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Characterization and regulation of phosphatidylglycerolphosphate phosphatase in *Saccharomyces cerevisiae*

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Phosphatidylglycerophosphatase (EC 3.1.3.27) activity was characterized in mitochondrial extracts from *Saccharomyces cerevisiae*. The enzyme has a pH optimum of 5.5. Maximum activity occurs in the presence of Triton X-100 (5 mM) and cobalt or magnesium ions (5 mM). The apparent K_m for PGP is 14.6 μM. The temperature optimum is between 50°C and 60°C. The enzyme is labile above 50°C. The presence of inositol in growth media results in a slight but reproducible increase in PGPase activity in mitochondrial extracts from glucose-grown cells but not glycerol-grown cells. The inositol effect is not seen in crude cell extracts. Carbon source does not affect PGPase activity in mitochondrial extracts or in crude cell extracts.

Introduction

The acidic phospholipid cardiolipin (CL) plays an important role in a variety of procaryotic and eucaryotic functions. In bacteria, it activates dnaA protein which is required for initiation of DNA replication [1–3]. In eucaryotic cells, CL is involved in at least two mitochondrial activities: it is required for cytochrome oxidase function [4,5] and participates in translocation of proteins across the mitochondrial membranes [6]. In addition to its importance in mitochondrial activity, CL can serve as a marker for the study of mitochondrion-specific membrane biogenesis, since the phospholipid is found only in the mitochondrial membrane [7,8].

To better understand the role of CL in mitochondrial function and biogenesis, we are studying the regulation of CL biosynthesis in the model eucaryote *Saccharomyces cerevisiae*. In eucaryotes the CL biosynthetic pathway involves the following sequential reactions [9–12]:

(1) Glycerol 3-Phosphate (G3P) + CDP-diacylglycerol (CDP-DG) → Phosphatidylglycerolphosphate (PGP) + CMP
(2) PGP → Phosphatidylglycerol (PG) + P_i
(3) PG + CDP-DG → CL + CMP

In bacteria the third step is 2 PG → CL + glycerol [13]. In yeast, PGP synthase (PGPS), the enzyme that catalyzes the first reaction of the CL biosynthetic pathway, is localized in the mitochondrial inner membrane [7]. Enzymological properties of PGPS in yeast mitochondrial extracts have been described [14]. Previous work in our laboratory has shown than PGPS expression is regulated by the phospholipid precursors inositol and choline in the same manner as are enzymes of the phosphatidylinositol (PI) and phosphatidylcholine (PC) pathways [15]. However, this process is not mediated by the same genetic regulatory circuit as that controlling PI and PC synthesis.

PGP phosphatase (PGPase), the enzyme that catalyzes the second step of the CL pathway, has not been extensively characterized in any eucaryotic system. In *Escherichia coli*, two different enzymes encoded by the genes *pgpA* and *pgpB* carry out this reaction [16]. The *pgpA* gene product is specific for PGP [17], while the *pgpB* gene product can dephosphorylate both phosphatidic acid (PA) and lysophosphatidic acid (LPA) [18]. An early biochemical analysis of PA and PGP phos-

Abbreviations: PGP, phosphatidylglycerolphosphate; PGPase, phosphatidylglycerolphosphate phosphatase; G3P, glycerol 3-phosphate; CDP-DG, CDP diacylglycerol; PG, phosphatidylglycerol; CL, cardiolipin; PGPS, PGP synthase; PA, phosphatidic acid; PI, phosphatidylinositol

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phatase activities in pig lung lamellar bodies, including sulfhydryl inhibition, heat inactivation, and substrate specificity studies, suggested that the same enzyme could conceivably carry out both activities [19]. However, PGPase has never been purified, nor has the gene encoding this enzyme been identified in any eucaryote.

This report is the first analysis of PGPase in the eucaryote *S. cerevisiae*. It lays the groundwork that is essential for the molecular analysis of PGPase expression. In this paper we describe the biochemical characterization of PGPase in yeast mitochondrial extracts. We show, further, that PGPase is not regulated in the same manner as PGPS, the first enzyme of the CL biosynthetic pathway.

Materials and Methods

Strains. The *E. coli* strain, JA200 (F$^+$/*pgsA*$^+$ *ΔtrpE5 recA thr leu thi*) [20], which carries the *pgsA* gene on the plasmid pPGL3008 [21] was kindly provided by William Dowhan. The *S. cerevisiae* strain used was ade 5 (ade5 MATα) [22,15].

Growth media. Yeast strains were maintained in 15% glycerol at −80°C for long term storage and on 1% yeast extract, 2% peptone, 2% glucose (YEPD) slants at 4°C for short-term storage. Minimal media for experiments consisted of salts in the indicated concentrations, [ammonium sulfate (2 g/l), boric acid (200 μg/l), copper sulfate (16 μg/l), potassium iodide (40 μg/l), ferric chloride (80 μg/l), manganese sulfate (160 μg/l), sodium molybdate (80 μg/l), zinc sulfate (160 μg/l), potassium phosphate, monobasic (0.4 g/l), magnesium sulfate (0.2 g/l), sodium chloride (0.04 g/l), and calcium chloride (0.04 g/l)], vitamins (6), 0.15 mM adenine, and either 2% glucose or 3% glycerol plus 0.95% ethanol. (The salts added are essentially the components of Difco vitamin-free yeast base, which also contains glucose.) Where indicated inositol was added to a concentration of 75 μM.

Bacterial strains were maintained in 15% glycerol at −80°C for long-term storage. Media for growth of bacteria contained 5% NaCl, 8% tryptone, 5% yeast extract and 12.5 μg/ml tetracycline.

Materials. All chemicals were reagent grade and were purchased from Sigma Chemical. Yeast extract, tryptone, and peptone were purchased from Difco. Other materials were purchased from the following sources: CDP-diacylglycerol (CDP-DG)-Life Sciences Resources; [γ-^{32}P]ATP (6000 Ci/mmol)-Dupont, New England Nuclear Research Products; glycerolkinase-Boehringer Mannheim; Biosafe II liquid scintillant-Research Products International; silica-gel 60 TLC plates-Merck Laboratories; Whatman #1 paper-Whatman International.

Growth conditions. For characterization of PGPase, minimal media was inoculated with yeast from YEPD slants or plates and grown overnight at 30°C. 2-l flasks containing 1.5 l of medium were inoculated from these overnight cultures, and cells were grown to mid-log stage. Cells were harvested by centrifugation at 4°C and washed once with a buffer containing 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 300 mM sucrose, and 10 mM β-mercaptoethanol (buffer 1), and were immediately frozen at −80°C. To study the effects of inositol and carbon source, experimental cultures were inoculated from overnight cultures and grown to mid-log stage, to the A_{550} indicated. Overnight cultures were grown in the same medium as the experimental cultures. Cells were harvested and frozen as described above.

Preparation of crude cell extracts. Extracts were prepared as described previously [14,15] with the following modifications. Pellets were suspended in a buffer containing 50 mM Tris-HCl (pH 7.5), 20% glycerol, and 10 mM β-mercaptoethanol (buffer 2) to a concentration of 1 g/ml (wet weight), and cells were broken by vortexing with glass beads for five 1-min intervals, with cooling of the cells on ice between intervals. Extracts were centrifuged in a Sorvall SS34 rotor at 5000 rpm for 5 min and supernatants were transferred to 15-ml Corex tubes. The 5000-rpm spin was repeated twice more, each time transferring the supernatants and discarding the pellets. After the third spin, aliquots of the supernatant were saved and stored at −80°C.

Preparation of mitochondrial extracts. Supernatants of the last 5000-rpm spin were transferred to small Oak Ridge tubes, and mitochondria were pelleted by centrifugation for 10 min at 15000 rpm in a Sorvall SS34 rotor. Mitochondrial pellets were washed twice with buffer 2 and then stored at −80°C at a final concentration of 2.5 mg/μl (wet weight).

Preparation of [^{32}P]G3P and [^{32}P]PGP. [^{32}P]G3P was synthesized from glycerol and [γ-^{32}P]ATP using glycerolkinase as described previously [23,24]. [^{32}P]G3P was checked for purity by paper chromatography in 1 M ammonium acetate (pH 7.5) absolute ethanol (35 : 65), solvent system A [25]. [^{32}P]PGP was synthesized as described previously [26] with the following modifications. PGPS was derived from the *E. coli* strain JA 200 which contains the bacterial PGPS gene on a high copy plasmid. PGPS was partially purified by the method of Gopalakrishnan et al [27]. This affinity-purified fraction was used to synthesize [^{32}P]PGP under conditions described previously [26] with the exception that the incubation time was shortened from 4 h to 20 min. To verify the identity of [^{32}P]PGP synthesized in this manner, the chloroform-soluble product was identified by TLC in chloroform/methanol/glacial acetic acid/water (50 : 30 : 4 : 8, v/v) [14], subjected to mild alkaline hydrolysis [28] and the water-soluble product was chromatographed as previously described [25]. A

single radioactive spot was detected with an R_F value corresponding to published R_F values for the alkaline hydrolysis of PGP [25].

[^{3}H]G3P was synthesized in the same manner as [^{32}P]G3P with the exception that [^{3}H]glycerol and unlabeled ATP were used as substrates. [^{3}H]PGP was synthesized from [^{3}H]G3P by the same procedure described above for preparation of [^{32}P]PGP.

Assays for protein and enzyme activity. Extracts were assayed for protein concentration by the method of Bradford [29] using a protein assay kit from Bio-Rad Laboratories with BSA as the standard.

PGPase activity was measured at 30°C for 10 min by following the release of water-soluble $^{32}P_i$ from 100 μM [^{32}P]PGP (20000 CPM/nmol) in the presence of 5 mM Triton X-100, 50 mM morpholinoethanesulfonic acid-HCl, (Mes, pH 5.5), 5% glycerol and mitochondrial extract corresponding to 1 μg protein in a total volume of 0.1 ml. The reaction was stopped after 10 min by the addition of 0.25 ml 0.1 M HCl in methanol. Chloroform (1 ml) and 1 M $MgCl_2$ (1.5 ml) were added. The tubes were vortexed and phases were separated by centrifugation at low speed for 3 min. 0.75-ml aliquots of the aqueous layer were transferred to scintillation vials, and 5 ml Biosafe II were added to each. The amount of product formed was quantified with a Beckman LS-3801 liquid scintillation counter. Triplicate tubes were included for each sample along with a blank tube containing extract inactivated before the other reaction components were added. 1 unit of PGPase activity is defined as the amount of protein required for the release of 1 nmol $^{32}P_i$/min. The reaction was linear with respect to protein from 0.5 to 12 μg in mitochondrial extracts and 5 to 40 μg in crude cell extracts. Activity was linear with respect to time from 5 to 30 min.

Results

Optimal assay conditions

PGPase activity was measured in citrate buffer from pH 3.0 to pH 6.0 and Mes buffer from pH 5.0 to pH 7.0. Maximal activity was observed at pH 5.5 (Fig. 1A). Activity was measured in the presence of a variety of divalent cations (Fig. 1B). Maximal activity was obtained in the presence of cobalt or magnesium ions (5 mM). Manganese did not significantly affect activity (data not shown). Zinc inhibited activity at all concentrations tested. PGPase activity was measured in the presence of various concentrations of Triton X-100 (Fig. 1C). Maximum activity occurred with 5 mM Triton X-100. At this concentration, the ratio of Triton X-100 to PGP is 50:1. At ratios greater than 50:1, activity decreased, indicating that the enzyme followed substrate dilution kinetics.

Enzyme activity was inhibited 100% and 70% by 1 mM *p*-chloromercuriphenylsulfonic acid (pCMPS) and 1 mM NaF, respectively (data not shown). Inhibition by pCMPS could be reversed by 10 mM β-mercaptoethanol.

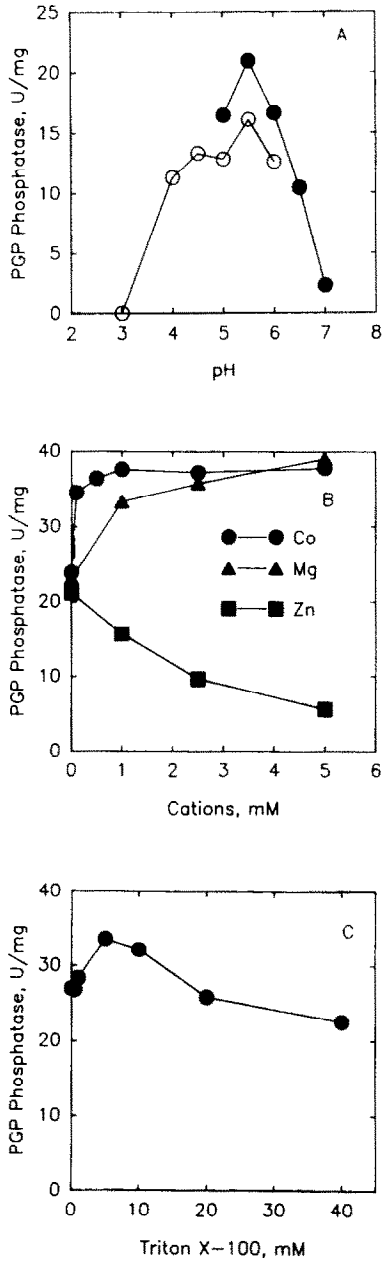

Fig. 1. Effect of pH (A), divalent cations (B) and Triton X-100 (C) on PGPase activity in mitochondrial extracts. Specific activity was determined as described in Materials and Methods. (A) Activity was measured in citrate buffer from pH 3 to pH 6 (○) and in Mes buffer from pH 5 to pH 7 (●). All other conditions are the standard assay conditions described in the text. (B) Extracts were passed over a Sephadex G-25 column which had been equilibrated with buffer 2. Samples were eluted with buffer 2 and assayed in the presence of the indicated divalent cations. All other assay conditions were standard as described in Materials and Methods. (C) Activity was measured in the presence of the indicated concentrations of Triton X-100. All other assay conditions were standard as described in Materials and Methods.

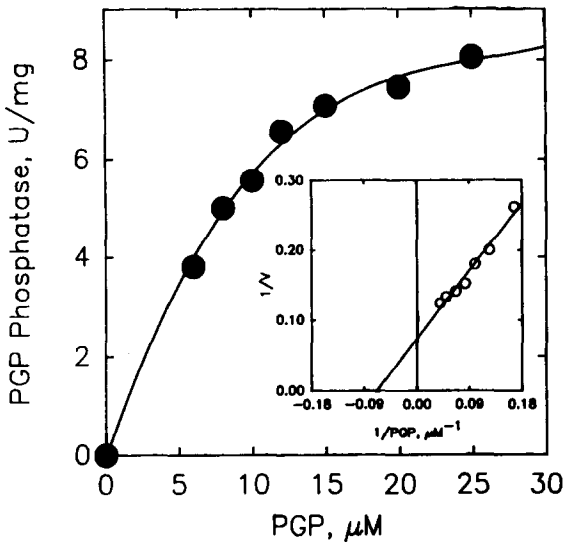

Fig. 2. Effect of PGP on PGPase activity in mitochondrial extracts. The concentration of PGP was varied while keeping the molar ratio of Triton X-100 to PGP constant at 50:1. The inset shows a double reciprocal plot of the data.

Substrate affinity and specificity

The affinity of PGPase for its substrate was determined by varying the concentration of PGP in the reaction mix. PGP was introduced to the reaction in a mixed micelle at a constant Triton X-100 to phospholipid ratio of 50:1. The apparent K_m for PGP was 14.6 μM (Fig. 2). Hydrolysis of PGP was not inhibited by the addition of G3P, PG, PA, or PI (data not shown).

Effect of temperature on catalysis and stability of PGPase

Enzyme activity was measured from 0°C to 80°C in a controlled temperature water bath under optimal conditions as described above. The temperature optimum for PGPase was between 50°C and 60°C (Fig. 3A). The energy of activation for the reaction was 18.3 kcal/mol between 4°C and 50°C (Fig. 3B). Stability of the enzyme to temperatures ranging from 20°C to 70°C was examined by heating extracts at the indicated temperatures for 10 min followed by assay under standard conditions. The enzyme was reasonably stable to temperatures up to 50°C with rapid inactivation at temperatures above 60°C (Fig. 3C).

Effect of phospholipid precursors and products

Water-soluble phospholipid precursors have been shown to affect activity of at least one phospholipid biosynthetic enzyme, phosphatidylserine synthase, which is non-competitively inhibited by inositol [30]. In contrast, water-soluble phospholipid precursors choline, ethanolamine, inositol, and serine, had no effect on PGPase activity at 1 mM concentrations (data not shown). P_i a product of the PGPase reaction had no effect on enzyme activity at a concentration of 1 mM (data not shown).

Identification of the reaction product

In order to identify the phospholipid product of the reaction, the PGPase assay was carried out under standard conditions using the substrate [^{3}H]PGP (115 Ci/mmol). Chloroform-soluble reaction products were retained, chromatographed on TLC plates, and identi-

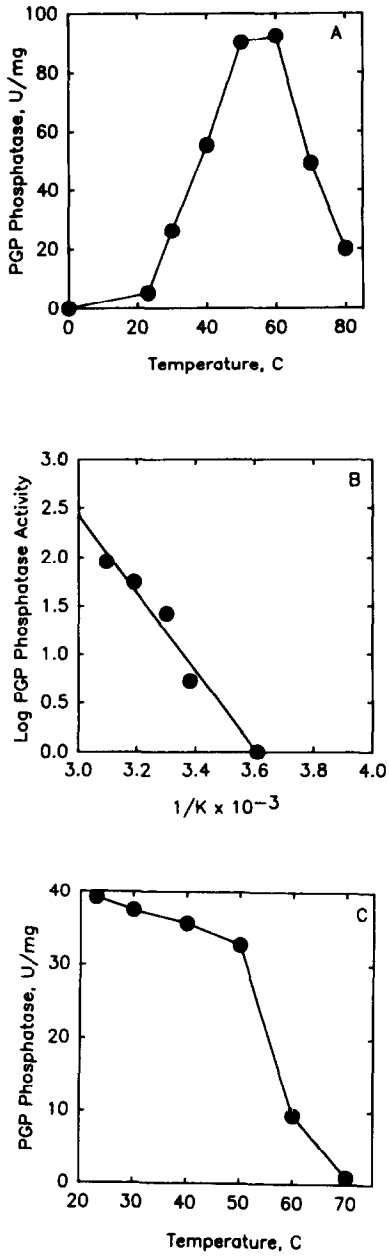

Fig. 3. Effect of temperature on PGPase activity in mitochondrial extracts. (A) Samples were incubated at the min in a controlled temperature water bath. PGPase activity was then measured under standard assay conditions as described in Materials and Methods. (B) The Arrhenius plot was constructed from the values in panel A. (C) Thermal stability of PGPase activity. Aliquots of mitochondrial extracts (1 μg protein/45 μl) were incubated for 10 min at the indicated temperatures in a controlled temperature water bath. After incubation, the samples were placed on ice, assay components were added and PGPase activity was measured at 30°C. under standard assay conditions.

fied by exposure to X-ray film. In addition to unreacted [^{3}H]PGP, a second radioactive spot was seen that co-migrated with a PG standard (Fig. 4).

Effect of inositol and carbon source

We have shown previously that expression of the first cardiolipin biosynthetic enzyme, PGPS, is inhibited by growth in the presence of inositol [15]. In order to determine whether PGPase is similarly regulated, cells were grown in the presence or absence of inositol and PGPase activity was measured in mitochondrial extracts and in crude cell extracts (Fig. 5). In mitochondrial extracts of glucose-grown cells a small but reproducible derepression of PGPase activity was seen in the presence of inositol (Fig. 5B). This difference was not observed in mitochondrial extracts from cells grown in glycerol (Fig. 5B), nor was the difference detected in crude cell extracts (Fig. 5A).

We have observed that the carbon source used for cell growth affects PGPS activity (unpublished data). While activity of PGPS in mitochondrial extracts is similar in glucose-grown versus glycerol-grown cells, PGPS specific activity in crude cell extracts of glycerol-grown cells is 4- to 5-fold higher than in glucose-grown cell extracts (unpublished data). In contrast, the specific activity of PGPase did not vary with carbon source in mitochondrial or crude cell extracts (Fig. 5A).

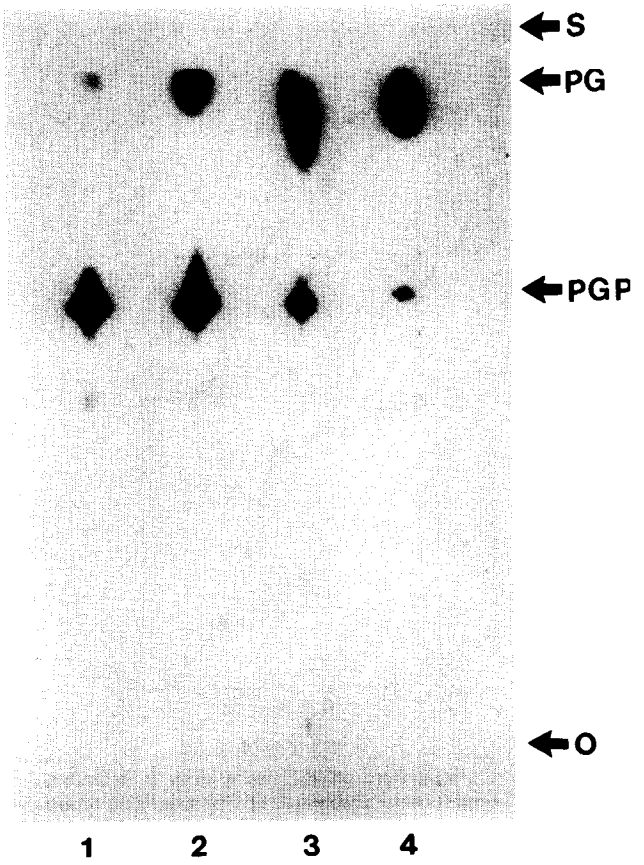

Fig. 4. Identification of the reaction products. [^{3}H]PGP was synthesized from [^{3}H]G3P and used as the substrate in a standard PGPase assay. Chloroform-soluble products were extracted, chromatographed on a TLC plate, exposed to X-ray film and compared to unlabelled phospholipid standards. Incubation times were 0 min (lane 1), 10 min (lane 2), 1 h (lane 3), 2 h (lane 4). Abbreviations: S, solvent front; PG, phosphatidlyglycerol; PGP, phosphatidylglycerolphosphate; O, origin.

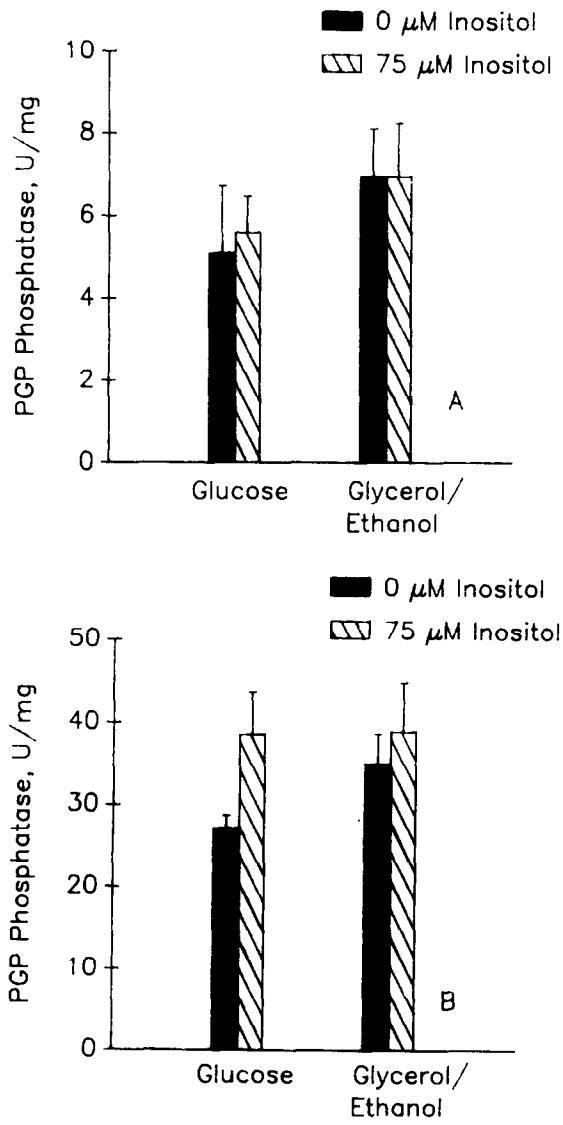

Fig. 5. Effect of inositol and carbon source on PGPase activity. Cells were grown in minimal media containing 2% glucose or 3% glycerol and 95% ethanol in the presence or absence of 75 UM inositol. Cells were harvested at an A_{550} of 0.4. Cell extracts (A) or mitochondrial extracts (B) were prepared, and PGPase activity was measured as described in the text. The specific activity of PGPase was calculated from a minimum of three independent growth studies.

Discussion

The number of enzymes which dephosphorylate phospholipid intermediates PA and PGP has not been definitively ascertained in any organism. PGPase activity has been biochemically characterized in *E. coli* [25], and subsequent genetic studies have resulted in the

identification of two genes, and pgpA and pgpB, that encode PGPase [16]. Furthermore, evidence for a third gene is that a strain with null mutations in both pgpA and pgpB genes still has PGPase activity (William Dowhan, personal communication). In eucaryotes, PGPase has been partially characterized only in rat liver extracts [31], but the enzyme has not been purified, and the system is not readily amenable to genetic analysis. The yeast system, in contrast, lends itself to biochemical as well as genetic analysis. It is, therefore, the ideal eucaryote in which to investigate the nature of these phospholipid biosynthetic enzymes.

This is the first report describing optimal conditions for PGPase activity in the yeast *S. cerevisiae*. The yeast enzyme shares similarities and differences with both the bacterial and mammalian enzymes [26,31]. It is similar to the bacterial enzyme(s) with regard to stimulation of enzyme activity by Triton X-100 (Fig. 1C) and divalent cations (Fig. 1B). However, the yeast enzyme is not totally dependent upon these factors, while the bacterial enzyme requires divalent cations for activity [26]. The mammalian enzyme, in contrast, is inhibited by both. The yeast enzyme exhibits an apparent K_m of 14.6 μM for PGP (Fig. 2), which is closer to that of the mammalian enzyme (2 μM) than that reported for *E. coli* (83 μM). The yeast PGPase temperature optimum of 50–60°C (Fig. 3A) is also similar to that reported for the mammalian enzyme (50°C). The pH optimum of the yeast enzyme is 5.5 (Fig. 1A) which is closer to that of the mammalian enzyme (6.3) than the bacterial enzyme (7.5).

In *E. coli* the product of the pgpB gene (but not the pgpA gene) can dephosphorylate PA as well as PGP [16–18]. In eucaryotic cells, however, characterization of the number and identity of enzymes that dephosphorylate PA and PGP is not definitive [19,31,32]. One study [19] suggests that PA and PGP phosphatase activities in rat lung are similar with regard to substrate specificities, heat inactivation, and sensitivity to sulfhydryl reagents; however, as these experiments were carried out in crude cell extracts, the number of enzymes that catalyze these reactions is uncertain. In contrast, PA phosphatase purified from yeast cell membranes is unable to dephosphorylate PGP (George Carman, personal communication). Furthermore, PA does not competitively inhibit PGP hydrolysis (data not shown). It appears therefore, that at least two different enzymes in yeast catalyze the dephosphorylation of PA and PGP.

The specific activity of PGPase that we observed in yeast mitochondrial extracts (35 U/mg) is 50- to 100-fold greater than that observed for PGPS, the first enzyme in the CL pathway [15]. This may explain the fact that PGP, the product of the reaction catalyzed by PGPS, is not detected in yeast membranes [8].

PGPase appears to be regulated differently from PGPS. We have shown previously that inositol represses expression of PGPS [15]. In contrast, PGPase activity is slightly higher in mitochondrial extracts from cells grown in glucose in the presence of inositol (Fig. 5B). A second difference between PGPS and PGPase regulation pertains to the effect of carbon source on enzyme activity. We have observed approximately 4-fold greater PGPS activity in crude cell extracts from glycerol-grown compared to glucose-grown cells (unpublished data). PGPase activity, on the other hand, does not differ in crude cell extracts from glucose-grown versus glycerol-grown cells (Fig. 5A). While these data suggest that PGPase expression is constitutive, they may also be explained by the existence of multiple genes that encode PGPase enzymes. This report lays the groundwork for genetic and molecular studies currently in progress to identify the gene(s) encoding this enzyme.

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