphate was examined, a CDP-diacylglycerol concentration of 25 $\mu$M gave maximum activity (Figure 4A). Double-reciprocal plots of PGP formation against CDP-diacylglycerol in the presence of excess L-glycerol 3-phosphate (100 $\mu$M) showed that the apparent $K_m$ for CDP-diacylglycerol was 288 $\mu$M and that the apparent $V_{max}$ was 13613 nmol of PGP/min per mg of protein (Figure 4B).

The effects of bivalent cations on the purified PGP synthase were determined. As shown in Figure 4(C), the enzyme activity in the presence of 6 mM $Ca^{2+}$ was 2.3-fold that in the absence of the bivalent cations. $Mg^{2+}$ slightly enhanced the enzyme activity; in contrast, the other bivalent cations, $Mn^{2+}$, $Co^{2+}$, $Cd^{2+}$ and $Zn^{2+}$, were inhibitory. Because PGP synthase is a mitochondrial enzyme (see below), the activity of PGP synthase might be
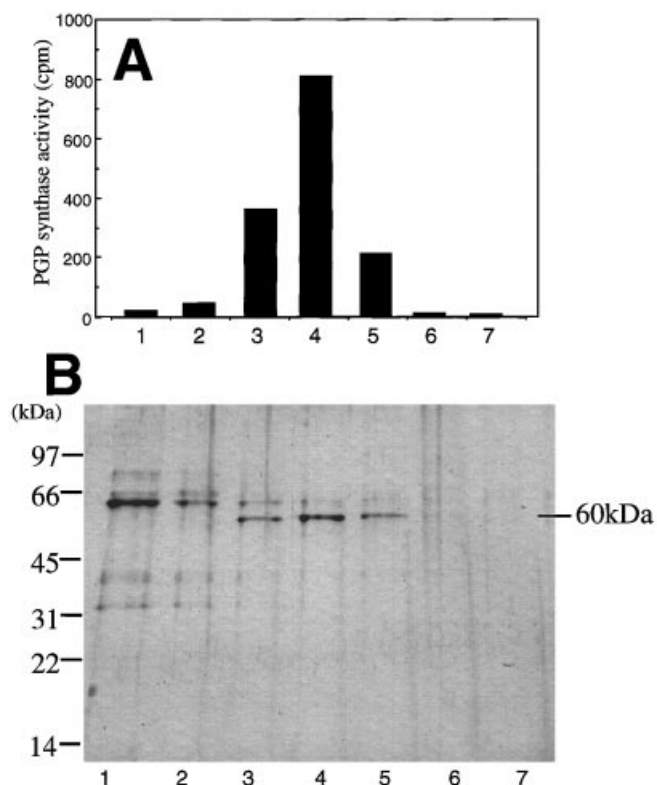
**Figure 2   Elution profiles of PGP synthase activity and 60 kDa protein on TSK-Ether chromatography**

(**A**) PGP synthase activity in TSK-Ether chromatography fractions (each 5 $\mu$l) was analysed under standard assay conditions. (**B**) Proteins in TSK-Ether chromatography fractions (each 100 $\mu$l), used for the analysis in (**A**), were analysed by SDS/PAGE [12.5% (w/v) gel] and then detected by staining with silver. The numbers indicate the fraction numbers on TSK-Ether chromatography. The positions of molecular mass markers are indicated at the left.
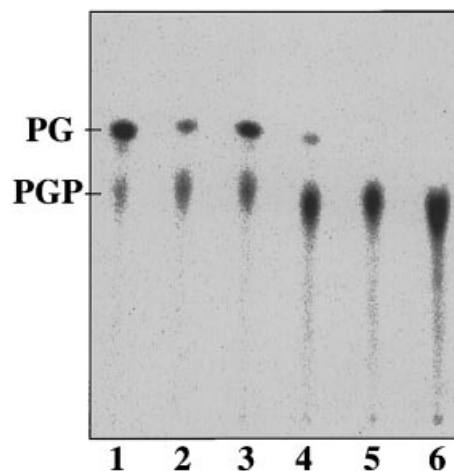


**Figure 3   Reaction products of the enzyme activity**

The purified and partly purified enzyme fractions, containing equal amounts of enzyme activity (35 pmol/min), were incubated under standard assay conditions. The radioactive lipids were separated by TLC on a 0.5 M oxalic acid-impregnated Silica gel 60 plate with chloroform/methanol/10 M hydroxyl chloride (87:13:0.2, by vol.). Lane 1, post-nuclear supernatant; lane 2, solubilized mitochondrial proteins; lanes 3–6, pooled active fractions from Blue-Sepharose (lane 3), Mono Q HR 5/5 (lane 4), Hi Load 16/60 Superdex 200 (lane 5) and TSK-Ether (lane 6). Abbreviation: PG, PtdGro.
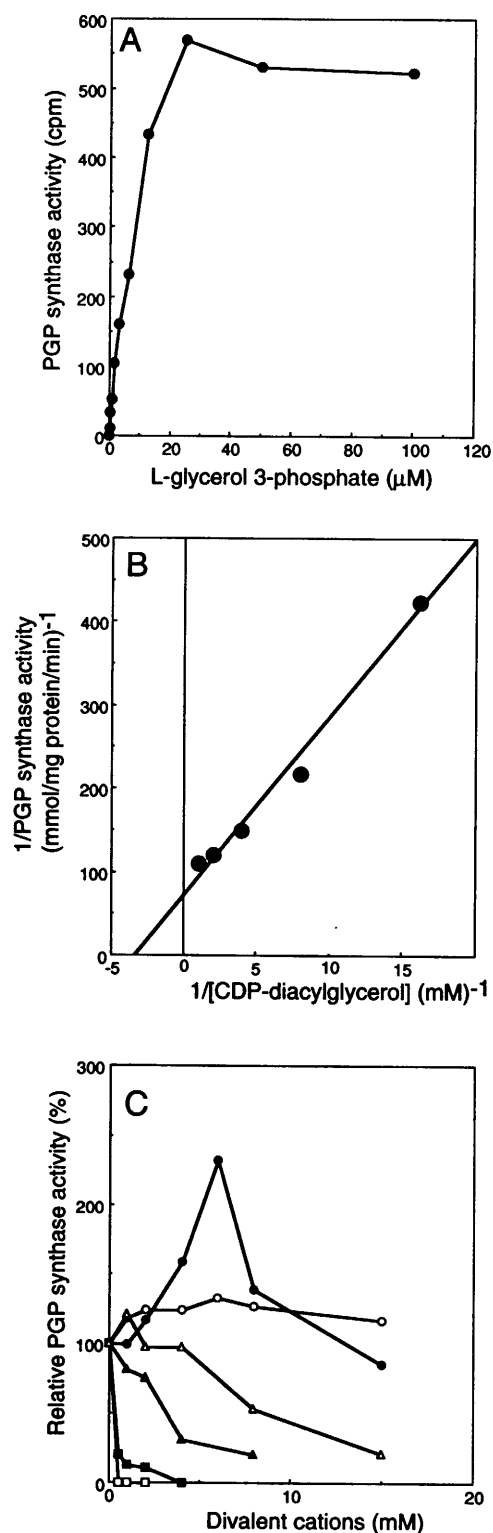
**Figure 4    Enzymic properties of purified PGP synthase**

(**A**) Dependence of activity on L-glycerol 3-phosphate. The activity of purified PGP synthase (0.3 ng) was measured with various concentrations of L-glycerol 3-phosphate by the standard assay method. (**B**) Dependence of activity on CDP-diacylglycerol. The activity of purified PGP synthase (0.3 ng) was measured with various concentrations of CDP-diacylglycerol by the standard assay method. The results obtained are shown as double-reciprocal plots. (**C**) Effects of bivalent cations on purified PGP synthase activity. The activity of purified PGP synthase (0.3 ng) was measured with the indicated concentrations of $ZnCl_2$ (□), $CdCl_2$ (■), $CoCl_2$ (▲), $MnCl_2$ (△), $CaCl_2$ (●) and $MgCl_2$ (○) by the standard assay method. Activities are shown relative to that obtained in the absence of the cations (100%).
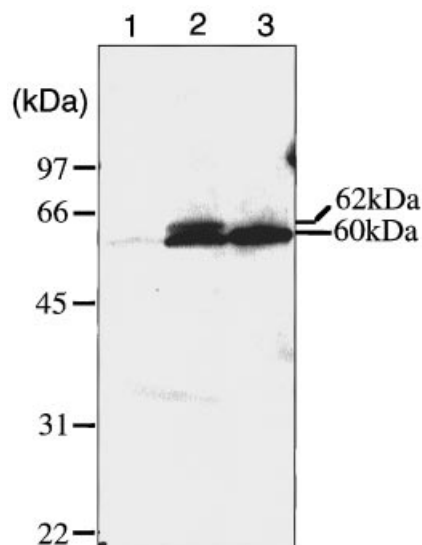


**Figure 5    Western blot analysis with anti-(PGS1 protein) antibodies**

Sonically disrupted cell lysates of CHO-K1 (50 μg, lane 1) and CHO/cPGS1 (50 μg, lane 2) cells, and purified PGP synthase (0.6 ng, lane 3) were separated by SDS/PAGE [10% (w/v) gel] and analysed by Western blotting with anti-(PGS1 protein) antibodies. The positions of molecular mass markers are indicated at the left.

regulated by $Ca^{2+}$, which has been shown to regulate mitochondrial metabolic processes [14].

In addition, the purified enzyme was shown to be incapable of using L-[$^{14}$C]serine and myo-[2-$^3$H]inositol as a substrate instead of L-glycerol 3-phosphate (results not shown).

### Immunochemical identification of the purified 60 kDa protein as a PGP synthase encoded by the CHO *PGS1* gene

We recently isolated a CHO *PGS1* cDNA that encodes a PGP synthase with a calculated molecular mass of 62329 Da [6]. To determine whether or not the purified 60 kDa protein was PGP synthase encoded by the CHO *PGS1* gene, polyclonal antibodies were raised against a recombinant PGS1 protein: 60 and 62 kDa proteins were detected by the anti-(PGS1 protein) antibodies in the cell extract of a stable transformant of CHO-K1 cells, CHO/cPGS1, which overproduced the PGS1 protein. The PGP synthase activity in the cell extract of CHO/cPGS1 was 7.5-fold that from wild-type CHO-K1 cells. Although the 60 and 62 kDa proteins were undetectable in the cell extract of wild-type CHO-K1 (Figure 5), the existence of both the 60 and 62 kDa proteins in wild-type CHO-K1 cells became evident on Western blot analysis of the mitochondrial fraction of CHO-K1 (Figure 6). In addition, we found that the amounts of the 60 and 62 kDa proteins recognized by anti-(PGS1 protein) antibody were very small in a PGP synthase-defective mutant (PGS-S) of CHO-K1 cells (Figure 6), the defect of which had been shown to be complemented by transfection with a *PGS1* cDNA [6]. These results indicated that the 60 and 62 kDa proteins recognized by the anti-(PGS1 protein) antibodies were CHO PGS1 products. Although PGP synthase activity in the CHO/cPGS1 transformant was 7.5-fold that in CHO-K1 cells, the increase in 60 and 62 kDa PGS1 proteins in CHO/cPGS1 cells seemed to be much more than 7.5-fold (Figure 5). A possible explanation for this discrepancy is that overproduction leads to the production of inactive forms of PGS1 protein, such as misfolded or aggregated
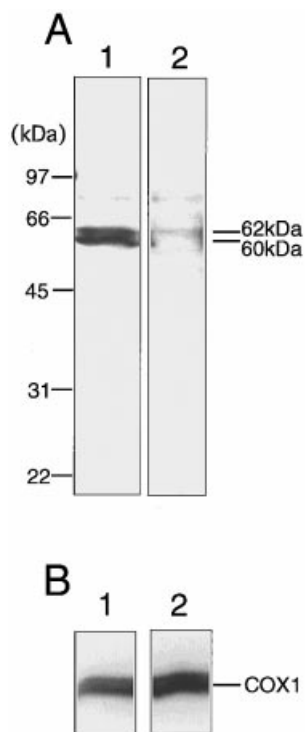
**Figure 6   Decreased expression of PGS1 protein in PGS-S cells**

CHO-K1 and PGS-S cells cultured at 40 °C for 4 days were disrupted sonically; the lysate was then centrifuged for 10 min at 700 **g**. The resultant supernatants were centrifuged for 20 min at 10 000 **g**; 33 μg portions of the precipitates (mitochondria fractions) prepared from CHO-K1 (lane 1) and PGS-S (lane 2) cells were separated by SDS/PAGE [10 % (w/v) gel] and analysed by Western blotting with anti-(PGS1 protein) antibodies (**A**) or anti-(COX I) antibodies (**B**). COX I is a marker protein for the mitochondrial inner membrane [18]. The positions of molecular mass markers are indicated at the left of (**A**).



**Figure 7   Enrichment of PGS1 protein in mitochondria**

Subcellular fractions were prepared from wild-type CHO-K1 cells as described in the Experimental section. Each lane contained 50 μg of protein. The post-nuclear supernatant (lane 1), cytosol (lane 2), microsomal (lane 3), crude mitochondrial (lane 4) and purified mitochondrial (lane 5) fractions were separated by SDS/PAGE [10 % (w/v) gel] and analysed by Western blotting with anti-(PGS1 protein) antibodies (**A**) or anti-(COX I) antibodies (**B**). The positions of molecular mass markers are indicated at the left of (**A**).

forms, although other possibilities cannot be excluded. As shown in Figure 5, the purified 60 kDa protein was recognized by the anti-(PGS1 protein) antibodies, and the mobility of the purified protein on SDS/PAGE was the same as that of the 60 kDa PGS1 protein. These results showed that the purified 60 kDa protein was a PGP synthase encoded by the CHO *PGS1* gene.

## Mitochondrial enrichment of the CHO PGS1 protein

It has been demonstrated that PGP synthase activity in mammalian cells is localized predominantly in the mitochondrial inner membrane [3]. Consistent with this was the observation that the PGS1 protein was in the mitochondria fraction of CHO-K1 cells (Figure 6). To determine more precisely the localization of PGS1-encoded PGP synthase, post-nuclear supernatant prepared from wild-type CHO-K1 cells was separated into crude mitochondrial, purified mitochondrial, microsomal and cytosol fractions; the same amount of protein in each fraction was then subjected to Western blot analysis with anti-(PGS1 protein) antibodies. As shown in Figure 7, 60 kDa PGP synthase was abundant in the mitochondria fraction. The other *PGS1* product, the 62 kDa protein, was also abundant in mitochondria. These results indicated that the PGP synthase encoded by the *PGS1* gene is a mitochondrial enzyme. The signal sequences for targeting to the mitochondrial intermembrane space have been shown to be located at the N-terminus of the precursor proteins and to have a bipartite structure [15–17]. Thus the N-terminal
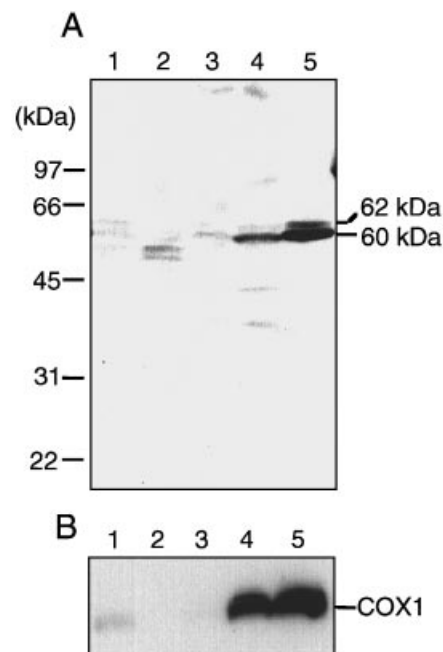
parts of the signal sequences exhibit the features of a typical mitochondrial targeting sequence and their C-terminal portions contain a hydrophobic stretch of approx. 20 amino acid residues that is preceded by basic residues [15–17]. The N-terminal sequence of the PGS1 gene product is rich in positively charged residues and poor in acidic amino acids [6]. These are common features of mitochondrial targeting sequences. A hydrophobic stretch of 28 residues preceded by basic residues is found at positions 47–74 in the PGS1 gene product [6]. Thus the primary structure of the N-terminus of the PGS1 gene product is consistent with the bipartite structure of the known signal sequences for the targeting of proteins to the intermembrane space, suggesting that PGS1-encoded PGP synthase is an inner-membrane protein that is exposed to the intermembrane space. It has also been shown that signal sequences with a bipartite structure are cleaved in two steps by peptidases in the matrix space and subsequently at the intermembrane-space surface of the inner membrane [15–17]. Because both the 60 and 62 kDa PGS1 proteins were enriched in mitochondria (Figure 7), we propose that the 62 kDa protein was produced by the processing of a precursor of PGP synthase in the matrix space and that 60 kDa protein was produced by the processing of 62 kDa protein at intermembrane-space surface of the inner membrane.

## PGP synthesis in CHO-K1 cells seems to be catalysed almost exclusively by PGS1-encoded PGP synthase

We previously demonstrated that the PGP synthase defect of PGS-S mutant cells is complemented by the introduction of CHO *PGS1* cDNA [6]. However, the molecular mechanism underlying the defect of PGP synthase in the mutant remained

uncertain. In the present study we found that the amounts of the PGS1 gene products of 60 and 62 kDa in the PGS-S mutant were much lower than those in CHO-K1 cells (Figure 6). The PGP synthase defect of the PGS-S mutant is therefore probably due to this decrease in PGS1 products. This, together with the fact that the PGP synthase activity in the homogenate of PGS-S mutant cells is only 1 % of that of CHO-K1 cells [5], suggested that PGP synthesis in CHO-K1 cells is catalysed almost exclusively by the *PGS1*-encoded PGP synthase, although the presence of another PGP synthase cannot be excluded.

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