

REGULATION OF THE *PIS1*-ENCODED PHOSPHATIDYLINOSITOL  
SYNTHASE BY ZINC, AND PURIFICATION AND  
CHARACTERIZATION OF THE *N*-ETHYLMALEIMIDE-SENSITIVE  
 $\text{Mg}^{2+}$ -DEPENDENT PHOSPHATIDATE PHOSPHATASE

by

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# **ABSTRACT OF THE DISSERTATION**

**Regulation of the *PIS1*-encoded Phosphatidylinositol Synthase by Zinc**

**AND**

**Purification and Characterization of the *N*-ethylmaleimide-Sensitive**

**Mg<sup>2+</sup>-Dependent Phosphatidate Phosphatase**

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Dissertation Director:

Dr. George M. Carman

In the yeast *Saccharomyces cerevisiae*, the mineral zinc is essential for growth and metabolism. The effects of zinc depletion on the regulation of the *PIS1*-encoded phosphatidylinositol synthase were examined. Phosphatidylinositol synthase activity increased when zinc was depleted from the growth medium. Analysis of a *zrt1Δ zrt2Δ* mutant defective in plasma membrane zinc transport indicated that the cytoplasmic levels of zinc were responsible for the regulation of phosphatidylinositol synthase. *PIS1* mRNA, its encoded protein Pis1p, and the  $\beta$ -galactosidase activity driven by the *P<sub>PIS1</sub>-lacZ* reporter gene were elevated in zinc-depleted cells. This indicated that the increase in phosphatidylinositol synthase activity was due to a transcriptional mechanism. The

regulation of *PIS1* gene expression by zinc depletion was mediated by the zinc-regulated transcription factor Zap1p. This work advances understanding of phospholipid synthesis regulation by zinc and the transcription control of the *PIS1* gene.

Mg<sup>2+</sup>-dependent phosphatidate phosphatase catalyzes the dephosphorylation of phosphatidate to synthesize diacylglycerol and inorganic phosphate. There are at least two genes that encode Mg<sup>2+</sup>-dependent phosphatidate phosphatase enzymes in yeast. The *PAH1* gene encodes an enzyme that is insensitive to *N*-ethylmaleimide whereas the gene that encodes the *N*-ethylmaleimide-sensitive enzyme has yet to be identified. The aim of this work was to purify and characterize the *N*-ethylmaleimide-sensitive Mg<sup>2+</sup>-dependent phosphatidate phosphatase. To purify the enzyme, a *pah1Δ dpp1Δ lpp1Δ* triple mutant that is devoid of all known phosphatidate phosphatase enzymes was used. The Mg<sup>2+</sup>-dependent phosphatidate phosphatase was extracted from the membrane fraction using 1 M NaCl. The salt extracted enzyme was then purified by chromatography with phosphocellulose, Mono Q and Superose 6. The enzyme was enriched 2,250 fold by this procedure. The basic enzymological properties of the purified Mg<sup>2+</sup>-dependent phosphatidate phosphatase were examined. The enzyme had a pH optimum from 7 to 7.5, and was dependent on Mg<sup>2+</sup> ions for activity. The enzyme activity followed surface dilution kinetics using Triton X-100-phosphatidate mixed micelles. The enzyme was inhibited by *N*-ethylmaleimide, phenylglyoxal and propranolol. In addition, the enzyme activity was regulated by nucleotides, sphingolipids and phospholipids. These studies provide information on the mode of action of this important enzyme in lipid metabolism.

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## DEDICATION

*To my parents, Eeung-Yeol Han, and Young-Ok Kim, without whose love  
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## LIST OF ABBREVIATIONS

ADP	adenosine diphosphate
ATP	adenosine triphosphate
CDP	cytidine diphosphate
CTP	cytidine triphosphate
CL	cardiolipin
DAG	diacylglycerol
DGPP	diacylglycerol pyrophosphate
GST	glutathione <i>S</i> -transferase
HA	hemagglutinin
kDa	kilo daltons
LPA	lysophosphatidate
NEM	<i>N</i> -ethylmaleimide
PA	phosphatidate
PAP1	NEM-sensitive $\text{Mg}^{2+}$ -dependent PA phosphatase
PAP2	NEM-insensitive $\text{Mg}^{2+}$ -independent PA phosphatase
PC	phosphatidylcholine
PE	phosphatidylethanolamine
PI	phosphatidylinositol
Pi	inorganic phosphate
PG	phosphatidylglycerol
PS	phosphatidylserine

PVDF	polyvinylidene difluoride
SC	synthetic complete
SDS	sodium dodecyl sulfate
TAG	triacylglycerol
TTP	thymine triphosphate
UAS <sub>INO</sub>	upstream activating sequence inositol-responsive element
UAS <sub>ZRE</sub>	UAS zinc responsive element
UTP	uridine triphosphate
Zap1p	zinc responsive activator protein

## INTRODUCTION

### Importance of Membrane Phospholipids

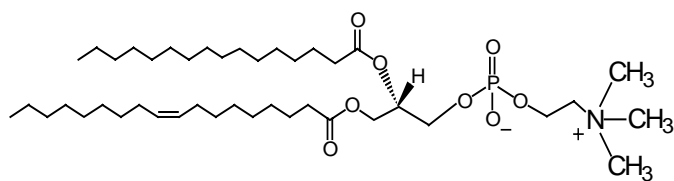
Cellular membranes consist of lipid bilayer in which proteins are associated. They function to protect the cell from the external environment and play a major role in numerous cellular processes (1). Peripheral proteins are bound only to the membrane surface, whereas integral proteins are buried within membranes. Phospholipids are essential amphipathic molecules that are major structural components in cellular membranes (1). Phospholipids also serve in protein modification for membrane association (2), as molecular chaperones (3) and as reservoirs of lipid signaling molecules (4). The yeast *Saccharomyces cerevisiae* is used to study the regulation of phospholipid synthesis, as it is a model eukaryotic system. The major phospholipids found in yeast membranes include phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS) and phosphatidylinositol (PI) (Fig. 1) (5, 6). The common fatty acids esterified to the glycerol phosphate backbone of the major phospholipids in yeast are palmitic acid (16:0), palmitoleic acid (16:1 $\Delta^{9,10}$ ), stearic acid (18:0) and oleic acid (18:1 $\Delta^{9,10}$ ) (6).

### Phospholipid Biosynthetic Pathways

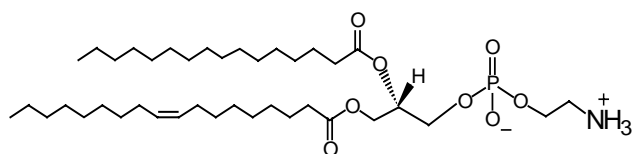
There are two pathways for the synthesis of the major phospholipids in yeast membranes (Fig. 2). One is the cytidine diphosphate-diacylglycerol (CDP-DAG) pathway and the other is the Kennedy pathway (CDP-choline and CDP-ethanolamine branches) (5, 6). When the cell is grown in the absence of exogenous choline and ethanolamine, the major phospholipids (PC, PE and PS) are predominantly synthesized



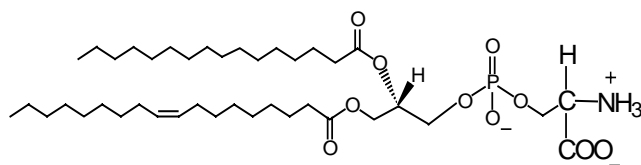
**Figure 1. Major phospholipids in *S. cerevisiae*.**



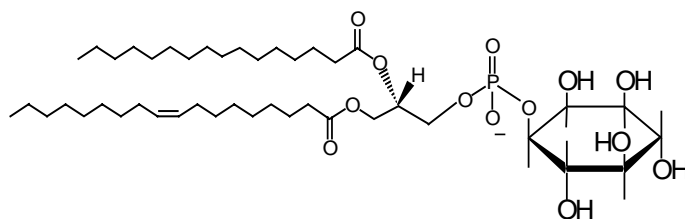
Phosphatidylcholine  
(PC)



Phosphatidylethanolamine  
(PE)



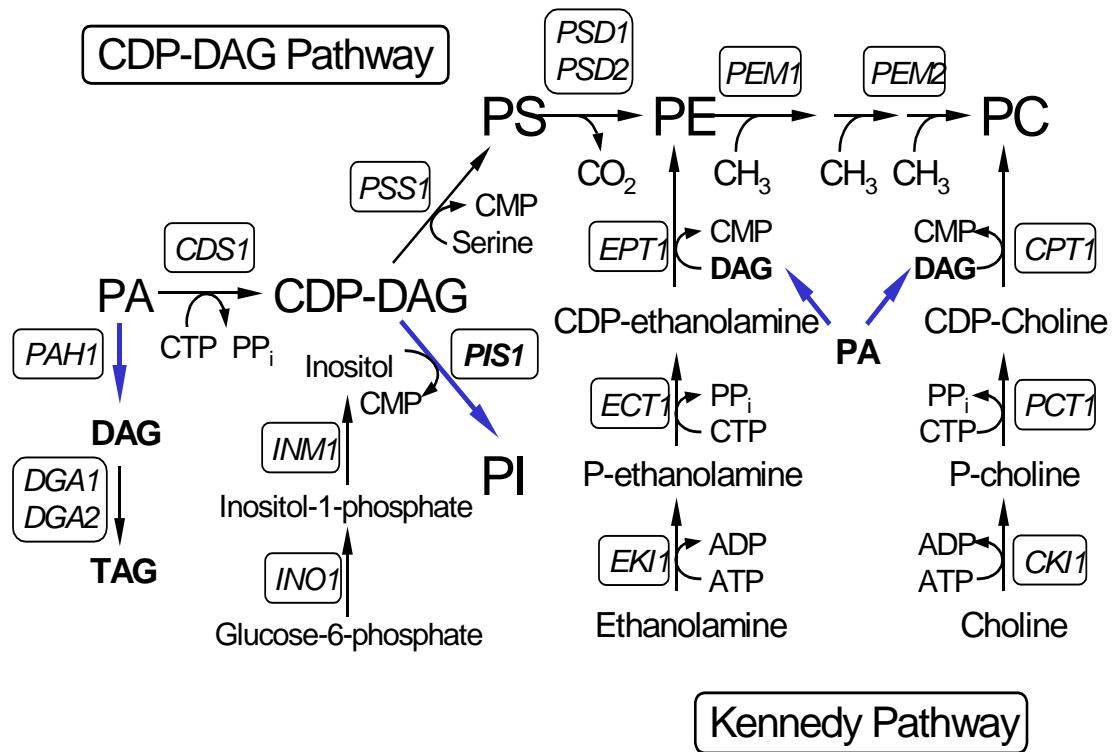
Phosphatidylserine  
(PS)



Phosphatidylinositol  
(PI)

**Figure 2. Phospholipid biosynthetic pathways in *S. cerevisiae*.**

The enzymes *CDS1*-encoded CDP-DAG synthase, *PSSI*-encoded PS synthase, *PSD1/PSD2*-encoded PS decarboxylase, *PEM1*-encoded PE methyltransferase and the *PEM2*-encoded PL methyltransferases catalyze reactions that lead to the formation of PC by the CDP-DAG pathway. *CK11*-encoded choline kinase, *PCT1*-encoded choline-P cytidylyltransferase, and *CPT1*-encoded choline phosphotransferase catalyze reactions that lead to the formation of PC by the CDP-choline branch of the Kennedy pathway. *EK11*-encoded ethanolamine kinase, *ECT1*-encoded ethanolamine-P cytidylyltransferase, and *EPT1*-encoded ethanolamine phosphotransferase catalyze reactions that lead to the formation of PE by the CDP-ethanolamine branch of the Kennedy pathway. *INO1*-encoded inositol 3-phosphate synthase and *INM1*-encoded inositol 3-phosphate phosphatase catalyze reactions that lead to the formation of inositol, which is utilized for the formation of PI. The *PAH1*-encoded PA phosphatase catalyzes reaction that leads to the formation of DAG and *DGAI* (*DGA2*)-encoded DAG acyltransferase catalyzes reaction that leads to the synthesis of TAG. Abbreviations: PA, phosphatidate; CDP-DAG, Cytidine 5'diphospho-diacylglycerol; PS, phosphatidylserine; PE, phosphatidylethanolamine; PC, phosphatidylcholine; PI, phosphatidylinositol; DAG, diacylglycerol; TAG, triacylglycerol



via the CDP-DAG pathway (7). When the cell is supplemented with exogenous choline and ethanolamine, PC and PE are primarily synthesized by the Kennedy pathway (7). This pathway is important when enzymes in the CDP-DAG pathway are repressed or defective (8). The inositol used for the PI synthase reaction is made from inositol-1-phosphate by inositol 3-phosphate synthase (9). The inositol-1-phosphate synthase produces inositol-1-phosphate from glucose-6-phosphate (10). PA serves as a precursor for the formation of CDP-DAG and diacylglycerol (DAG). DAG is used to make PE and PC by the Kennedy pathway and is also used to make triacylglycerol (TAG). PA phosphatase catalyzes the formation of DAG and inorganic phosphate (Pi) by dephosphorylating PA (11). PA can be phosphorylated by membrane-associated enzyme PA kinase to form diacylglycerol pyrophosphate (DGPP) (12, 13). DGPP can be dephosphorylated back to PA by DGPP phosphatase (14).

### **Genetic and Biochemical Regulation of Phospholipid Synthesis**

The regulation of phospholipid synthesis in *S. cerevisiae* is complex being controlled by transcriptional and biochemical mechanisms (15). The water-soluble precursor inositol plays a major role in the transcriptional regulation of phospholipid synthesis (15). Many structural genes encoding proteins that are involved with the production of yeast phospholipids contain a 10-base pair consensus sequence (5'CATGTGAAAT3') in their promoters (15, 16). This sequence is known as an inositol-sensitive upstream activation sequence (UAS<sub>INO</sub>) or an inositol choline responsive element (ICRE) (6, 15, 17). The transcription occurs by the interaction of

DNA-binding proteins to the core sequence in UAS<sub>INO</sub> elements (6, 18, 19). The products of *INO2* and *INO4* regulatory genes are the Ino2p and Ino4p, respectively (15). These proteins bind to the core sequences of UAS<sub>INO</sub> elements. Maximal gene expression occurs when these proteins bind to these elements (15). Inositol-mediated regulation involves the expression of genes and the modulation of enzyme activities. The transcription factor Opi1p negatively regulates genes containing UAS<sub>INO</sub> elements in their promoters (15). Under normal growth conditions, Opi1p is localized to the endoplasmic reticulum (ER). When inositol is added to the growth medium, the level of PA is reduced by a result of CDP-DAG synthesis and ultimate utilization by PI synthase to make PI (20, 21). The decreased PA causes the removal of Opi1p interaction with the ER and results in the translocation into the nucleus (20). *In vitro* analysis shows that Opi1p binds to Ino2p (22). However, the mechanism by which Opi1p moves into the nucleus to repress target genes is still unclear. UAS<sub>INO</sub> containing genes are also regulated through out the different phases of cell growth (23). Maximum expression occurs in exponential phase whereas these genes are repressed in stationary phase. (23, 24).

The mRNA stability of genes involved in the synthesis of phospholipids affects the regulation of phospholipid synthesis. For example, the expression of the *CHO1*-encoded PS synthase is regulated by mRNA stability (24). Additionally, several phospholipid biosynthetic enzymes are also regulated by biochemical mechanisms. Phosphorylation plays a major role in the regulation of phospholipid synthesis (25). Key phospholipid biosynthetic enzymes, such as CTP synthetase, choline kinase, and Mg<sup>2+</sup>-dependent PA phosphatase have been identified as targets of phosphorylation (25).

Much of this phosphorylation is mediated by protein kinases A and C. For instance, choline kinase activity is up-regulated by phosphorylation via protein kinase A (25, 26).

### **Importance of Zinc**

Zinc is an essential element and is critical for vital cellular processes (27). Zinc is required for the proper function of over 300 enzymes (28). These enzymes include RNA polymerase, alcohol dehydrogenase, alkaline phosphatase and superoxide dismutase (27, 28). Also, zinc has three main functions with enzymes, which include catalytic, co-catalytic, and structural roles (28). It is also important in maintaining the structure of other proteins, especially stabilizing the tertiary or quaternary structures of proteins (27, 28). Because of its importance with a variety of enzymes, zinc is involved with the metabolism of nucleic acids, proteins, lipids and carbohydrates (27) and it plays a role in processes involved with growth, development, reproduction, regulation, differentiation, and immune function (27, 28). Because zinc is involved with many cellular processes, deficiency of zinc can lead to many diseases caused by damages to DNA, lipids and proteins (29). For example, zinc deficiency can cause growth retardation, male hypogonadism, mental retardation and delayed wounded healing (27, 28, 30). Excess zinc can be toxic to the cell, so the mechanism to maintain moderate levels of zinc is critical for cell viability (31). However, the roles of zinc in phospholipids synthesis in yeast are not fully understood.

### Regulation of Zinc Homeostasis

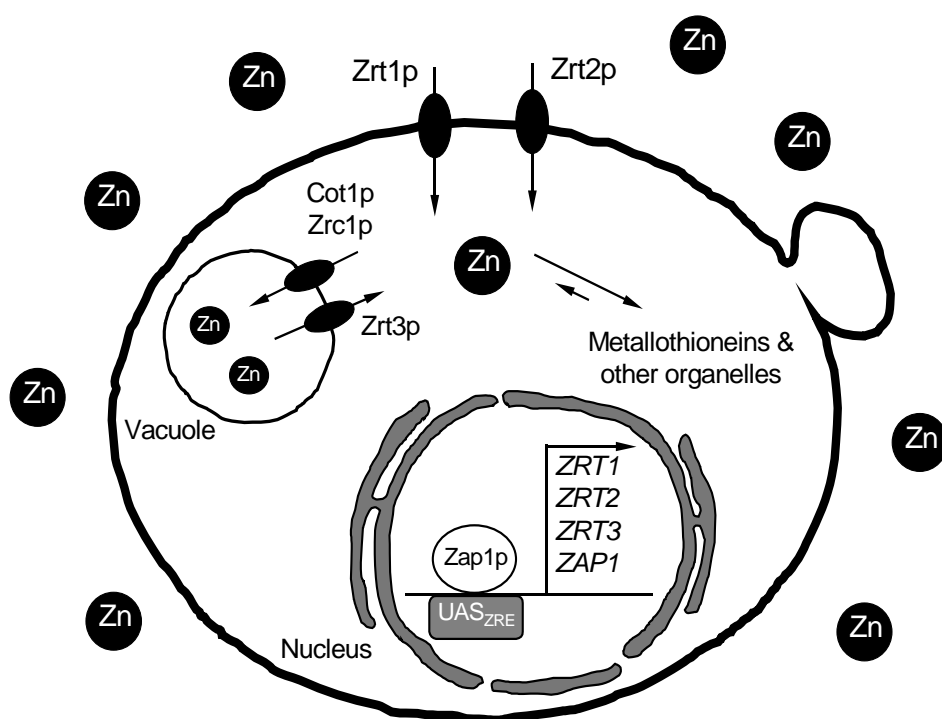
Zinc homeostasis in yeast is primarily controlled by zinc transporters. Zinc transporters play a major role in maintaining adequate level of zinc in the cell (28). Yeast has several zinc transporters that are regulated by transcriptional and post-translational mechanisms (Fig. 3) (32). Zrt1p and Zrt2p are the high-affinity zinc transporter (33) and low-affinity zinc transporter (34), respectively. Zrt1p is encoded by the *ZRT1* gene and Zrt2p is encoded by the *ZRT2* gene (31). Both zinc transporter proteins are members of Zrt-Irt-like protein (ZIP) family and they are close to each other in amino acid sequence identity and similarity (32). Analysis of the *zrt1Δ zrt2Δ* mutant shows that the mutant is viable (32), indicating that there are additional zinc uptake systems in yeast, such as a Fet4p (31). However, those systems are not major sources of zinc homeostasis because the *zrt1Δ zrt2Δ* mutant requires 105-fold zinc in order to grow as the same as the wild type cell grows (33). There are three zinc transporters on the vacuolar membrane in yeast. Zrc1p and Cot1p have been shown to exist in the vacuolar membrane. These zinc transporters are influx zinc transporters and members of cation diffusion facilitator (CDF) family (32, 35). Zrt3p is an efflux zinc transporter on vacuolar membrane and it works as the same manner of Zrt1p and Zrt2p (36).

A transcriptional mechanism is involved in the regulation of zinc transporters, Zrt1p and Zrt2p (36, 37). When the concentration of zinc in the cytosol is low, the zinc-responsive activation protein (Zap1p) is induced and binds to the zinc-responsive elements (UAS<sub>ZRE</sub>), which are located on the upstream region of the promoter. This



**Figure 3. Zinc homeostasis in *S. cerevisiae*.**

Model of zinc transport and its regulation in *S. cerevisiae*. Zrt1p is the high affinity zinc transporter protein and Zrt2p is the low affinity zinc transporter protein. Zrc1p and Cot1p are influx zinc transporter proteins in vacuole. Zrt3p is efflux zinc transporter protein in vacuole. The transcription factor Zap1p is induced and binds to UAS<sub>ZRE</sub>-containing genes (*ZRT1*, *ZRT2*, *ZRT3*, *ZAP1*). The induction by zinc depletion results in increased expression of the zinc transporters to increase availability of cytosolic zinc.



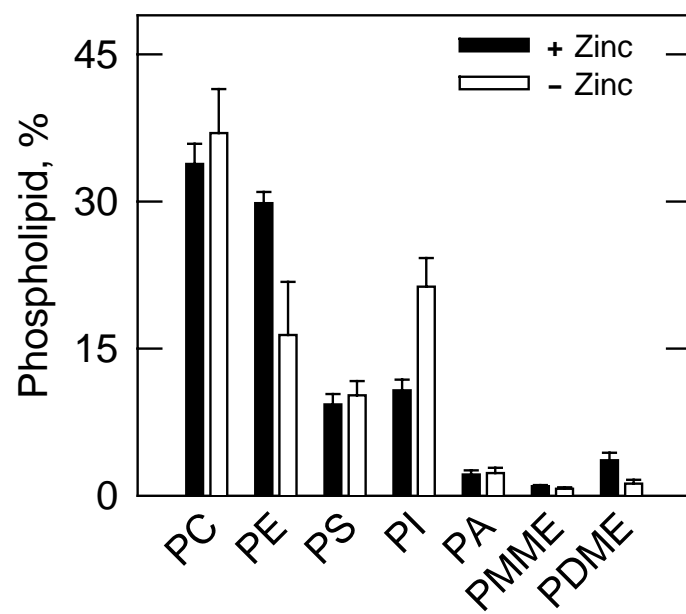
causes an increase in expressions of several zinc-sensitive genes, such as *ZRT1*, *ZRT2*, *ZRT3* and *ZAP1* (36, 37). This induction results in an increase amount of zinc transporters to increase zinc transport from extracellular medium into the cytosol. Besides, Zrt1p is also regulated by post-translational mechanism (32). When the cell is exposed to high amount of zinc, the protein and activity levels of Zrt1p are abolished. This is caused by ubiquitination, zinc-induced endocytosis and vacuolar degradation (38, 39). Excess zinc inside cells is toxic to the cells (27), so the post-translational regulation of zinc transporter is important to avert zinc uptake when the cell is under high level of zinc. It is known that transcriptional and post-translational regulations of zinc transporters work simultaneously to maintain moderate intracellular levels of zinc in yeast (31).

### **Regulation of Phospholipid Composition by Zinc**

Recent studies indicate that zinc depletion results in the changes of phospholipid composition in the vacuole, and total cellular phospholipid composition in yeast are changed by zinc depletion (40). Zinc depletion causes a 45% decrease in the level of PE and an 100 % increase in level of PI (Fig. 4) (40). Moreover, in the neutral lipid analysis, the levels of triacylglycerol (TAG) and ergosterol ester (ErgE) are increased when zinc was depleted from the growth medium (41). Also, zinc depleted cells show reduced activity levels of the CDP-DAG pathway enzymes. However, the activity of PI synthase, which competes with PS synthase for the substrate CDP-DAG, is elevated in zinc-depleted cells (40). To explore the mechanism for the increase of PI synthase by zinc depletion is one of the aims in this dissertation.

**Figure 4. Effect of zinc on phospholipid composition.**

The abbreviations used are: PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PI, phosphatidylinositol; PA, phosphatidate; PMME, phosphatidylmonomethyl-ethanolamine; PDME, phosphatidylmethylethanolamine (Iwanyshin, W.M., Han G.S., and Carman G.M. (2004) Regulation of phospholipid synthesis in *Saccharomyces cerevisiae* by zinc *J.Biol.Chem.* **279**, 21976-21983)



### Importance of PI and Regulation of PI Synthase

PI is the third most abundant phospholipid in yeast membranes (5). PI is an essential phospholipid in all eukaryotic cells, therefore it is essential for the growth and metabolism of yeast (42). Besides its role as a major structural component of the membrane, PI is a precursor for the phosphoinositides, inositol polyphosphates and complex sphingolipids (43). These molecules are important for vital cellular processes. For example, phosphoinositides play roles in membrane trafficking events, endocytosis and vacuolar protein sorting (43). Alteration of PI metabolism is associated with many diseases, such as Lowe's oculocerebrorenal syndrome, which is related with a defect in inositol polyphosphate phosphatase (44).

PI is synthesized by the ER-associated enzyme, PI synthase (CDP-diacylglycerol:myo-inositol 3- phosphatidyltransferase, EC 2.7.8.11) from CDP-DAG and inositol (45). PI synthase is encoded by the essential *PIS1* gene on chromosome XVI. The PI synthase is regulated by both transcriptional and biochemical mechanisms (43). The expression of the *PIS1*-encoded PI synthase is not regulated by inositol supplementation and growth phase (23, 46). The expression of the *PIS1* gene is increased in fermentable carbon sources, such as glucose and galactose when compared with non-fermentable carbon sources (47). Also, the expression of the *PIS1* gene is elevated under anaerobic growth conditions (48). PI synthase activity is also regulated by a biochemical mechanism with substrate availability, such as inositol and CDP-DAG (8). Taking into consideration of the essential nature of PI, the study of the regulation of the *PIS1* gene is significant. As stated above, zinc depletion causes an increase in PI content in yeast (40). In view of previous findings that the level of PI is regulated by zinc, the mechanism that PI synthase is regulated by zinc is warranted. Also, an understanding of the mechanism

that PI synthase is regulated by zinc may impact on health promotion and nutrition, because it sheds light on how normal cells regulate the synthesis of essential PI in response to the stress condition of zinc deprivation.

### **Importance of Phosphatidate in Yeast**

Cellular PA is produced from glycerol-3-phosphate by two successive acyltransferase reactions, and also generated by the action of phospholipase D from PC (1,15). The various pathways govern the level of PA in *S. cerevisiae* (Fig. 2). PA is utilized for phospholipids synthesis by CDP-DAG synthase and PA phosphatase, and is an important precursor for triacylglycerols synthesis. PA is a common substrate between CDP-DAG synthase reaction and PA phosphatase reaction, therefore the different levels of PA affect the levels of individual phospholipids and also change the proportions of phospholipids and the neutral lipids, such as DAG and TAG (50).

In addition, the amount of PA in yeast is associated with the transcriptional repressor Opi1p to control phospholipid biosynthetic gene expression in yeast. The concentration of PA in ER membrane is responsible for the localization of Opi1p (20). Addition of inositol results in the consumption of PA and CDP-DAG, the accumulation of PI, and the translocation of Opi1p from ER into the nucleus (20). The translocation of Opi1p negative transcription factor causes the repression of UAS<sub>INO</sub>-containing genes, such as the *CHO1* gene (20).

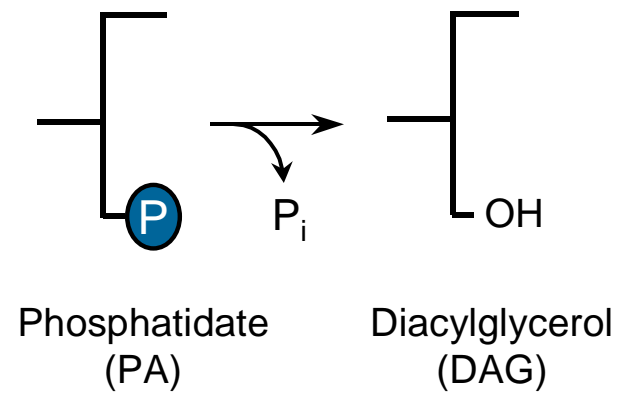
### **Phosphatidate Phosphatase**

PA phosphatase (PAP, 3-*sn*-phosphatidate phosphohydrolase, EC 3.1.3.4) catalyzes the dephosphorylation of PA, yielding DAG and inorganic phosphate (Fig. 5) (11). PA phosphatase was first identified from *S. cerevisiae* by Hosaka and Yamasita (51). In yeast, there are two kinds of PA phosphatases,  $Mg^{2+}$ -dependent (referred to as PAP1) and  $Mg^{2+}$ -independent (referred to as PAP2) (52). The majority of PA phosphatase activity in yeast is attributed to the  $Mg^{2+}$ -independent forms of the enzyme (53).  $Mg^{2+}$ -independent PA phosphatases are encoded by *DPPI* and *LPPI* genes (53, 54). These  $Mg^{2+}$ -independent PA phosphatases are localized in the membrane (53, 54). *LPPI*-encoded PAP2 enzyme is *N*-ethylmaleimide (NEM) sensitive, while *DPPI*-encoded PAP2 enzyme is insensitive to NEM (54, 13). *LPPI* gene and *DPPI* gene products encoded nearly all of the  $Mg^{2+}$ -independent PA phosphatase activities in yeast (54, 13). Analysis of the *dpp1Δ lpp1Δ* mutant indicates that the *DPPI*- and *LPPI*-encoded PA phosphatases are not required for cell viability (53, 54). Even if this mutant lacks these  $Mg^{2+}$ -independent PA phosphatases, it still shows residual PA phosphatase activity (53, 54). This activity is attributed to the  $Mg^{2+}$ -dependent PA phosphatase (53).

$Mg^{2+}$ -dependent PA phosphatases have been purified and characterized from the membrane and cytosolic fractions of yeast (52). Recently, the *PAHI* gene that encodes  $Mg^{2+}$ -dependent PA phosphatase has been identified in yeast (55). The *PAHI*-encoded PA phosphatase is localized in the cytosolic and membrane fractions of the cell (55). The membrane-associated enzyme is salt-extractable and this result indicates that the *PAHI*-encoded enzyme could be a peripheral membrane protein (56).



**Figure 5. PA phosphatase reaction.**



In addition, the *PAH1*-encoded  $\text{Mg}^{2+}$ -dependent PA phosphatase activity is NEM-insensitive (55). The study of the *pah1Δ dpp1Δ lpp1Δ* mutant showed that it still has  $\text{Mg}^{2+}$ -dependent PA phosphatase activity and the activity is NEM-sensitive (55). This finding indicates the presence of another gene encoding a PA phosphatase enzyme that is a NEM-sensitive  $\text{Mg}^{2+}$ -dependent PA phosphatase. The second work in this dissertation is focused on this enzyme.

### **Regulation of PA Phosphatase Activity in Yeast**

PA phosphatase activities are regulated by genetic and biochemical mechanisms (52). The membrane-associated PA phosphatase activity is genetically regulated by growth phase and inositol supplementation, whereas the enzyme activity is regulated by nucleotides, sphingoid bases and phospholipids with a biochemical mechanism (52, 143, 144, 162). Those regulations of PA phosphatase activity might correlate with changes in the synthesis of phospholipids and triacylglycerols (52). The enzyme activity is inhibited by nucleotides in a dose-dependent manner (143). The most potent nucleotide inhibitors are ATP and CTP. A kinetic study shows that the mechanism of enzyme inhibition by nucleotides with respect to PA is complex (143). Moreover, the study shows that the inhibition on enzyme activity by ATP is explained by a  $\text{Mg}^{2+}$  ion chelation mechanism (143). The PA phosphatase activity is also regulated by sphingoid bases, and the enzyme activity is inhibited by sphingosine, phytosphingosine and sphinganine (144). The most potent sphingoid base inhibitor is sphingosine (144). The inhibitory effect on the PA phosphatase enzyme by sphingoid bases might be involved in the proportional synthesis of phospholipids and triacylglycerols (144). Phospholipids regulate the activities of

several enzymes of lipid metabolism in yeast (15). PA phosphatase activity is inhibited by phospholipids (162). Anionic phospholipids (CL, CDP-DAG, PI, PG and PS) activate the enzyme activity, whereas zwitterionic phospholipids (PE and PC) inhibit the enzyme activity (162). The most potent phospholipid activators are CL, CDP-DAG and PI. (162). In addition, the regulation of PA phosphatase activity is correlated with the regulation of PS synthase activity (52). PS synthase is an enzyme that catalyzes the committed step for the synthesis of phospholipids via CDP-DAG pathway, and PS synthase is highly regulated enzyme in yeast (15). The study of regulation of PA phosphatase and PS synthase enzyme activity indicate that differential regulation of PA phosphatase and PS synthase by phospholipids has an important role in controlling both phospholipids and neutral lipids synthesis pathways. (52).

### **Roles of PA Phosphatase in Lipid Metabolism**

PA phosphatase catalyzes the committed step in the synthesis of triacylglycerol (TAG) in yeast (52). The reaction product DAG is used for the synthesis of PE and PC and for the synthesis of TAG (Fig. 2) (57-59). The substrate PA is utilized for the synthesis of all membrane phospholipids (and the derivative inositol-containing sphingolipids) through the intermediate CDP-DAG (58, 59). Therefore, regulation of PA phosphatase activity might govern whether cells make storage lipids and phospholipids through DAG or phospholipids through CDP-DAG (60). PA phosphatases are also involved in lipid signaling in mammalian cells, so the PA phosphatase activity can attenuate the bioactive functions of PA, such as cell proliferation, vesicular trafficking, secretion and endocytosis (61, 62). Moreover, DAG that is produced by PA phosphatase

is well known as a signaling molecule, and it activates protein kinase C (4, 63). In both yeast and mammalian cells, PAP2 enzymes have the role in lipid signaling (52, 62, 64), whereas PAP1 enzymes are involved in *de novo* lipid synthesis (55).

### **Differentiation of PAP1 and PAP2 Enzymes**

As mentioned earlier, nearly all  $Mg^{2+}$ -independent PA phosphatases are encoded by *DPPI* (54) and *LPPI* (53) genes. The *DPPI*- and *LPPI*- encoded PAP2 enzymes have broad substrate specificity, so these enzymes use various lipid phosphate substrates including PA, diacylglycerol pyrophosphate (DGPP), lyso PA, sphingoid base phosphates and isoprenoid phosphates (13, 53, 54, 65). Whereas, the *PAHI*-encoded PAP1 enzyme is specific for PA as its substrate (55). Also, the PAP2 enzymes are integral membrane proteins with six transmembrane spanning regions and they are localized to the vacuole (14, 66) and Golgi (67). However, the *PAHI*-encoded enzyme is a peripheral membrane protein and localized to both membrane and cytosol (55). In addition, the catalytic motifs that govern the phosphatase reaction for these enzymes are different (60). The *PAHI*-encoded PAP1 enzyme has a DxDxT catalytic motif present in a haloacid dehalogenase (HAD)-like domain and the motif is found in a superfamily of  $Mg^{2+}$ -dependent phosphatase enzymes (Fig. 6A) (60). However, the *DPPI*- and *LPPI*- encoded PAP2 enzymes have a three-domain lipid phosphatase motif (Fig. 6B) (14, 53, 54, 68, 69). These motifs are conserved sequences found in a superfamily of  $Mg^{2+}$ -independent lipid phosphatases (68, 70, 71).

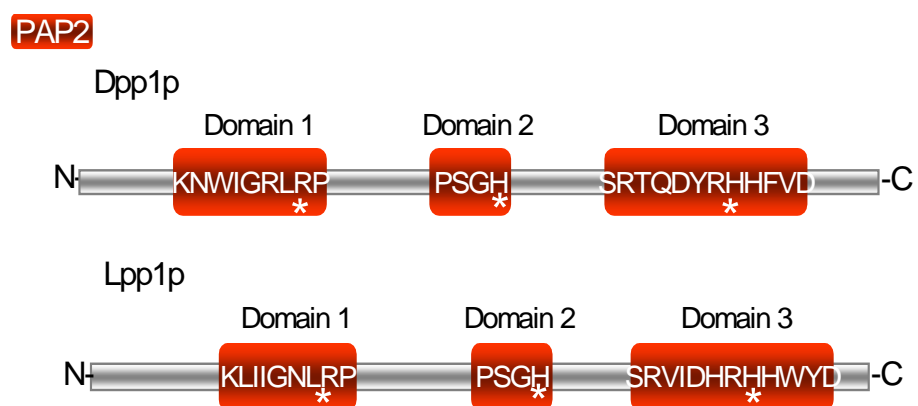
**Figure 6. Catalytic motifs of PA phosphatase enzymes.**

A. The reaction catalyzed by the PAP1 type enzyme uses a DxDxT motif present in a HAD-like domain. Asterisk indicates the conserved aspartate residue responsible for phosphate binding in the phosphatase reaction. B. The reaction catalyzed by PAP2 type enzyme uses a three-domain lipid phosphatase motif. Asterisk indicates the amino acids that are conserved in each domain and required for phosphatase activity.

A



B



### **The *PAH1*-encoded $Mg^{2+}$ -dependent PA Phosphatase and Lipin Protein**

The study of lipid analysis in the *pah1Δ* mutant shows that the mutant accumulates PA and has reduced amounts of DAG and its derivative TAG (55). This result indicates this enzyme is responsible for *de novo* lipid synthesis. Also, the *pah1Δ* mutation affects phospholipid composition because of the reduction in PC content (55). In addition, the *pah1Δ* mutant has a temperature sensitivity phenotype (55, 72).

The basic enzymological study of the purified recombinant  $Mg^{2+}$ -dependent PA phosphatase enzyme shows that optimum enzyme activity is found at pH 7.5 (55). The PA phosphatase activity absolutely requires  $MgCl_2$  with maximum activity at a 1 mM (55). Also, the *PAH1*-encoded PAP1 enzyme follows surface dilution kinetics when Triton X-100 is added to the assay mixture, and it exhibits cooperative kinetics with respect to the surface concentration of PA (55). Analysis of kinetic data for this enzyme indicates a Hill number of 3 and a  $K_m$  value for PA of 3 mol % (55). In addition, PA phosphatase activity of the *PAH1*-encoded enzyme is not inhibited by NEM (55).

Lipin 1 is a protein that regulates fat metabolism in mammalian cells (73-76). The study of lipin 1 in mice shows that lipin 1 deficiency causes lipodystrophy and insulin resistance, otherwise an excess of lipin 1 results in obesity and insulin sensitivity (73, 74). The *PAH1*-encoded PAP1 protein has a sequence homology with the lipin 1 protein in evolutionarily conserved N-terminal and C-terminal regions of the protein. (55). A recent biochemical study of recombinant human lipin 1 shows that lipin 1 is a  $Mg^{2+}$ -dependent PA phosphatase enzyme (55). Also, Lipin1 is phosphorylated in response to insulin treatment in rat adipocytes (77). In mammals, there are three lipin genes, and each of three lipin family members show  $Mg^{2+}$ -dependent phosphatase



activity (78). The three different lipin show distinct tissue expression patterns and this finding suggests that unique physiological role for each in lipid synthesis (78).

# **CHAPTER I**

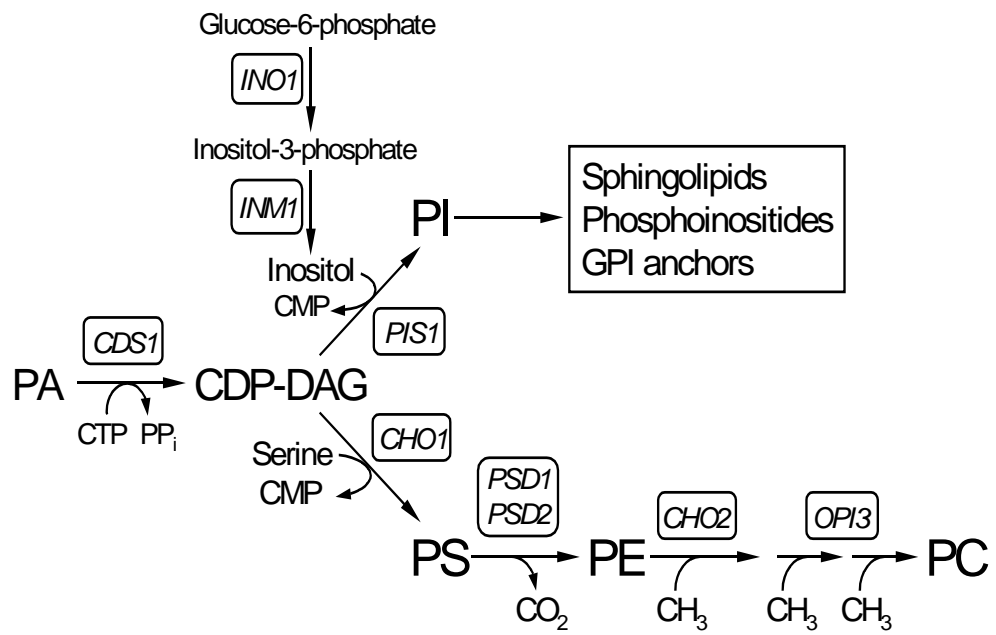
## **REGULATION OF THE *PIS1*-ENCODED PHOSPHATIDYLINOSITOL SYNTHASE BY ZINC**

### **BACKGROUND**

In addition to being a major structural component of the membrane, PI serves as the precursor for sphingolipids (84,85), the D-3, D-4, and D-5 phosphoinositides (6, 86-89), and glycosyl PI anchors (90, 91) (Fig. 7). As shown in Figure 7, CDP-DAG is a branch point for the synthesis of PS and PI. Thus, the levels of CDP-diacylglycerol are controlled through its utilization by the PI synthase enzyme itself and the competing activity of PS synthase (8, 20, 58, 103). PS synthase catalyzes the committed step in the synthesis of PC via the CDP-diacylglycerol pathway (58) (Fig. 7). Indeed, the coordinate regulation of the PI synthase and PS synthase enzymes is part of an overall mechanism by which the synthesis of PI is coordinately regulated with the synthesis of PC (6, 15, 17, 58, 104-106). Despite its essential nature, zinc is toxic to cells when accumulated in excess amounts (28). Cells have mechanisms to maintain intracellular zinc homeostasis to survive under stress condition. Recent studies have revealed that the synthesis of

**Figure 7. Pathways for the synthesis of PI and PC in *S. cerevisiae*.**

The pathways shown for the synthesis of PI and PC (CDP-diacylglycerol pathway) include the relevant steps discussed throughout this dissertation. The genes encoding enzymes responsible for the reactions in the pathways are indicated in the figure. A more detailed description of phospholipid synthesis that includes the Kennedy pathway for PC synthesis can be found in Figure 2. PA, phosphatidate; CDP-DAG, CDP-diacylglycerol; GPI, glycosyl phosphatidylinositol.



phospholipids in *S. cerevisiae* is influenced by zinc deficiency (40). In particular, PI synthase activity is elevated in zinc-depleted cells whereas several enzyme activities (e.g., PS synthase, PS decarboxylase, PE methyltransferase, and phospholipid methyltransferase) in the CDP-diacylglycerol pathway for PC synthesis are reduced in response to zinc depletion (40). The regulation of these activities by zinc availability contributes to alterations in the cellular levels of the major membrane phospholipids PI (elevated) and PE (reduced) (40). For the PS synthase enzyme, the reduction in activity in response to zinc depletion is controlled at the level of transcription through the UAS<sub>INO</sub> element the *CHO1* promoter and by the transcription factors Ino2p, Ino4p, and Opi1p (40). In this work, I explored the mechanism by which PI synthase activity is regulated in response to zinc depletion. The data indicated that this regulation occurred by a transcriptional mechanism that was mediated by the transcriptional activator Zap1p.

## EXPERIMENTAL PROCEDURES

### Materials

All chemicals were reagent grade. Growth medium supplies were purchased from Difco laboratories. Yeast nitrogen base lacking zinc sulfate was purchased from BIO 101. Restriction endonucleases, modifying enzymes, and the NEBlot kit were purchased from New England Biolabs, Inc. RNA size markers were purchased from Promega. The Yeastmaker yeast transformation kit was obtained from Clontech. Plasmid DNA purification and DNA gel extraction kits were from Qiagen, Inc. The QuikChange site-directed mutagenesis kit was from Stratagene. Oligonucleotides for PCRs and electrophoretic mobility shift assays were prepared by Genosys Biotechnology, Inc. ProbeQuant G-50 columns, polyvinylidene difluoride membranes, an enhanced chemifluorescence Western blotting detection kit, and glutathione-Sepharose 4 fast flow were purchased from GE Healthcare. DNA markers for agarose gel electrophoresis, protein molecular mass standards for SDS-PAGE, Zeta Probe blotting membranes, protein assay reagents, electrophoretic reagents, immunochemical reagents, isopropyl 1-thio- $\beta$ -galactopyranoside, and acrylamide solutions were purchased from Bio-Rad. Ampicillin, aprotinin, benzamidine, bovine serum albumin, leupeptin, 0-nitrophenyl  $\beta$ -d-galactopyranoside, pepstatin, phenylmethylsulfonyl fluoride, reduced glutathione, IGEPAL CA-630, and Triton X-100 were purchased from Sigma. Mouse monoclonal anti-HA antibodies (12CA5) and immunoPure goat anti-mouse IgG (H+L) antibodies were purchased from Roche Applied Science and Pierce, respectively. Radiochemicals and scintillation counting supplies were purchased from PerkinElmer Life Sciences and National Diagnostics, respectively. Liqui-Nox detergent was from Alconox, Inc.

### **Strains Plasmids and Growth Conditions**

The strains and plasmids used in this work are presented in Table I. Yeast cells were grown according to standard methods (110, 111) at 30 °C in YEPD medium (1% yeast extract, 2% peptone, 2% glucose) or in synthetic complete medium containing 2% glucose. Appropriate nutrients were omitted from synthetic complete medium for the selection of cells bearing plasmids. Zinc-depleted medium was synthetic complete medium prepared with yeast nitrogen base lacking zinc sulfate. For zinc-depleted cultures, cells were first grown for 24 h in synthetic complete medium supplemented with 1.5  $\mu$ M zinc sulfate. Saturated cultures were harvested, washed in deionized distilled water, diluted to  $1 \times 10^6$  cells/ml in medium containing 0 or 1.5  $\mu$ M zinc sulfate, and grown for 24 h. Cultures were then diluted to  $1 \times 10^6$  cells/ml and grown again in medium containing 0 or 1.5  $\mu$ M zinc sulfate. This growth routine with medium lacking was used to deplete internal stores of zinc (14). Cells in liquid medium were grown to the exponential phase ( $1 \times 10^7$  cells/ml), and cell numbers were determined spectrophotometrically at an absorbance of 600 nm. Plasmids were maintained and amplified in *Escherichia coli* strain DH5a grown in LB medium (1% tryptone, 0.5 % yeast extract, 1% NaCl, pH 7.4) at 37 °C. Ampicillin (100  $\mu$ g/ml) was added to bacterial cultures that contained plasmids. Yeast and bacterial media were supplemented with 2% and 1.5 % agar, respectively, for growth on plates. Glassware were washed with Liqui-Nox, rinsed with 0.1 mM EDTA, and then rinsed several times with deionized distilled water to prevent zinc contamination.

TABLE I  
Strains and Plasmids used in PI synthase Work

Strain or plasmid	Relevant characteristics	Ref.
<i>S. cerevisiae</i>		
W303-1A	<i>MATa ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1</i>	(112)
DY1457	<i>MATα ade6 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-52</i>	(113)
ZHY6	<i>MATa ade6 can1-100oc his3 leu2 ura3 zap1Δ::TRP1</i>	(113)
ZHY3	<i>MATα ade6 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-52</i> <i>zrt1Δ::LEU2 zrt2Δ::HIS3</i>	(34)
SH303	<i>MATa his3Δ200 leu2Δ1 trp1Δ63 ura3-52 ino2Δ::TRP1</i>	S.A. Henry
SH307	<i>MATα his3Δ200 leu2Δ1 trp1Δ63 ura3-52 ino4Δ::LEU2</i>	S.A. Henry
SH304	<i>MATa his3Δ200 leu2Δ1 trp1Δ63 ura3-52 opi1Δ::LEU2</i>	S.A. Henry
<i>E. coli</i>		
DH5α	F <sup>-</sup> φ80dlacZΔM15 Δ( <i>lacZYA-argF</i> )U169 <i>deoR</i> , <i>recA1 endA1</i> <i>hdR17(r<sub>k</sub><sup>-</sup> m<sub>k</sub><sup>+</sup>) phoA supE44 λ<sup>-</sup> thi-1 gyrA96 relA1</i>	(111)
Plasmids		
pWMI1	HA-tagged <i>PIS1</i> gene ligated into the <i>XmaI/XbaI</i> sites of pRS416	This study
pRS416	Single-copy <i>E. coli</i> /yeast shuttle vector containing <i>URA3</i>	(114)
pPI514	<i>PIS1</i> gene on a multicopy plasmid with <i>LEU2</i>	(98)
pGEX-687	<i>E. coli</i> expression plasmid for recombinant GST-Zap1p <sup>687-880</sup>	(115)
pMA109	P <sub><i>PIS1</i></sub> - <i>lacZ</i> reporter plasmid containing the <i>PIS1</i> promoter with <i>URA3</i>	(47)
pPZM1	Derivative of pMA109 with mutations in UAS <sub>ZRE</sub> <sup>1</sup>	This study
pPZM2	Derivative of pMA109 with mutations in UAS <sub>ZRE</sub> <sup>2</sup>	This study
pPZM3	Derivative of pMA109 with mutations in UAS <sub>ZRE</sub> <sup>3</sup>	This study



## DNA Manipulations and Amplification of DNA by PCR

Plasmid and genomic DNA preparation, restriction enzyme digestion, and DNA ligation were performed by standard methods (111). Conditions for the amplification of DNA by PCR were optimized as described previously (116). Transformation of yeast (117) and *E.coli* (111) was performed using standard protocols.

### Construction of Plasmids

Plasmid pWM11 contains a 2.2-kb DNA fragment for the *PIS1* gene with sequences for an HA epitope tag inserted after the start codon. Genomic DNA prepared from strain W303-1A was used as a template to produce a 5'-fragment of *PIS1*<sup>HA</sup> (primers 5'-CCCCCGGGCTAATGCATGAGCCAATAGAG-3' and 5'-AGCGTAGTCTGGGACGTCGTATGGGTACATCTTGTACTATCACACTTTCCCTCTTAT-3') and a 3' fragment of *PIS1*<sup>HA</sup> (primers 5'-TACCCATACGACGTCCCAGACTACACGCTAGTTCGAATTCAACACCAGAAAA GGTTACT-3' and 5'-CGTCTAGAGTGCAAGTTGGAGAGAATCGCTTCCG-3'). The 5'- and 3'-fragments of *PIS1*<sup>HA</sup> were digested with XmaI/AatII and AatII/XbaI, respectively, and inserted into the XmaI/XbaI sites of pRS416 to generate the plasmid pWM11. The Stratagene QuikChange site-directed mutagenesis kit was utilized according to the manufacturer's instructions to generate plasmids pPZM1-pPZM3. These plasmids were derivatives of pMA109 (*P<sub>PIS1</sub>-lacZ*) and contained mutations in UAS<sub>ZRE</sub><sup>1</sup>, UAS<sub>ZRE</sub><sup>2</sup> and UAS<sub>ZRE</sub><sup>3</sup> of the *PIS1* promoter. Plasmids pPZM1 (mutagenic primers 5'-TTTTTCTTCCTTTTCCCTAACAATTCCAATTGCTTCTCTTCTCTTCTCCTT-3' and 5'-AAGGAGAAGAGAAGAGAAGCAATTGGAATTGTTAGGGAAAAGGAAGAA

AAA-3') were constructed by amplification of plasmid reactions by digestion with DpnI. The mutant plasmids were amplified in *E.coli*, and the purified plasmids were sequenced to confirm the mutations in the *PIS1* promoter.

### **RNA Isolation and Northern Blot Analysis**

Total RNA was isolated from cells (118, 119), resolved by agarose gel electrophoresis (120), and then transferred to Zeta Probe membranes by vacuum blotting. The *PIS1* and *CMD1* probes were labeled with [ $\alpha$ -<sup>32</sup>P]dTTP using the NEBlot random primer labeling kit, and unincorporated nucleotides were removed using ProbeQuant G-50 columns. Prehybridization, hybridization with the probes, and washes to remove nonspecific binding were carried out according to the manufacturer's instructions. Images of the radiolabeled mRNAs were acquired by phosphorimaging analysis.

### **Anti-PI Synthase Antibodies and Immunoblotting**

The peptide sequence AALILADNDAKNANE (residues 201-215 at the C-terminal end of the deduced amino acid sequence of *PIS1*) was synthesized and used to raise antibodies in New Zealand White rabbits by standard procedures at Bio-Synthesis, Inc. The IgG fraction was isolated from the antiserum using protein A-Sepharose CL-4B (121). SDS-PAGE (122) using 10% slab gels and transfer of proteins to polyvinylidene difluoride membranes (123) were performed as described previously. The membrane was probed with 12.5  $\mu$ g/ml purified anti-PI synthase IgG fraction. Mouse monoclonal anti-HA antibodies were used at a dilution of 1:1,000. Goat anti-rabbit and anti-mouse IgG-alkaline phosphatase conjugates were used as secondary antibodies at a dilution of

1:5,000. The PI synthase protein (Pis1p) was detected using the enhanced chemifluorescence Western blotting detection kit, and the signals were acquired by FluorImaging. The relative density of the signal was analyzed using ImageQuant software. Immunoblot signals were in the linear range of detectability.

### **Preparation of Cell Extracts and Protein Determination**

Cell extracts were prepared as described previously (124). Cells were suspended in 50 mM Tris-maleate buffer, pH 7.0, containing 1 mM EDTA, 0.3 M sucrose, 10 mM 2-mercaptoethanol, 0.5 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, 5 µg/ml aprotinin, 5 µg/ml leupeptin, and 5 µg/ml pepstatin. Cells were disrupted by homogenization with chilled glass beads (0.5 mm diameter) using a Biospec Products Mini-Bead-Beater-8. Samples were homogenized for 10 times of 1 min bursts followed by 2-min cooling between bursts at 4 °C. The cell extract (supernatant) was obtained by centrifugation of the homogenate at 1,500 X g for 10 min. The protein concentration was determined by the method of Bradford (125) using bovine serum albumin as the standard.

### **Enzyme Assays**

All assays were conducted in triplicate at 30 °C in a total volume of 0.1 ml. PI synthase activity was measured by following the incorporation of [2-<sup>3</sup>H]inositol (10,000 cpm/nmol) into PI as described previously (45). The assay mixture contained 50 mM Tris-HCl, pH 8.0, 2 mM MnCl<sub>2</sub>, 0.5 mM inositol, 0.2 mM CDP-diacylglycerol, 2.4 mM Triton X-100, and enzyme protein.  $\beta$ -galactosidase activity was measured by following the formation of 0-nitrophenyl from 0-nitrophenyl  $\beta$ -D-galactopyranoside

spectrophotometrically at a wavelength of 410 nm (126). The assay mixture contained 100 mM sodium phosphate, pH 7.0, 3 mM 0-nitrophenyl  $\beta$ -D-galactopyranoside, 1 mM  $\text{MgCl}_2$ , 100 mM 2-mercaptoethanol, and enzyme protein. All assays were linear with time and protein concentration. The average standard deviation of all assays was  $\pm 5\%$ . A unit of PI synthase activity was defined as the amount of enzyme that catalyzed the formation of 1 nmol of product/min, whereas a unit of  $\beta$ -galactosidase activity was defined in  $\mu\text{mol/min}$ . Specific activity was defined as units/mg of protein.

### **Expression and Purification of GST-Zap1p<sup>687-880</sup> from *E. coli*.**

This experiment was conducted by Gil-Soo Han. The GST-Zap1p<sup>687-880</sup> fusion protein was expressed in *E. coli* BL21(DE3)pLysS bearing plasmid pGEX-687. A 500 ml culture was grown to  $A_{600} \sim 0.8$  at 28 °C, and the expression of GST-Zap1p<sup>687-880</sup> was induced for 1 h with 0.1 mM isopropyl 1-thio- $\beta$ -D-galactopyranoside. The culture was harvested, and the resulting pellet was resuspended in 20 ml of phosphate-buffered saline (10 mM  $\text{Na}_2\text{HPO}_4$ , 1.8 mM  $\text{KH}_2\text{PO}_4$ , 140 mM  $\text{NaCl}$ , 2.7 mM  $\text{KCl}$ , pH 7.3). Cells were disrupted with a French press at 20,000-pounds/square inch, and unbroken cells and cell debris were removed by centrifugation at 12,000 X g for 30 min at 4 °C. The supernatant (cell lysate) was mixed for 1 h with 1 ml of a 50 % slurry of glutathione-Sepharose with gentle shaking. The glutathione-Sepharose resin was then packed in a 10 ml Poly-Prep disposable column and was washed with 25 ml of phosphate-buffered saline. Proteins bound to the column were eluted (0.5-ml fractions) with 50 mM Tris-HCl, pH 8.0, buffer containing 10 mM reduced glutathione. SDS-PAGE analysis indicated that the 48-kDa GST-Zap1p<sup>687-880</sup> was purified  $\sim 90\%$  of homogeneity. The purified GST-Zap1p<sup>687-880</sup>

preparation was dialyzed against phosphate-buffered saline containing 10 % glycerol and 2.5 mM dithiothreitol.

### **Electrophoretic Mobility Shift Assays**

This experiment was conducted by Gil-Soo Han. Double-stranded oligonucleotides (Table II) were prepared by annealing 25  $\mu$ M complementary single-stranded oligonucleotides in a total volume of 0.1 ml containing 10 mM Tris-HCl, pH 7.5, 100 mM NaCl, and 1 mM EDTA. The reaction mixtures were incubated for 5 min at 100 °C in a heat block and then for 2 h in the heat block that was turned off. Annealed oligonucleotides were designed to contain a 5'-overhanging end, and they were labeled by incorporating [ $\alpha$ -<sup>32</sup>P]dTTP to the ends. Annealed oligonucleotides (100 pmol) were incubated with 5 units of Klenow fragment and [ $\alpha$ -<sup>32</sup>P]dTTP (400-800 Ci/nmol) for 30 min at room temperature. Labeled oligonucleotides were purified from unincorporated nucleotides using ProbeQuant G-50 spin columns. Formation of the protein-DNA complexes was allowed for 15 min at room temperature in a total volume of 10  $\mu$ l containing 1 pmol of radiolabeled DNA probe ( $2.5 \times 10^5$  cpm/pmol), 10 mM Tris-HCl, pH 8.0, 10 mM MgCl<sub>2</sub>, 50 mM KCl, 1 mM dithiothreitol, 0.025 mg/ml poly(dI-dC)poly(dI-dC), 0.2 mg/ml bovine serum albumin, 0.04 % IGEPAL CA-630, 10 % glycerol, and the indicated concentrations of purified GST-Zap1p<sup>687-880</sup>. The reaction mixtures were resolved on 6 % polyacrylamide gels (1.5-mm thickness) in 0.5 X Tris-borate-EDTA buffers at 100 V for 45 min. Gels were dried onto blotting paper, and the radioactive signals were visualized by phosphorimaging analysis.

TABLE II  
Oligonucleotides used for Electromobility Shift Assays

Element	Annealed oligonucleotides <sup>a</sup>
<i>PIS1</i> UAS <sub>ZRE</sub> <sup>1</sup>	5' -TTCCCTAAACCTTTTCAGAGCTTCtct-3' 3' -aagGGATTTGGAAAAGTCTCGAAGAGA-5'
<i>PIS1</i> UAS <sub>ZRE</sub> <sup>2</sup>	5' -GACACTTCTATCTTAGAAGTGTTGata-3' 3' -ctgTGAAGATAGAATCTTCACAACTAT-5'
<i>PIS1</i> UAS <sub>ZRE</sub> <sup>3</sup>	5' -CATAAAAAACATGAGAGGTGGTATggt-3' 3' -gtaTTTTTTGTACTCTCCACCATAACCA-5'
<i>PIS1</i> UAS <sub>ZRE</sub> <sup>3</sup> (M1)	5' -CATAAAAA <b>CAATTCCAAT</b> TGGTATggt-3' 3' -gtaTTTTTTGTTAAGGTTAACCATAACCA-5'
<i>PIS1</i> UAS <sub>ZRE</sub> <sup>3</sup> (M2)	5' -CATAAAAAAC <b>CTTGA</b> AGGTGGTATggt-3' 3' -gtaTTTTTTGGAACCTCCACCATAACCA-5'

<sup>a</sup>Underlined sequences are putative UAS<sub>ZRE</sub> sites. The mutations (*M1* and *M2*) in UAS<sub>ZRE</sub><sup>3</sup> are shown in bold letters. The small letters indicate the nucleotides filled with the Klenow fragment.

### **Analysis of Data**

Statistical significance was determined by performing Student's *t* test using SigmaPlot software. *p* values < 0.05 were taken as a significant difference.

## RESULTS

### **Effect of the *zrt1* $\Delta$ *zrt2* $\Delta$ Mutations on the Expression of PI Synthase Activity in Response to Zinc Depletion**

Iwanyshyn *et al.* (40) identified PI synthase as an enzyme whose activity increased in wild type cells when zinc was depleted from the growth medium. To confirm that this regulation was governed by the intracellular levels of zinc, the expression of PI synthase activity was examined in a *zrt1* $\Delta$  *zrt2* $\Delta$  double mutant (34). This mutant lacks both the high-affinity (Zrt1p) and low-affinity (Zrt2p) plasma membrane zinc transporters that are primarily responsible for regulating the cytoplasmic levels of zinc in *S. cerevisiae* (33, 34). For this and subsequent experiments, the growth medium lacked inositol and choline supplementation to preclude the regulatory effects that these phospholipid precursors have on phospholipid synthesis (6, 15, 58, 104). As described previously (40), depletion of zinc from the growth medium of wild type cells caused a 2-fold increase in the expression of PI synthase activity (Fig. 8). The level of PI synthase activity in the *zrt1* $\Delta$  *zrt2* $\Delta$  mutant grown in the presence of zinc was similar to that expressed in the wild type control cells that were depleted for zinc (Fig. 8). This result indicated that the intracellular levels of zinc were responsible for regulating the expression of PI synthase activity.

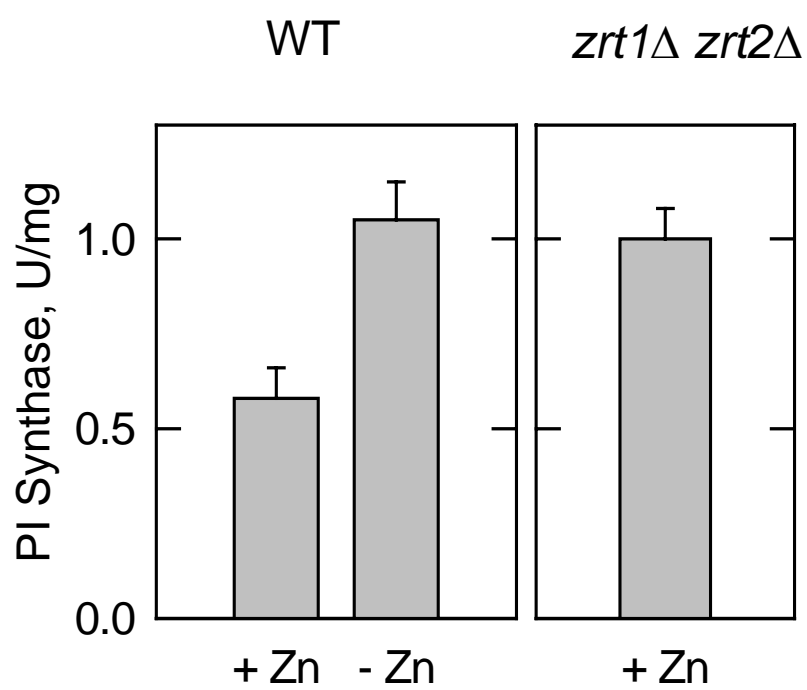
### **Effect of Zinc Depletion on the Expression of PI Synthase Protein and *PIS1* mRNA**

Antibodies were generated against a peptide sequence found at the C-terminal end of the PI synthase protein. These antibodies recognized a protein with a subunit molecular mass of 24 kDa, the predicted size of the *PIS1* gene product (83).



**Figure 8. Effect of the *zrt1* $\Delta$  *zrt2* $\Delta$  mutations on the expression of PI synthase activity in response to zinc depletion.**

Wild type and *zrt1* $\Delta$  *zrt2* $\Delta$  mutant cells were grown in the presence (1.5  $\mu$ M) and absence of zinc as indicated. Cell extracts were prepared and used for the assay of PI synthase activity. Each data point represents the average of triplicate enzyme determinations from a minimum of two independent experiments  $\pm$  S.D.

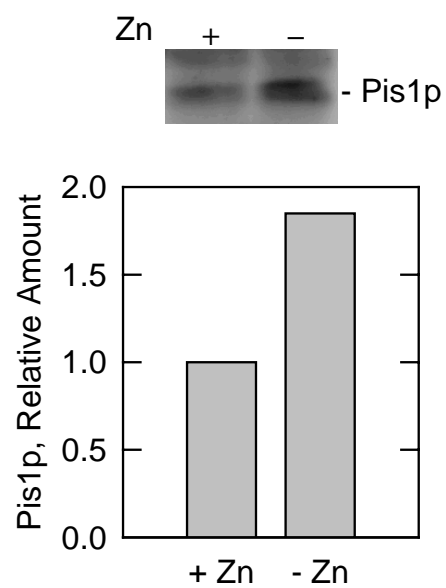


To confirm the identity of this 24-kDa protein as the PI synthase protein, an immunoblot experiment was performed using a cell extract derived from wild type cells that overexpressed the *PIS1* gene on a high copy plasmid. Consistent with the overexpression of the *PIS1* gene, the amount of the 24-kDa protein that was recognized by the anti-PI synthase antibodies was elevated ~7-fold. As a further confirmation, an immunoblot experiment was performed using a cell extract from wild type cells that expressed the *PIS1*<sup>HA</sup> gene on a single copy plasmid. The anti-PI synthase antibodies recognized both the native and HA-tagged versions of the PI synthase protein. HA-tagged PI synthase migrated with a molecular mass of 25 kDa because of the HA epitope. The identity of the HA-tagged PI synthase protein was confirmed by immunoblot analysis using anti-HA antibodies. The expression of the PI synthase protein was analyzed by immunoblotting to examine the mechanism by which PI synthase activity was regulated in response to zinc depletion. Zinc depletion resulted in nearly a 2-fold increase in the amount of the PI synthase protein when compared with cells grown with zinc (Fig. 9A). This indicated that the increase in PI synthase activity was a result of an increase in enzyme level. We next examined the level of *PIS1* mRNA to determine if the increase in enzyme content was due to an increase in gene expression. *CMD1* mRNA (encodes calmodulin) was measured in this analysis as a loading control because its expression level is not affected by zinc availability (127, 128). Northern blot analysis of total RNA isolated from exponential phase cells showed that the relative amount of *PIS1* mRNA in zinc-depleted cells was almost 2-fold greater when compared with that found in cells grown with zinc (Fig. 9B). These results indicated that a transcriptional mechanism was responsible for the regulation of PI synthase in zinc-depleted cells.

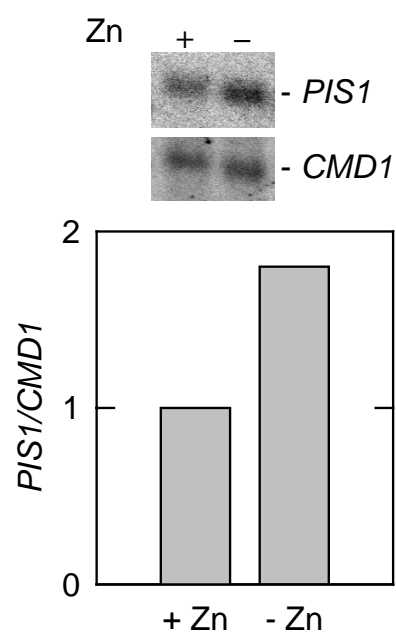
**Figure 9. Effect of zinc depletion on the expression of PI synthase protein.**

Wild type cells were grown in the presence (1.5  $\mu$ M) and absence of zinc. A, cell extracts were prepared and 50- $\mu$ g samples were used for immunoblot analysis using anti-PI synthase antibodies (12.5  $\mu$ g/ml). A portion of the immunoblot is shown, and the position of the PI synthase (Pis1p) protein is indicated. The signals of the PI synthase protein from cells grown with and without zinc were quantified using ImageQuant software. The amount of PI synthase protein found in cells grown with zinc was arbitrarily set at 1. The data shown is representative of two independent experiments. B, total RNA was extracted and 25- $\mu$ g samples were used for Northern blot analysis to determine the abundance of *PIS1* mRNA. Portions of Northern blots are shown and the positions of *PIS1* and *CMD1* (loading control) mRNAs are indicated. The relative amounts of *PIS1* and *CMD1* mRNAs from cells grown with and without zinc were determined by ImageQuant analysis of the data. The relative amount of *PIS1* to *CMD1* mRNA in cells grown with zinc was arbitrarily set at 1. The data shown are representative of two independent experiments.

A



B



## Effect of Zinc Depletion on the Expression of $\beta$ -galactosidase Activity in Cells Bearing the $P_{PIS1}$ -*lacZ* Reporter Gene

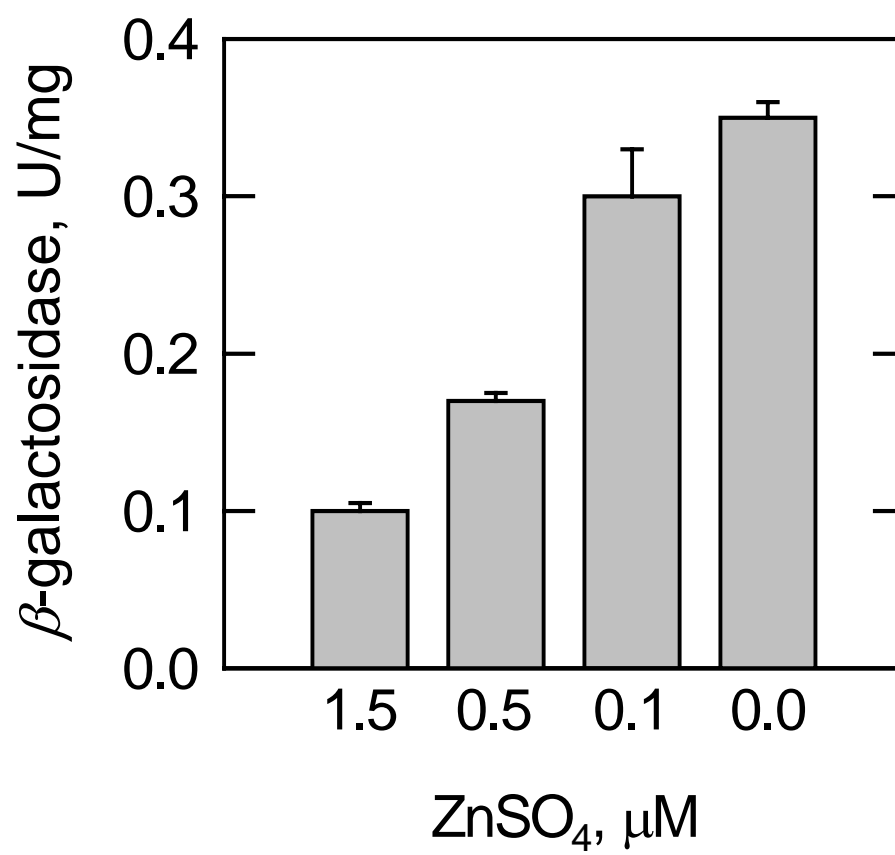
The analysis of *PIS1* expression was facilitated by the use of plasmid pMA109 that bears a  $P_{PIS1}$ -*lacZ* reporter gene where the expression levels of  $\beta$ -galactosidase activity are dependent on transcription driven by the *PIS1* promoter (47). To further examine the effect of zinc depletion on the expression of the *PIS1* gene, we measured  $\beta$ -galactosidase activity from wild type cells bearing plasmid pMA109 that were grown with various concentrations of zinc. Reduction for zinc in the growth medium resulted in a dose-dependent increase in  $\beta$ -galactosidase activity (Fig. 10). The activity found in cells grown in the absence of zinc was 3.5-fold greater than the activity in cells grown in the presence of 1.5  $\mu$ M zinc (Fig. 10). Concentrations of zinc above 1.5  $\mu$ M did not result in a further reduction in  $\beta$ -galactosidase activity.

## Effects of *ino2* $\Delta$ , *ino4* $\Delta$ , and *opi1* $\Delta$ Mutations on the Regulation of PI synthase by Zinc Depletion

The PI synthase enzyme is found at a branch point in phospholipid synthesis where it competes with another enzyme, PS synthase, for the common liponucleotide substrate CDP-diacylglycerol (58). Unlike *PIS1*, the expression of the PS synthase gene (*CHO1*) is repressed in wild type cells when zinc is depleted from the growth medium (40). The regulation of PS synthase expression by zinc depletion is mediated through a  $UAS_{INO}$  element in the *CHO1* promoter and by the positive transcription factors Ino2p and Ino4p, and the negative transcription factor Opi1p (40). Owing to the fact that the *PIS1* promoter contains a  $UAS_{INO}$  element (47) and that the synthesis of PI and PS is

Figure 10. **Dose-dependent induction of  $\beta$ -galactosidase activity in cells bearing the  $P_{PIS1}$ -*lacZ* reporter gene in response to zinc depletion.**

Wild type cells bearing the  $P_{PIS1}$ -*lacZ* reporter plasmid pMA109 were grown in the absence and presence of the indicated concentrations of zinc sulfate. Cell extracts were prepared and used for the assay of  $\beta$ -galactosidase activity. Each data point represents the average of triplicate enzyme determinations from a minimum of two independent experiments  $\pm$  S.D.





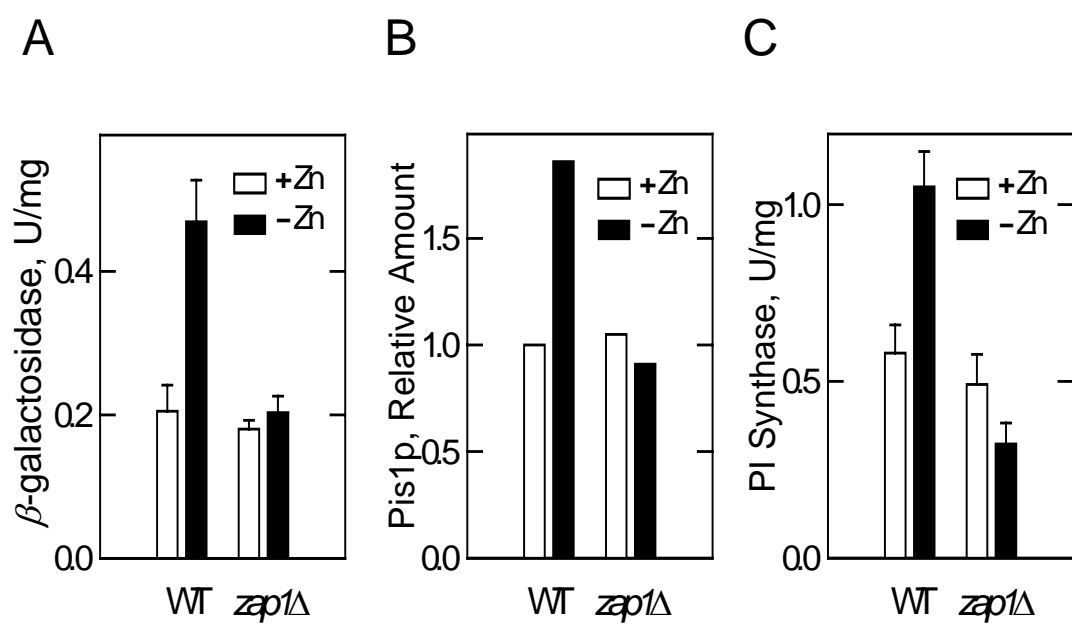
coordinately regulated in *S. cerevisiae* (6, 15, 58, 58, 104), we questioned whether the regulation of PI synthase expression by zinc depletion was mediated by Ino2p, Ino4p, and Opi1p. To address this question, PI synthase activity was measured in *ino2Δ*, *ino4Δ*, and *opi1Δ* mutant cells that were grown in the presence and absence of zinc. In all three regulatory mutants, the PI synthase enzyme was elevated in response to zinc depletion similar to that observed in wild type cells (data not shown). These results indicated that the induction of PI synthase in zinc-depleted cells was not mediated by Ino2p, Ino4p, and Opi1p.

### **Effects of the *zap1Δ* Mutation on the Regulation of PI synthase by Zinc Depletion**

Zap1p is a positive transcription factor that is maximally expressed in zinc-deplete cells and repressed in zinc-replete cells (113). Zap1p directly regulates UAS<sub>ZRE</sub>-containing genes (e.g., *ZRT1*, *ZRT2*, *ZRT3*, *ZRC1*, *FET4*, *DPPI*) whose expression is induced by zinc depletion (14, 127, 129-131). Inspection of the *PIS1* promoter revealed that it contains sequences that bear resemblance to the consensus UAS<sub>ZRE</sub> (see below). Accordingly, we questioned whether the regulation of PI synthase expression by zinc was dependent on Zap1p function. In the first set of experiments, the *zap1Δ* mutant bearing the *P<sub>PIS1</sub>-lacZ* reporter gene was grown in the presence and absence of zinc followed by the measurement of  $\beta$ -galactosidase activity. In contrast to wild type cells, zinc depletion did not result in the induction of  $\beta$ -galactosidase activity (Fig. 11A). In a second set of experiments, PI synthase protein and activity levels were measured in cell extracts derived from *zap1Δ* mutant cells grown in the presence and

**Figure 11. Effect of the *zap1Δ* mutation on the regulation of PI synthase by zinc depletion.**

Wild type and *zap1Δ* mutant cells were grown in the presence (1.5  $\mu$ M) and absence of zinc. *Panel A*, cell extracts were prepared from cells bearing the  $P_{PIST-lacZ}$  reporter plasmid pMA109 and used for the assay of  $\beta$ -galactosidase activity. Each data point represents the average of triplicate enzyme determinations from a minimum of two independent experiments  $\pm$  S.D. *Panel B*, 50- $\mu$ g samples of cell extracts were used for immunoblot analysis using anti-PI synthase antibodies (12.5  $\mu$ g/ml). The signals of the PI synthase protein from wild type and *zap1Δ* mutant cells grown with and without zinc were quantified using ImageQuant software. The amount of PI synthase protein found in wild type cells grown with zinc was arbitrarily set at 1. The data shown are representative of two independent experiments. *Panel C*, cell extracts were prepared and assayed for PI synthase activity. Each data point represents the average of triplicate enzyme determinations from a minimum of two independent experiments  $\pm$  S.D.



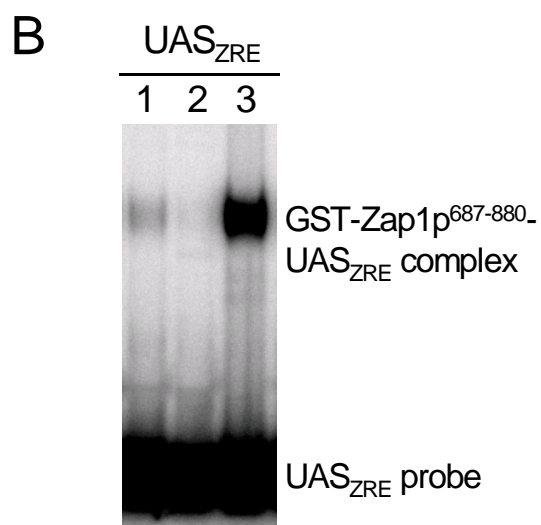
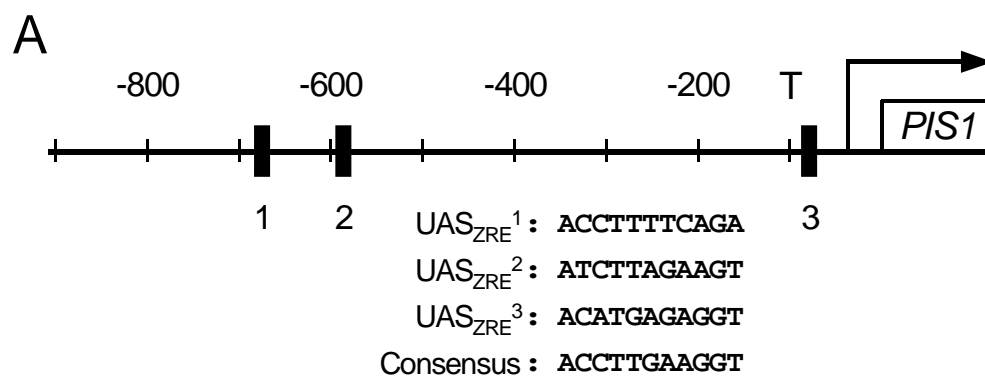
absence of zinc. Unlike wild type cells, the depletion of zinc from the growth medium of the *zap1Δ* mutant did not result in elevated levels of PI synthase protein (Fig. 11B) and activity (Fig. 11C). These results indicated that the zinc-mediated regulation of *PIS1* expression was dependent on the Zap1p transcription factor.

### **Interactions of GST-Zap1p<sup>687-880</sup> with Putative UAS<sub>ZRE</sub> Sites in the *PIS1* Promoter**

We sought evidence that Zap1p mediates the regulation of *PIS1* expression in response to zinc depletion by direct interaction with the *PIS1* promoter. The *PIS1* promoter contains three putative UAS<sub>ZRE</sub> sites (UAS<sub>ZRE</sub><sup>1</sup>, UAS<sub>ZRE</sub><sup>2</sup>, and UAS<sub>ZRE</sub><sup>3</sup>) with sequences that resemble the consensus UAS<sub>ZRE</sub> sequence for Zap1p binding (Fig. 12A). Electrophoretic mobility shift assays were performed by Gil-Soo Han with labeled oligonucleotides containing the putative UAS<sub>ZRE</sub> sites using recombinant GST-Zap1p<sup>687-880</sup> purified from *E. coli*. Zap1p<sup>687-880</sup> contains the UAS<sub>ZRE</sub> binding domain (amino acids 687-880) of Zap1p (115). Of the three probes, the oligonucleotide containing UAS<sub>ZRE</sub><sup>3</sup> showed the strongest interaction with GST-Zap1p<sup>687-880</sup> (Fig. 12B). The interaction of GST-Zap1p<sup>687-880</sup> with UAS<sub>ZRE</sub><sup>1</sup> was ~20-fold lower when compared with UAS<sub>ZRE</sub><sup>3</sup> whereas an interaction with UAS<sub>ZRE</sub><sup>2</sup> was hardly detectable (Fig. 12B). The interaction of GST-Zap1p<sup>687-880</sup> with UAS<sub>ZRE</sub><sup>3</sup> was examined further using the same assay. The formation of the GST-Zap1p<sup>687-880</sup>-UAS<sub>ZRE</sub><sup>3</sup> complex was dependent on the concentration of GST-Zap1p<sup>687-880</sup> (Fig. 13A).

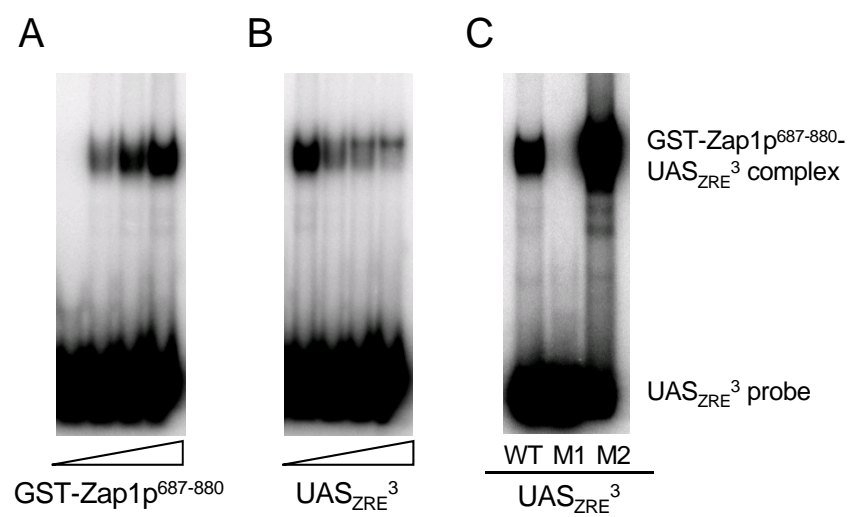
Figure 12. **Interactions of GST-Zap1p<sup>687-880</sup> with putative UAS<sub>ZRE</sub> sites in the *PIS1* promoter.**

*Panel A*, the locations, and sequences of the putative UAS<sub>ZRE</sub> sites in the *PIS1* promoter are shown in the figure. *T*, TATA box. *Panel B*, samples (1 pmol) of double-stranded synthetic oligonucleotides ( $2.5 \times 10^5$  cpm/pmol) with sequences for UAS<sub>ZRE</sub><sup>1</sup> (1), UAS<sub>ZRE</sub><sup>2</sup> (2), and UAS<sub>ZRE</sub><sup>3</sup> (3) in the *PIS1* promoter were incubated with 0.6 µg of recombinant GST-Zap1p<sup>687-880</sup> purified from *E. coli*. Interaction of GST-Zap1p<sup>687-880</sup> with the labeled oligonucleotides was determined by electrophoretic mobility shift assay using a 6% polyacrylamide gel. The data shown are representative of two independent experiments. (This experiment was conducted by Gil-Soo Han.)



**Figure 13. Interactions of GST-Zap1p<sup>687-880</sup> with UAS<sub>ZRE</sub><sup>3</sup>.**

Samples (1 pmol) of radiolabeled double-stranded synthetic oligonucleotide ( $2.5 \times 10^5$  cpm/pmol) with the sequence for UAS<sub>ZRE</sub><sup>3</sup> in the *PIS1* promoter were incubated with recombinant GST-Zap1p<sup>687-880</sup>. *Panel A*, the experiment was performed with 0, 0.15, 0.3, and 0.6  $\mu$ g of recombinant GST-Zap1p<sup>687-880</sup>. *Panel B*, the experiment was performed with 0.6  $\mu$ g of recombinant GST-Zap1p<sup>687-880</sup> and 0, 25, 50, and 100 pmol of unlabeled oligonucleotide with the sequence for UAS<sub>ZRE</sub><sup>3</sup>. *Panel C*, the experiment was performed with 0.6  $\mu$ g of recombinant GST-Zap1p<sup>687-880</sup> and sequences for wild type and mutated forms of UAS<sub>ZRE</sub><sup>3</sup>. The wild type (*WT*) UAS<sub>ZRE</sub><sup>3</sup> sequence was mutated from 5'-ACATGAGAGGT-3' to the nonconsensus sequence 5'-CAATTCCAATT-3' (*M1*) and to a consensus sequence 5'-ACCTTGAAGGT-3' (*M2*). Interaction of GST-Zap1p<sup>687-880</sup> with the labeled oligonucleotides was determined by electrophoretic mobility shift assay using a 6% polyacrylamide gel. The data shown are representative of two independent experiments. (This experiment was conducted by Gil-Soo Han.)





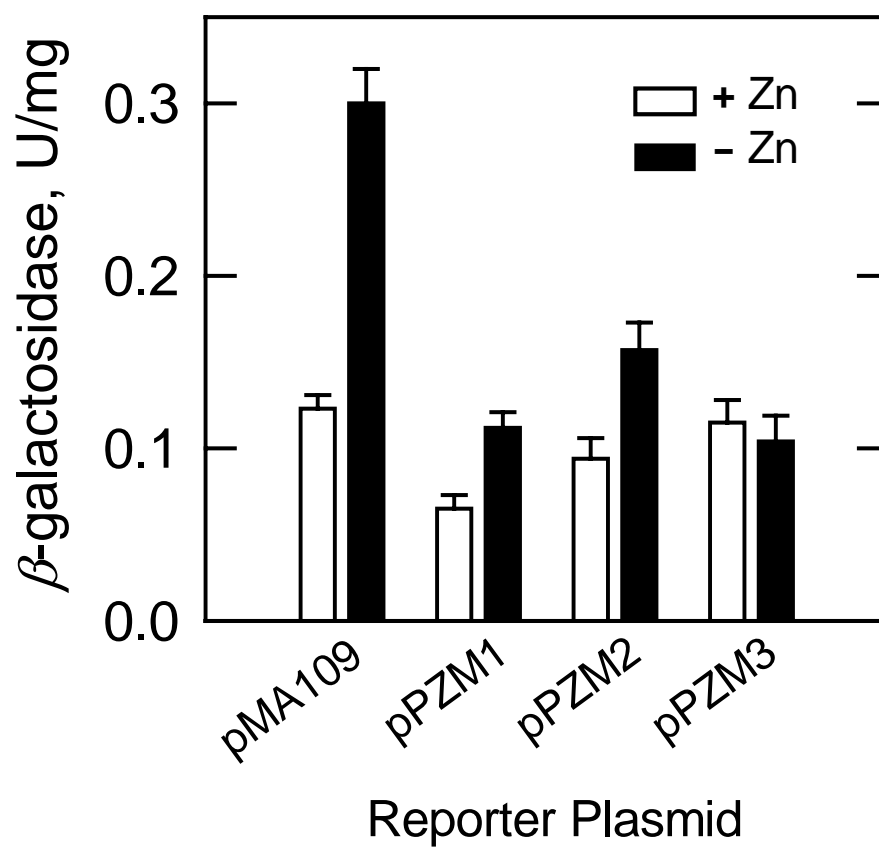
In addition, the unlabeled UAS<sub>ZRE</sub><sup>3</sup> probe competed with the labeled probe for binding to GST-Zap1p<sup>687-880</sup> in a dose-dependent manner (Fig. 13B). Moreover, this interaction was abolished when the UAS<sub>ZRE</sub><sup>3</sup> sequence was mutated (*M1*) to a nonconsensus sequence (Fig. 13C). When the UAS<sub>ZRE</sub><sup>3</sup> sequence was mutated (*M2*) to the consensus UAS<sub>ZRE</sub>, the extent of interaction with GST-Zap1p<sup>687-880</sup> was 10-fold greater than the interaction with the wild type UAS<sub>ZRE</sub><sup>3</sup> sequence (Fig. 13C).

### **Effects of Mutations in the Putative UAS<sub>ZRE</sub> Elements in the *PIS1* Promoter on the Zinc-mediated Regulation of *PIS1* Expression**

The effects of mutations in UAS<sub>ZRE</sub><sup>1</sup>, UAS<sub>ZRE</sub><sup>2</sup>, and UAS<sub>ZRE</sub><sup>3</sup> in the *PIS1* promoter on the zinc-mediated regulation of *PIS1* expression were examined. P<sub>*PIS1*</sub>-*lacZ* reporter genes were constructed with mutations in each of the three putative UAS<sub>ZRE</sub> elements. For each element, the core sequences were changed to the nonconsensus sequence of 5'-CAATTCCAATT-3'. Cells bearing the wild type or mutant P<sub>*PIS1*</sub>-*lacZ* reporter genes were grown in the presence and absence of zinc; cell extracts were prepared and assayed for  $\beta$ -galactosidase activity. The mutations in UAS<sub>ZRE</sub><sup>3</sup> in the reporter plasmid pPZM3 abolished the induction of  $\beta$ -galactosidase activity that was observed in zinc-depleted cells bearing the wild type P<sub>*PIS1*</sub>-*lacZ* reporter plasmid pMA109 (Fig. 14). Although the expression of the  $\beta$ -galactosidase activities found in cells bearing the reporter plasmids with mutations in UAS<sub>ZRE</sub><sup>1</sup> (pPZM1) and UAS<sub>ZRE</sub><sup>2</sup> (pPZM2) was somewhat attenuated, the *PIS1* gene was still induced when cells were depleted for zinc (Fig. 14). These data indicated that the zinc-mediated regulation of *PIS1* expression was primarily mediated by the UAS<sub>ZRE</sub><sup>3</sup> sequence in its promoter.

**Figure. 14. Effects of mutations in UAS<sub>ZRE</sub><sup>1</sup>, UAS<sub>ZRE</sub><sup>2</sup>, and UAS<sub>ZRE</sub><sup>3</sup> in the *PIS1* promoter on the zinc-mediated regulation of  $\beta$ -galactosidase activity in cells bearing the P<sub>*PIS1*</sub>-*lacZ* reporter gene.**

Wild type cells bearing the indicated P<sub>*PIS1*</sub>-*lacZ* reporter plasmids were grown in the presence (1.5  $\mu$ M) and absence of zinc. The UAS<sub>ZRE</sub><sup>1</sup>, UAS<sub>ZRE</sub><sup>2</sup>, and UAS<sub>ZRE</sub><sup>3</sup> sequences in the *PIS1* promoter of plasmid pMA109 were mutated to the nonconsensus sequence 5'-CAATTCCAATT-3' in plasmids pPZM1, pPZM2, and pPZM3, respectively. Cell extracts were prepared and used for the assay of  $\beta$ -galactosidase activity. Each data point represents the average of triplicate enzyme determinations from a minimum of two independent experiments  $\pm$  S.D.



## DISCUSSION

The yeast *S. cerevisiae* has the ability to cope with a variety of stress conditions (e.g., nutrient deprivation) by regulating the expression of enzyme activities including those involved in phospholipid synthesis (14, 23, 40, 40, 58, 81, 132). In particular, the stress condition of zinc depletion results in an increase in PI content that is attributed to elevated expression of PI synthase activity (40). Analysis of the *zrt1Δ zrt2Δ* mutant defective in the major plasma membrane zinc transporters Zrt1p and Zrt2p indicated that a decrease in the intracellular levels of zinc was responsible for the induction of PI synthase activity. That *PISI* mRNA, its encoded protein Pis1p, and the  $\beta$ -galactosidase activity driven by the  $P_{PISI}$ -*lacZ* reporter gene were elevated in zinc-depleted cells indicated that the increase in PI synthase activity was due to a transcriptional mechanism.

The zinc-mediated induction of the  $P_{PISI}$ -*lacZ* reporter gene, and PI synthase protein and activity was lost in *zap1Δ* mutant cells. These data indicated that the regulation of *PISI* gene expression by zinc was mediated by the Zap1p transcription factor. Zap1p is a zinc-sensing and zinc-inducible regulatory protein that binds to a UAS<sub>ZRE</sub> found in the promoter of zinc-regulated genes to drive their transcription (37, 113, 127, 133-135). Zap1p plays a major role in regulating the intracellular levels of zinc in *S. cerevisiae* (113, 135). For example in zinc-depleted cells, Zap1p mediates increased expression and activity of the high-affinity (Zrt1p) and low-affinity (Zrt2p, Fet4p) zinc transporters in the plasma membrane and of the efflux zinc transporter Zrt3p in the vacuole membrane to elevate the cytoplasmic levels of zinc (33, 34, 36, 37, 129, 135).

The promoter of the *PISI* gene does not contain a consensus UAS<sub>ZRE</sub>. However, three putative UAS<sub>ZRE</sub> sites were identified in the *PISI* promoter sequence by a motif

search using the Vector NTI computer program. Electrophoretic mobility shift assays with DNA probes containing the putative UAS<sub>ZRE</sub> sites and purified recombinant GST-Zap1p<sup>687-880</sup> showed that UAS<sub>ZRE</sub><sup>3</sup> in the *PIS1* promoter was required for GST-Zap1p<sup>687-880</sup> binding *in vitro*. Moreover, mutations in UAS<sub>ZRE</sub><sup>3</sup> to a nonconsensus sequence abolished the GST-Zap1p<sup>687-880</sup>-DNA interactions *in vitro* and abolished the induction of *PIS1* gene expression (as reflected in  $\beta$ -galactosidase activity) in response to zinc depletion. A genome-wide cDNA microarray analysis of gene expression identified 46 direct Zap1p target genes that are induced by zinc depletion (127). The *PIS1* gene was not identified in that microarray study (127). This might be attributed to the relatively modest level of *PIS1* induction (~ 2-fold) when compared with the > 10-fold inductions of other Zap1p target genes (e.g., *ZRT1*, *DPPI*) (14, 127). The differences between the magnitudes of induction of the *PIS1* gene and other Zap1p target genes correlated with the relative binding efficiencies of GST-Zap1p<sup>687-880</sup> with the *PIS1* promoter UAS<sub>ZRE</sub><sup>3</sup> sequence when compared with this sequence mutated to a consensus UAS<sub>ZRE</sub> sequence. Notwithstanding, the 2-fold induction of the *PIS1* gene in response to zinc depletion correlated with the ~ 2-fold increase in the PI content of yeast cells depleted for zinc (40). The steady state composition of PI in *S. cerevisiae* is tightly regulated (~2- to 3-fold changes) (6, 58, 80). In this regard, we found that the expression of PI synthase did not respond to zinc depletion when the *PIS1* gene was overexpressed from a plasmid.

Inositol, the water-soluble substrate of the PI synthase enzyme reaction, plays a major role in the regulation of phospholipid synthesis and composition in *S. cerevisiae* (6, 58, 80-82). The addition of inositol to the growth medium of wild type cells causes an increase in the level of PI and a decrease in the levels of PS, PE, and PC (103, 124). The

decreased levels of PS, PE, and PC are primarily due to a repression mechanism that involves the positive transcription factors Ino2p and Ino4p, the negative transcription factor Opi1p, and a UAS<sub>INO</sub> element found in the promoter of genes (i.e., *CHO1*, *PSD1*, *CHO2*, and *OPI3*) encoding the enzymes in the CDP-diacylglycerol pathway for PC synthesis (6, 15, 17, 58, 104) (Fig. 1). The coordinate repression of the CDP-diacylglycerol pathway enzymes by inositol requires the ongoing synthesis of PC (136, 137), and is enhanced by the inclusion of choline in the growth medium (6, 15, 17, 58, 104). The increased level of PI in response to inositol/choline supplementation is not due to increased expression of *PIS1* mRNA (138) and the PI synthase enzyme (46). Transcription of the *PIS1* gene is insensitive to inositol/choline, and it does not require the UAS<sub>INO</sub> element in its promoter or the transcription factors Ino2p and Opi1p (47). The regulation of PI synthesis by inositol is due to a biochemical mechanism (103). Given the low intracellular levels of inositol and the relatively high  $K_m$  value for inositol, the synthesis of PI by the PI synthase enzyme is regulated by the availability of inositol (103). Moreover, inositol is an inhibitor of the PS synthase enzyme, and this regulation also contributes to the decrease in the synthesis of PS and ultimately PE and PC (103). These observations raised the suggestion that PI synthase is a constitutively expressed enzyme (6, 15, 104). However, as shown here, the level of the PI synthase enzyme is regulated by zinc availability.

This is not the first study to show that the expression of the *PIS1* gene is subject to transcriptional regulation. Anderson and Lopes (47) have shown that expression of *PIS1* is regulated in response to growth medium carbon source. When compared with glucose, glycerol represses *PIS1* expression whereas galactose induces expression (47).

The transcription factor Mcm1p mediates the glycerol-dependent repression of *PIS1* gene expression whereas the transcription factor Sln1p mediates the galactose-mediated induction of gene expression (47). The expression of the *PIS1* gene is also regulated by oxygen availability (48). Gene expression is induced when cells are grown under anaerobic conditions and repressed under aerobic conditions. Repression is dependent on transcription factor Rox1p and its binding site in the *PIS1* promoter (48). Similar to that observed in cells deprived for zinc (40), a reduction in oxygen availability results in elevated levels of PI (48). The induction of *PIS1* gene expression may represent one of the mechanisms by which cells cope with the stress conditions of zinc and oxygen deficiencies given that PI is a precursor to several lipid molecules (sphingolipids, phosphoinositides, and glycosyl PI anchors) that are essential to the growth and metabolism of this eukaryotic organism (6, 86-97).

## FUTURE DIRECTIONS

The regulation of PI synthase by zinc depletion is primarily governed by the action of the Zap1p transcription factor, which binds to the UAS<sub>ZRE</sub><sup>3</sup> sequence in the *PIS1* promoter. If UAS<sub>ZRE</sub><sup>3</sup> sequence in the *PIS1* promoter is mutated, Zap1p cannot bind to the element in a zinc-depleted cell. Therefore, cells containing mutated UAS<sub>ZRE</sub><sup>3</sup> sequences in the *PIS1* promoter should not up-regulate the expression of *PIS1* in response to zinc depletion. Ultimately, this may result in the inability of the cell to elevate the level of PI, which is an essential phospholipid.

In future study, the effects of mutations in UAS<sub>ZRE</sub><sup>3</sup> of the *PIS1* promoter on cell growth and viability in response to zinc will be conducted. To examine the effects of mutations in UAS<sub>ZRE</sub><sup>3</sup> of the *PIS1* promoter on the growth and viability of yeast cells under zinc-depleted conditions, a mutant cell that can no longer regulate the expression of *PIS1* will be constructed by two-step gene replacement technique and homologous recombination. Then, wild type and *pis1* mutant (cell contains a mutation in UAS<sub>ZRE</sub><sup>3</sup> of the *PIS1* promoter) with promoter mutation will be cultured in the media containing 1.5  $\mu$ M zinc sulfate or without zinc sulfate. Cell growth will be determined by measuring the change in optical density at 600 nm over time. If the *pis1* mutants grow slower than wild type cells in the absence of zinc, cell viability will be examined by making a serial dilution of cultures and spreading onto YEPD (1% yeast extract, 2% peptone, 2% glucose) plates, followed by incubating at 30 °C. The number of viable cells in each culture will be determined by counting the colonies on plates. If the number of colonies in *pis1* mutant cells is less than that of wild type cell, this result will indicate that the regulation of *PIS1* through UAS<sub>ZRE</sub><sup>3</sup> under zinc-limited conditions will affect the cell



viability of yeast. Moreover, the phospholipids composition in wild type and *pis1* mutant cells should be analyzed to examine the effect of mutation in  $\text{UAS}_{\text{ZRE}}^3$  of the *PIS1* promoter on the PI level because the mutant cell can no longer up-regulate the expression of *PIS1* when the cell is depleted for zinc. This work could provide information on the contribution of PI synthase in cell physiology in response to zinc depletion.

## **CHAPTER II**

### **PURIFICATION AND CHARACTERIZATION OF THE NEM-SENSITIVE $Mg^{2+}$ -DEPENDENT PA PHOSPHATASE IN YEAST**

#### **BACKGROUND**

In 1984,  $Mg^{2+}$ -dependent PA phosphatase activity was first identified in yeast by Hosaka and Yammasita (139) and cytosolic and membrane-associated forms of the  $Mg^{2+}$ -dependent PA phosphatase have been purified and characterized (13, 52, 140-144). The cytosolic-associated PA phosphatase is partially purified from the soluble fraction about 600-fold and the purification procedures include ammonium sulfate fractionation and column chromatography on DEAE-Sepharose, Sephadex G-100 and Blue-Sepharose (139). The purified enzyme absolutely requires  $Mg^{2+}$  ions for activity and the molecular weight of the enzyme is 75,000 and the enzyme is highly specific of phosphatidate (139). The purification of the membrane-associated PA phosphatase from yeast includes the preparation of sodium cholate extract and column chromatography on DE-53, Affi-Gel Blue, Hydroxylapatite, Mono Q and Superose 12 (140). The enzyme is purified 9,833-fold over the crude extract and the subunit molecular weight is 91,000 (140). The PA

phosphatase activity is dependent on  $Mg^{2+}$  ions and Triton X-100, and the activation energy for the reaction is 11.9 kcal/mol (140). The enzyme is labile above 30 °C and sensitive to thioreactive reagents (140). Additionally, immunoblot analysis of cell extracts derived from wild type and protease-deficient cells find the existence of a 45-kDa form of PA phosphatase, and the study also shows that the 91-kDa form of PA phosphatase is a proteolytic product of a 104-kDa protein (142). The 45- and 104-kDa proteins are localized differentially in the cell, and the purified membrane associated  $Mg^{2+}$ -dependent PA phosphatases are inhibited by phenylglyoxal and propranolol compounds, that are shown to inhibit PA phosphatase activity from animal tissues (142, 145, 146). The *PAHI*-encoded  $Mg^{2+}$ -dependent PA phosphatase that is a peripheral membrane-associated protein is involved in the synthesis of lipids, but its enzyme activity is sulfhydryl reagent resistant (55). A recent study of the *PAHI*-encoded  $Mg^{2+}$ -dependent PA phosphatase shows that the predicted size of Pah1p is 95 kDa, and the enzymological properties of the recombinant  $Mg^{2+}$ -dependent PA phosphatase are very similar to those of the 91-kDa  $Mg^{2+}$ -dependent enzyme previously purified from yeast (142, 55).

The study of the *pah1Δ dpp1Δ lpp1Δ* mutant provides the evidence for the presence of the NEM-sensitive  $Mg^{2+}$ -dependent PA phosphatase which its gene has not been identified (55). Owing to the lack of information of the NEM-sensitive  $Mg^{2+}$ -dependent PA phosphatase in yeast, the physiological function of  $Mg^{2+}$ -dependent PA phosphatase enzyme in lipid metabolism is still unclear. For that reason, the aim of this work was the purification and characterization of the NEM-sensitive  $Mg^{2+}$ -dependent PA phosphatase. As a reference of the study of purification of PA phosphatase by Hosaka

and Yamashita (148), a new purification scheme for the NEM-sensitive  $Mg^{2+}$ -dependent PA phosphatase was made using results from preliminary experiments. The purified enzyme was utilized for characterization of the enzymological properties and for determination of the native molecular mass of the enzyme. Also, the enzymological properties of the NEM-sensitive  $Mg^{2+}$ -dependent PA phosphatase can be compared with those of other PA phosphatase enzymes to clarify the role of the enzyme in lipid metabolism.

## EXPERIMENTAL PROCEDURES

### Materials

All chemicals were reagent grade. Growth medium supplies were purchased from Difco laboratories. Nucleotides, PMSF, benzamidine, aprotinin, leupeptin, pepstatin, gel filtration protein standard markers, bovine serum albumin, *N*-ethylmaleimide, phenylglyoxal, propranolol, Triton X-100, phosphocellulose, *D-erythro*-sphingosine, *D*-phytosphingosine, sphingomyelin and ceramide were purchased from Sigma. Phospholipids were purchased from Avanti Polar Lipids and Sigma. Dioleoyl-diacylglycerol and dioleoyl-PA were purchased from Avanti Polar Lipids. Protein assay reagents, electrophoretic reagents, and protein standards were purchased from Bio-Rad. The Centricon® Centrifugal Filter device was purchased from Millipore. Mono Q and Superose 6 were purchased from Pharmacia Biotech Inc. Radiochemicals were purchased from PerkinElmer Life Sciences. [<sup>32</sup>P]Pi was purchased from ICN. Scintillation counting supplies were purchased from National Diagnostics. *E. coli* diacylglycerol kinase was obtained from Lipidex Inc.

### Strains and Growth Conditions

Mutant *S.cerevisiae* strain *pah1Δ dpp1Δ lpp1Δ* (55) was used for the purification of the Mg<sup>2+</sup>-dependent PA phosphatase. Cells were grown in YEPD medium (1% yeast extract, 2% peptone, 2% glucose) at 30 °C, harvested by centrifugation, and stored at –80 °C. Yeast cell numbers in liquid media were determined spectrophotometrically at *A*<sub>600</sub> nm.

### **Purification of $Mg^{2+}$ -dependent PA Phosphatase**

All steps were performed at 4 °C.

Step 1: Preparation of Cell Extract - Yeast cells were suspended in 50 mM Tris-maleate (pH 7.5), 0.3 M sucrose, 10 mM 2-mercaptoethanol, 0.5 mM phenylmethanesulfonyl fluoride, 1 mM benzamide, 5 µg/ml aprotinin, 5 µg/ml leupeptin, 5 µg/ml pepstatin. Cells were disrupted with glass beads (0.5 mm diameter) using a Biospec Products Bead Beater. Unbroken cells and glass beads were removed by centrifugation at 1,500 X g for 10 min.

Step 2: Preparation of Membrane and Salt-extractable Fractions - The cell extract was centrifuged at 100,000 X g for 1h to separate cytosolic from the total membrane fraction. The membrane was then suspended in the same buffer containing 1M NaCl. After incubation with shaking for 5 min at 4 °C, the suspension was centrifuged at 100,000 X g for 1h at 4 °C.

Step 3: Preparation of Aggregated Proteins - The salt-extractable fraction was dialyzed in same buffer without salt for overnight at 4 °C to remove the salt. The dialyzed sample was centrifuged at 10,000 X g to separate the aggregated proteins.

Step 4: Preparation of Solubilized Aggregated Proteins - The aggregated proteins were solubilized in 50 mM Tris-HCl buffer (pH 7.5) containing 10 mM  $MgCl_2$ , 10 mM 2-mercaptoethanol, 20 % glycerol, 1 mM PMSF and 1% sodium cholate.

Step 5: Phosphocellulose Chromatography - A phosphocellulose column (6 X 34 cm) was equilibrated with Buffer A (50 mM Tris-HCl (pH 7.5), 10 mM  $MgCl_2$ , 10 mM 2-mercaptoethanol, 20 % glycerol, 1 mM PMSF and 1 % sodium cholate). The solubilized aggregated protein was applied to the column, and the column was washed with 5 column

volumes of Buffer A. The PA phosphatase was then eluted from the phosphocellulose column with Buffer A containing 0.1 M NaCl at a flow rate of 14 ml/h. Fractions containing the PA phosphatase activity were combined and immediately used for the next step of the purification scheme.

Step 6: Mono Q Chromatography I – A Mono Q (1 X 5 cm) column was equilibrated with Buffer A containing 0.1 M NaCl. The enzyme preparation from the previous step was applied to the column. The column was washed with 5 column volumes of Buffer A containing 0.1 M NaCl followed by elution of the enzyme with 30 column volumes of a linear NaCl gradient (0.1 - 1 M) in Buffer A at a flow rate of 30 ml/h. The peak of PA phosphatase activity eluted from the column at NaCl concentration of about 0.3 M. The most active fractions were pooled and concentrated with Centricon® Centrifugal Filter device at 4 °C to a final volume of 1 ml. The concentrated enzyme was stored at 4 °C and used for the next step in the purification scheme.

Step 7: Superose 6 Chromatography - A Superose 6 column (1 X 30 cm) was equilibrated with Buffer A. Purified enzyme from the Mono Q column chromatography was applied to the Superose 6 column at a flow rate of 22 ml/h. The enzyme was then eluted from the column with 4 column volumes of Buffer A. The peak of activity was eluted with 0.6 column volumes of Buffer A. The most active fractions were pooled and applied to the next step in the purification scheme.

Step 8: Mono Q Chromatography II - Mono Q (1X 5 cm) was equilibrated with Buffer A. Enzyme purified from the Superose 6 chromatography was applied to the column at a flow rate of 30 ml/h. The column was washed with 5 column volumes of Buffer A followed by elution of the enzyme with 40 column volumes of a linear NaCl

gradient (0 - 0.5 M) in Buffer A at a flow rate of 30 ml/h. The peak of PA phosphatase activity eluted from the column at NaCl concentration of 0.25 M. The most active fractions were pooled and stored at -80°C.

### **Preparation of Labeled Substrates**

[<sup>32</sup>P]PA was synthesized enzymatically from DAG (diacylglycerol) and [ $\gamma$ -<sup>32</sup>P]ATP with *E.coli* DAG kinase as described by Carman and Lin (149).

### **Enzyme Assays and Protein Determination**

Mg<sup>2+</sup>-dependent PA phosphatase was measured for 20 min by following the release of water-soluble [<sup>32</sup>P]P<sub>i</sub> from the chloroform-soluble [<sup>32</sup>P]PA (10,000 cpm/nmol) at 30 °C as described by Carman and Lin (149). The reaction mixture contained 50 mM Tris-HCl buffer (pH 7.5), 10 mM MgCl<sub>2</sub>, 0.1 mM PA, 10 mM 2-mercaptoethanol, and enzyme protein in a total volume of 0.1 ml. All enzyme assays were conducted in triplicate at 30 °C. The average standard deviation of the assays was  $\pm$  5%. The reactions were linear with time and protein concentration. A unit of enzyme activity was defined as the amount of enzyme that catalyzed the formation of 1 nmol of product per minute. Protein concentration was determined by the method of Bradford (125) using bovine serum albumin as the standard.



## Electrophoresis

To check the purity of purified proteins, SDS-polyacrylamide gel electrophoresis (122) was performed with 10% slab gels. Proteins on polyacrylamide gels were visualized with silver (150) or with Coomassie blue.

### Determination of Native Molecular Mass of $\text{Mg}^{2+}$ -dependent PA Phosphatase

The native molecular mass of the  $\text{Mg}^{2+}$ -dependent PA phosphatase was determined by gel filtration chromatography with Superose 6. The column was equilibrated with Buffer A, using the standard molecular mass proteins  $\beta$ -amylase (200 kDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa), and carbonic anhydrase (29 kDa). The enzyme was eluted with Buffer A at a flow rate of 22 ml/h. The molecular mass was calculated from a plot of log molecular mass (kDa) *versus* the  $V_e/V_o$  (elution volume/void volume).

### Preparation of Triton X-100/Lipid-mixed Micelles

Lipids in chloroform were transferred to a test tube, and solvent was removed *in vacuo* for 40 min. Triton X-100/lipid-mixed micelles were prepared by adding Triton X-100 to the dried lipids. After the addition of Triton X-100, the mixture was vortexed. The surface concentration of lipids in mixed micelles was varied by the addition of Triton X-100. The total lipid concentration in Triton X-100/lipid-mixed micelles did not exceed 15 mol % to ensure that the structure of the mixed micelles were similar to the structure of pure Triton X-100 (151, 152). The mole percent of a lipid in a mixed micelle was calculated using the formula:

$$\text{Mol \%}_{\text{lipid}} = ([\text{lipid (bulk)}]/[\text{lipid (bulk)}] + [\text{Triton X-100}]) \times 100$$

### **Analyses of Data**

Kinetic data were analyzed according to the Michaelis-Menten and Hill equations using the EZ-FIT enzyme kinetic model-fitting program developed by Perrella (153). Statistical analyses were performed with SigmaPlot 7.0 software.

## RESULTS

### Purification of the $Mg^{2+}$ -dependent PA Phosphatase

The *pah1Δ dpp1Δ lpp1Δ* mutant facilitated the purification of the NEM-sensitive  $Mg^{2+}$ -dependent PA phosphatase, since the mutant lacks nearly all  $Mg^{2+}$ -independent PA phosphatases and the NEM-insensitive  $Mg^{2+}$ -dependent PA phosphatase. The  $Mg^{2+}$ -dependent PA phosphatase activity in the *pah1Δ dpp1Δ lpp1Δ* mutant cell extract is 60 % lower than the activity in the cell extract of wild type cells (55). The remaining  $Mg^{2+}$ -dependent PA phosphatase activity in the triple mutant should be attributed yet to another gene that codes for a PA phosphatase enzyme (55). In addition, previous data indicated that this activity is associated with both cytosolic and membrane fractions of the cell (55). The activity in the membrane fraction is higher than the activity in the cytosolic fraction (55). Moreover, about 80 % of the  $Mg^{2+}$ -dependent PA phosphatase present in the membrane fraction can be extracted with 0.5 M NaCl (55). This result indicated that the  $Mg^{2+}$ -dependent PA phosphatase in the *pah1Δ dpp1Δ lpp1Δ* mutant is a peripheral membrane protein (55). Accordingly, the salt extractable fraction of the membrane fraction in the *pah1Δ dpp1Δ lpp1Δ* mutant was utilized for the purification of the NEM-sensitive  $Mg^{2+}$ -dependent PA phosphatase. Interestingly, the salt extractable enzyme aggregated during dialysis. This aggregated protein was solubilized in Tris HCl buffer (pH 7.5) containing 1% sodium cholate. Based on preliminary experiments, a purification scheme using phosphocellulose, Mono Q, and Superose 6 was developed. A summary of the final purification scheme for PA phosphatase is shown in Table III. The  $Mg^{2+}$ -dependent PA phosphatase activity was solubilized from the salt extractable fraction with 1% sodium cholate.

TABLE III  
Purification of PA Phosphatase

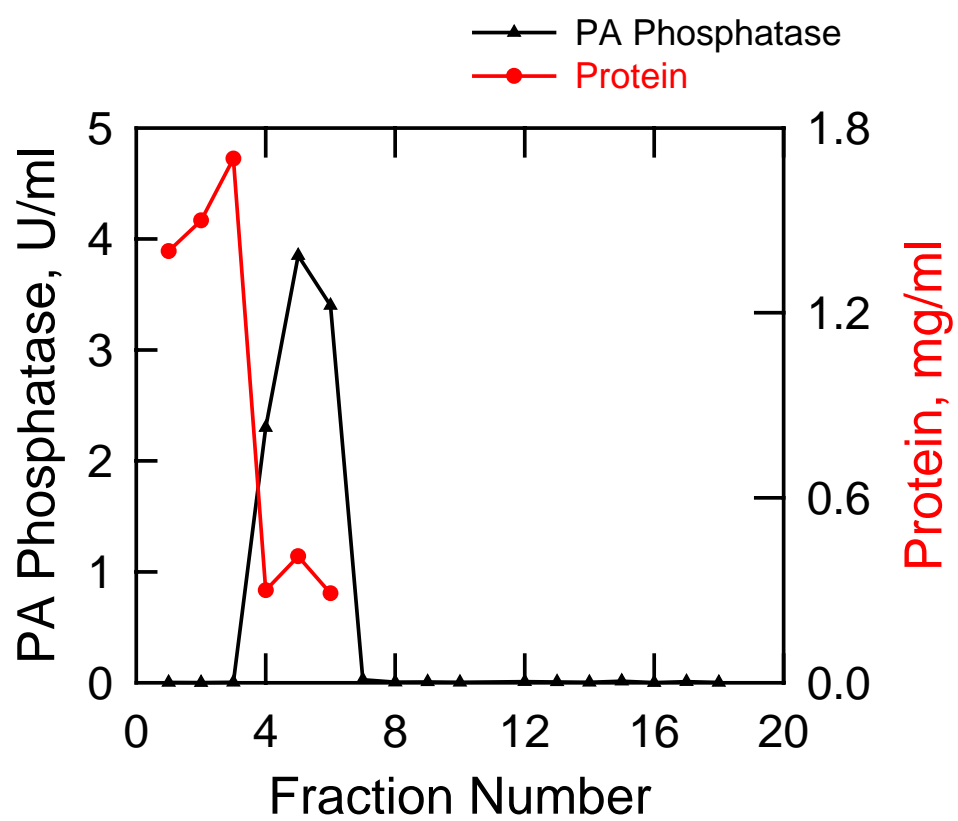
The data were based on starting with 165 g (wet weight) of cells.

Purification step	Total units	Total Protein	Specific activity	Yield	Purification
	<i>nmol/min</i>	<i>mg</i>	<i>units/mg</i>	<i>%</i>	<i>-fold</i>
1. Cell extract	1896	5550	0.34	100	1
2. Membrane	1257	2232	0.56	66	3
3. Salt extract	843	763	1.03	40	5.2
4. Aggregated protein	720	280	2.7	36	13.5
5. Phosphocellulose	117	3.2	36.4	19	182
6. Mono Q I	47	0.2	235	17	691
7. Superose 6	19	0.05	350	3.5	1029
8. Mono Q II	7.5	0.01	750	1.2	2250

Following protein aggregation and solubilization of the aggregated protein, the solubilized enzyme was applied to the phosphocellulose column. The enzyme was eluted from the column with a step elution of 0.1 M NaCl (Fig. 15). The phosphocellulose chromatography step afforded a 13.4-fold purification of the enzyme (Table III). The purified enzyme from phosphocellulose column was purified with Mono Q chromatography. The enzyme was eluted from the Mono Q column with a NaCl gradient from 0.1 to 1M (Fig. 16). The peak of PA phosphatase activity was eluted at 0.3 M NaCl. Mono Q chromatography resulted in a 6-fold increase in specific activity over the previous step (Table III). The purified enzyme from the Mono Q was applied the Superose 6, and the peak of activity was eluted with 16 ml of chromatography buffer (Fig. 17). This step provided about 2-fold increase in specific activity over the previous step (Table III). The Superose 6 purified enzyme activity lost about 50 % of activity when compared with the Mono Q purified enzyme. This result might be due to the concentration of the enzyme to 1 ml using Centricon® Centrifugal Filter after Mono Q chromatography. To get a higher purity of the enzyme, the Superose 6 purified enzyme was purified with a second Mono Q column. The peak of PA phosphatase activity was eluted from the Mono Q column at 0.25 M NaCl with a single sharp peak (Fig. 18). Overall, the  $Mg^{2+}$ -dependent PA phosphatase was purified 2,250-fold over the cell extract to a final specific activity of 750 U/mg with an activity yield of 1.2 % (Table III). The purity of the purified proteins from second Mono Q chromatography was checked by SDS-polyacrylamide gel electrophoresis (Fig. 19).

**Figure 15. Elution profiles of the  $Mg^{2+}$ -dependent PA phosphatase activity after chromatography with phosphocellulose.**

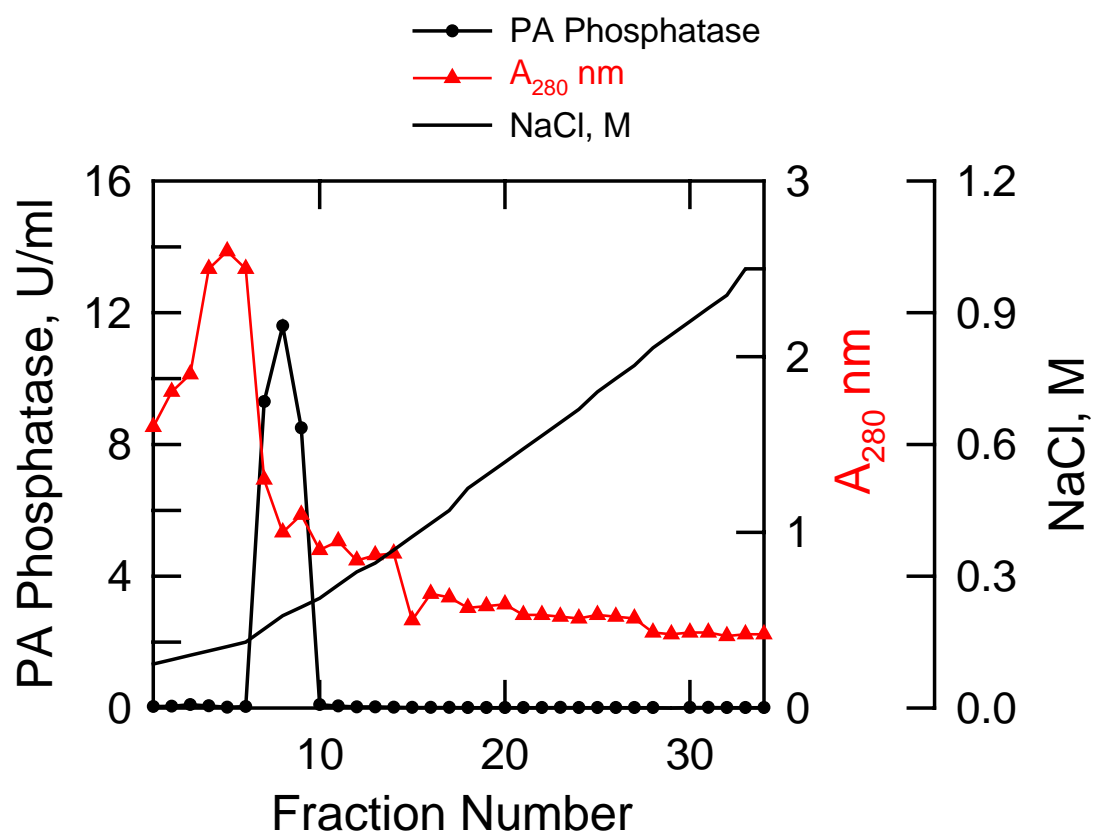
The  $Mg^{2+}$ -dependent PA phosphatase was subjected to phosphocellulose chromatography. Fractions containing PA phosphatase activity were collected and assayed for the  $Mg^{2+}$ -dependent PA phosphatase activity. The protein concentration was measured the method described under “Experimental Procedures”.



**Figure 16. Elution profile of the  $\text{Mg}^{2+}$ -dependent PA phosphatase activity after Mono Q I chromatography.**

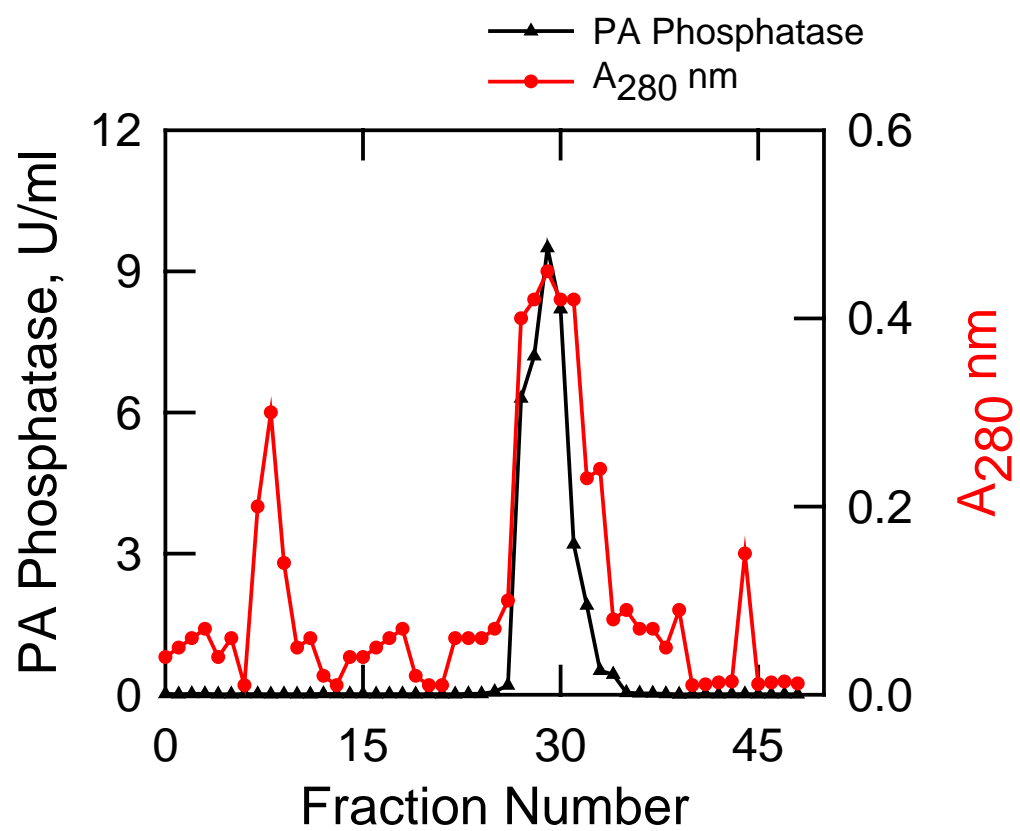
The most active fractions from the phosphocellulose column were subjected to Mono Q I chromatography. Fractions were collected and assayed for the  $\text{Mg}^{2+}$ -dependent PA phosphatase activity and protein concentrations were measured by the absorbance at 280 nm. The NaCl gradient profile is indicated by the solid line.





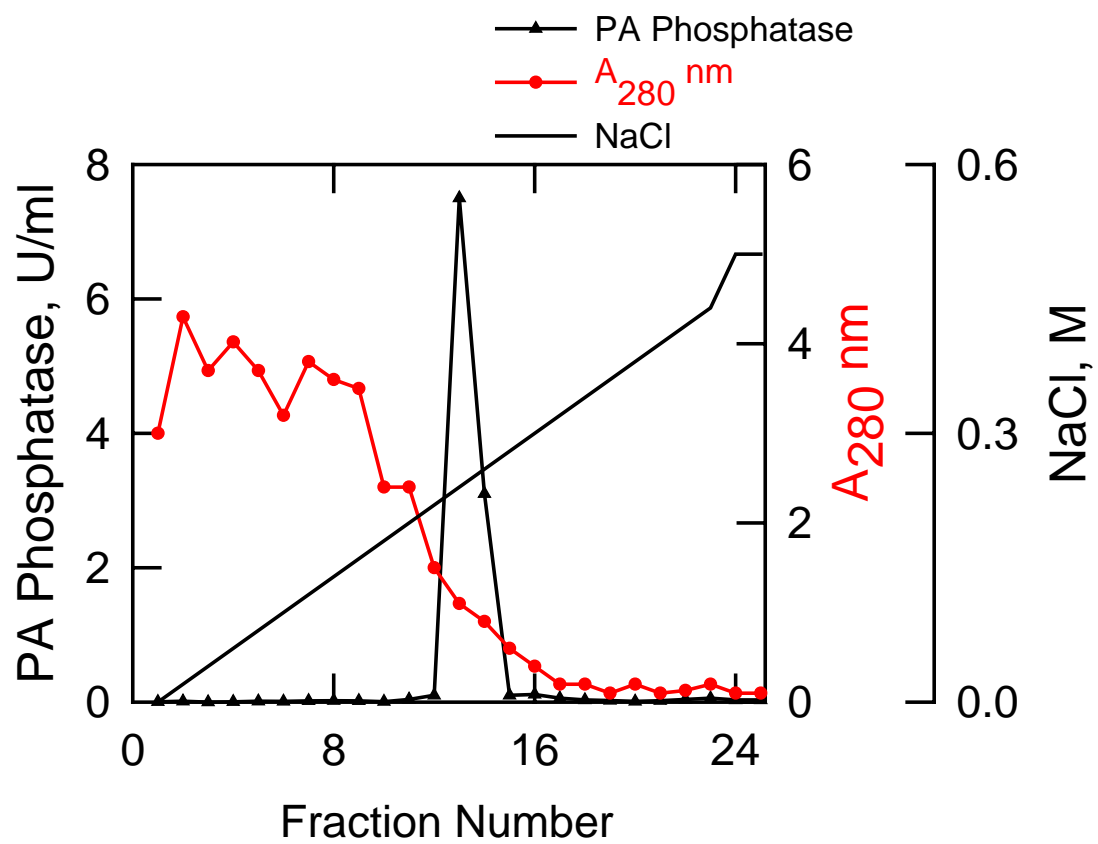
**Figure 17. Elution profile of the  $\text{Mg}^{2+}$ -dependent PA phosphatase activity after Superose 6 gel filtration chromatography.**

The  $\text{Mg}^{2+}$ -dependent PA phosphatase was subjected to Superose 6 gel filtration chromatography. Fractions containing PA phosphatase activity were collected and assayed for PA phosphatase activity. The protein concentrations were measured by absorbance at 280 nm.



**Figure 18. Elution profile of the  $Mg^{2+}$ -dependent PA phosphatase activity after Mono Q II chromatography.**

Fractions containing PA phosphatase activity were collected and assayed for PA phosphatase activity. The protein concentrations were measured by absorbance at 280 nm. The NaCl gradient profile is indicated by solid line.



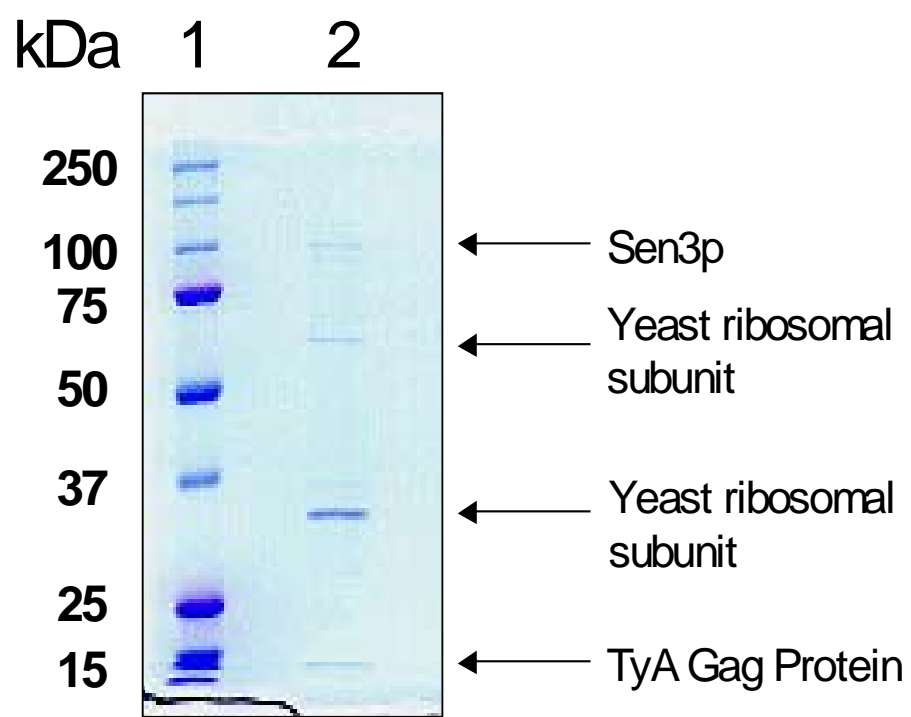
The purified enzyme was not a homogeneous protein, and several protein bands appeared in the gel stained with Coomassie blue (Fig. 19). The sequences of these proteins were determined at the Proteomics lab at UMDNJ in Newark, NJ. Unfortunately, all of these proteins have been previously identified with functions unrelated to PA phosphatase (Fig. 19). Although the PA phosphatase enzyme was not purified to homogeneity, it was utilized to characterize its enzymological properties. Since characteristics of the NEM-sensitive  $\text{Mg}^{2+}$ -dependent PA phosphatase are lacking so far, information on the enzymological properties should be important to figure out the role of the enzyme in lipid metabolism in yeast.

#### **Determination of the Native Molecular Mass of the $\text{Mg}^{2+}$ -dependent PA Phosphatase**

The native molecular mass of the  $\text{Mg}^{2+}$ -dependent PA phosphatase was determined by Superose 6 gel filtration chromatography. The column was calibrated with the marker proteins. The values of the ratio of elution volume to the void volume ( $V_e/V_o$ ) for each protein standard including the purified PA phosphatase were calculated. Analysis of data indicated a linear relationship between log of molecular mass (kDa) and  $V_e/V_o$  (Fig. 20). The value of  $V_e/V_o$  for the PA phosphatase enzyme was 1.88, which corresponded to a molecular mass of 74.3 kDa (Fig. 20).

**Figure 19. SDS-polyacrylamide gel electrophoresis of purified PA phosphatase.**

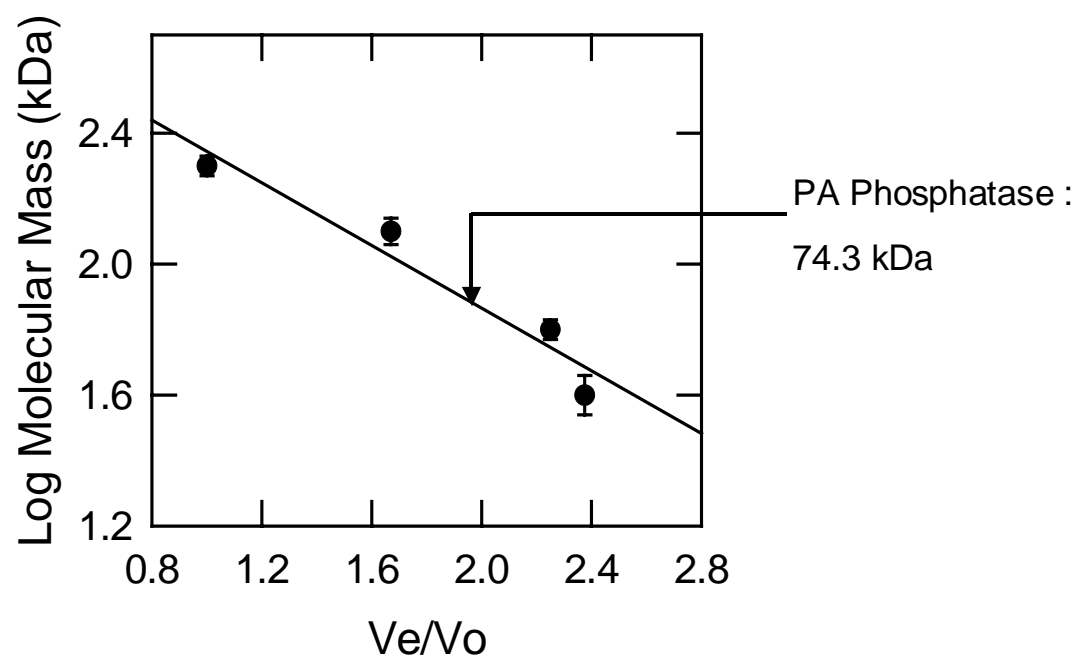
Purified enzyme was subjected to SDS-polyacrylamide gel electrophoresis as described in the text. Lane 1 shows a Coomassie blue-stained polyacrylamide gel of protein molecular mass standards: (Precision plus dual color standards, Bio-Rad). Lane 2 shows a Coomassie blue-stained polyacrylamide gel of purified PA phosphatase from the second Mono Q II chromatography. The names of identified proteins by sequencing analysis are indicated.





**Figure 20. Calibration curve for the determination of native molecular mass of the  $\text{Mg}^{2+}$ -dependent PA phosphatase**

The protein standards ( $\beta$ -amylase (200 kDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa), carbonic anhydrase (29 kDa)) were applied to Superose 6 column, and the values of ratio elution volume ( $V_e$ ) to the void volume ( $V_o$ ) were determined. The void volume was determined by the elution of blue dextran (2,000 kDa). The curve was plotted as log known molecular mass (kDa) of protein standards *versus*  $V_e/V_o$ . The data shown were determined from triplicate experiments  $\pm$  S.D.



## **Enzymological Properties of the $\text{Mg}^{2+}$ -dependent PA Phosphatase**

### ***Dependence of the $\text{Mg}^{2+}$ -dependent PA Phosphatase on pH and Magnesium***

The effect of pH on the  $\text{Mg}^{2+}$ -dependent PA phosphatase activity was examined. The enzyme had an optimum pH from 7 to 7.5 (Fig. 21). The enzyme activity was measured in the absence and presence of  $\text{MgCl}_2$ . PA phosphatase activity was absolutely dependent on  $\text{Mg}^{2+}$  ions for activity with maximum activity at a final concentration of 1 mM (Fig. 22). The magnesium ions dependence of PA phosphatase activity could not be substituted by  $\text{Mn}^{2+}$  ions (Fig. 22).

### ***Effects of Divalent Cations on the PA Phosphatase Activity***

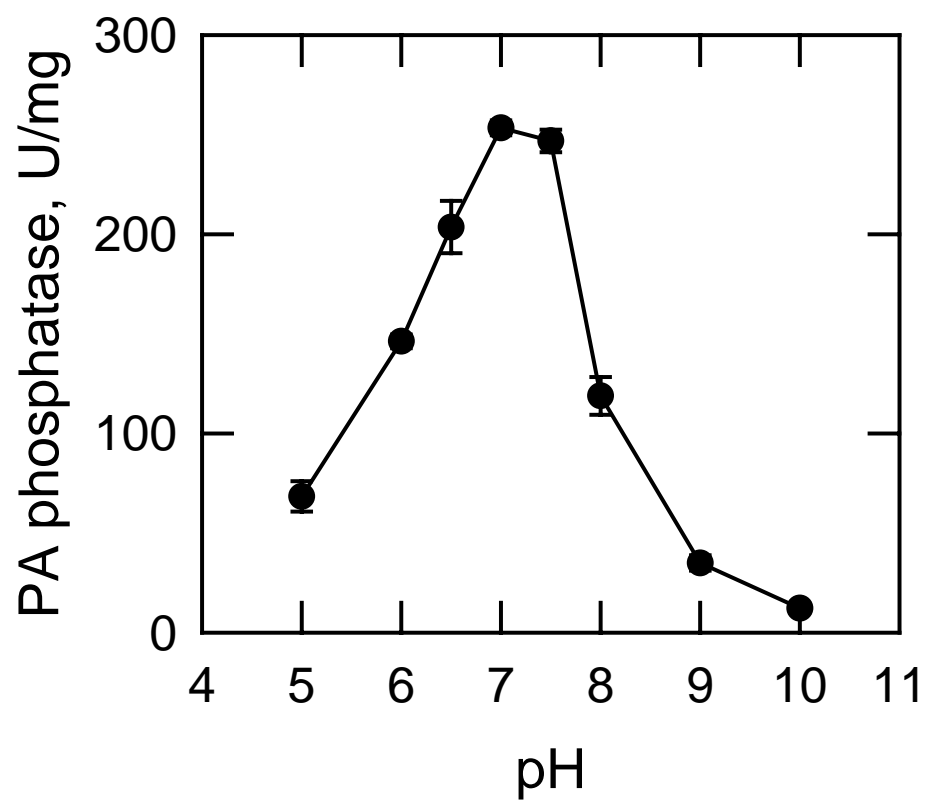
The  $\text{Mg}^{2+}$ -dependent PA phosphatase activity was measured in the presence of a variety of divalent cations (Fig. 23). The addition of  $\text{Ca}^{2+}$ ,  $\text{Zn}^{2+}$  and  $\text{Mn}^{2+}$  ions to the assay system resulted in a dose-dependent inhibition of  $\text{Mg}^{2+}$ -dependent PA phosphatase activity (Fig. 23). The  $\text{IC}_{50}$  values for  $\text{Zn}^{2+}$  (0.2 mM) and  $\text{Mn}^{2+}$  (0.5 mM) ions were calculated from a replot of the log of relative PA phosphatase activity *versus* the concentration of divalent cations.

### ***Dependence of the $\text{Mg}^{2+}$ -dependent PA Phosphatase on PA***

The effect of Triton X-100 on the  $\text{Mg}^{2+}$ -dependent PA phosphatase activity was examined (Fig. 24). The addition of Triton X-100 to the assay mixture resulted in the apparent inhibition of activity characteristic of surface dilution kinetics (Fig. 24) (154). The function of Triton X-100 in the assay for  $\text{Mg}^{2+}$ -dependent PA phosphatase as well as

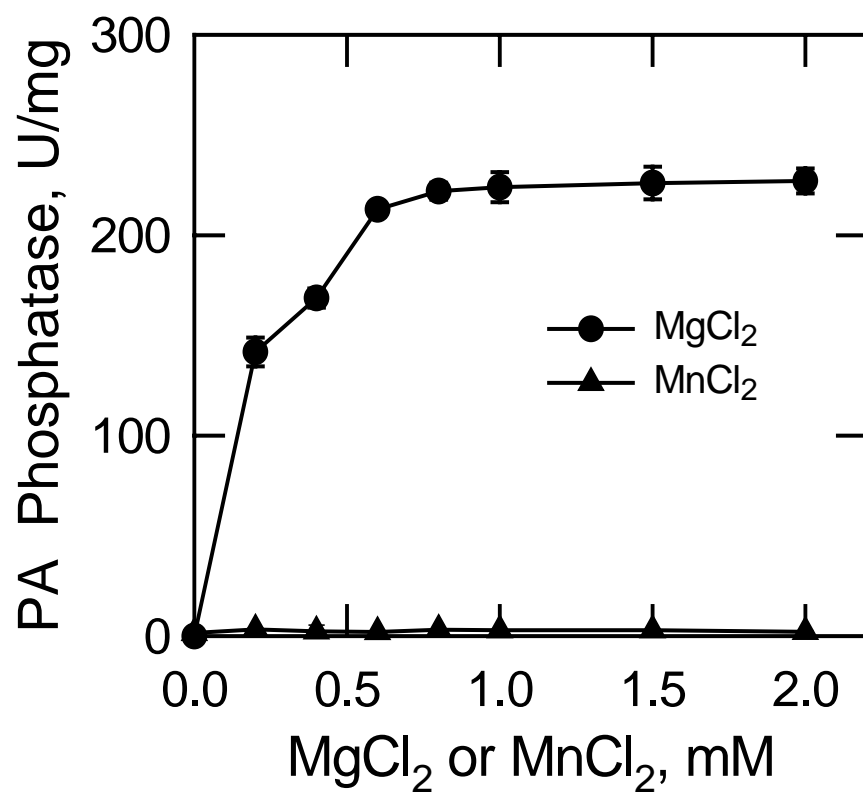
**Figure 21. Effect of pH on the  $\text{Mg}^{2+}$ -dependent PA phosphatase activity.**

$\text{Mg}^{2+}$ -dependent PA phosphatase activity was measured at the indicated pH values with 50 mM Tris-maleate-glycine buffer. The data shown were determined from triplicate enzyme determinations  $\pm$  S.D.



**Figure 22. Effects of magnesium or manganese on the  $\text{Mg}^{2+}$ -dependent PA phosphatase activity.**

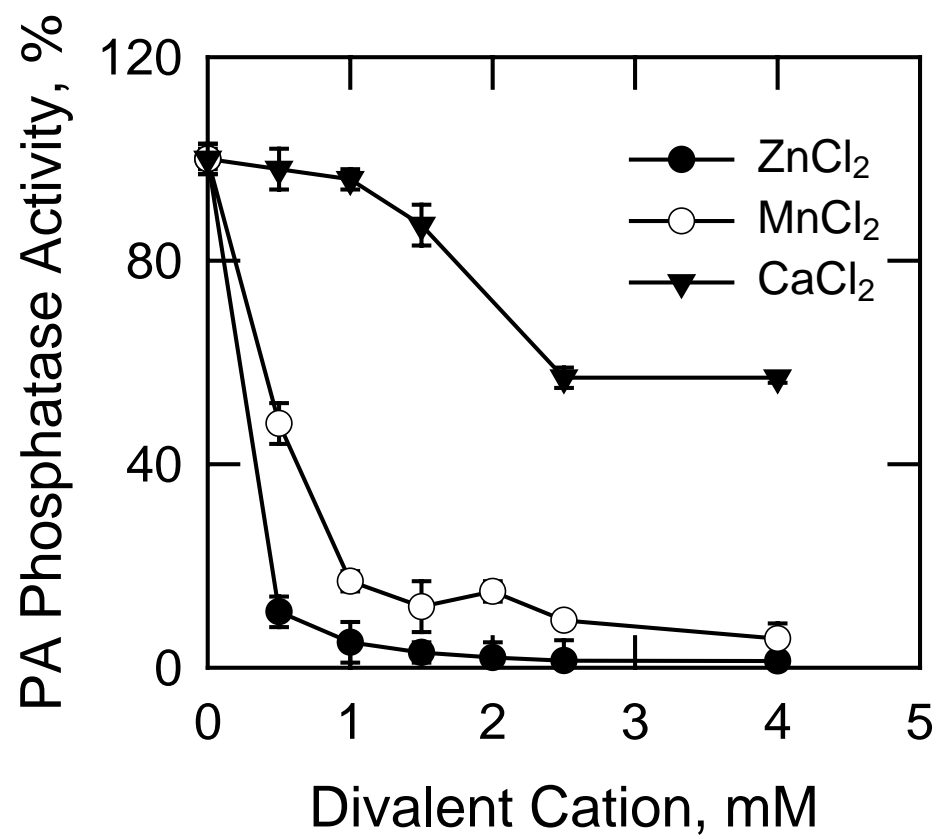
$\text{Mg}^{2+}$ -dependent PA phosphatase activity was measured at the indicated concentrations of  $\text{MgCl}_2$  or  $\text{MnCl}_2$ . The data shown were determined from triplicate enzyme determinations  $\pm$  S.D.



**Figure 23. Effects of divalent cations on the  $\text{Mg}^{2+}$ -dependent PA phosphatase activity.**

$\text{Mg}^{2+}$ -dependent PA phosphatase activity was measured at the indicated concentrations of  $\text{CaCl}_2$ ,  $\text{MnCl}_2$ , and  $\text{ZnCl}_2$ . The data shown were determined from triplicate enzyme determinations  $\pm$  S.D.





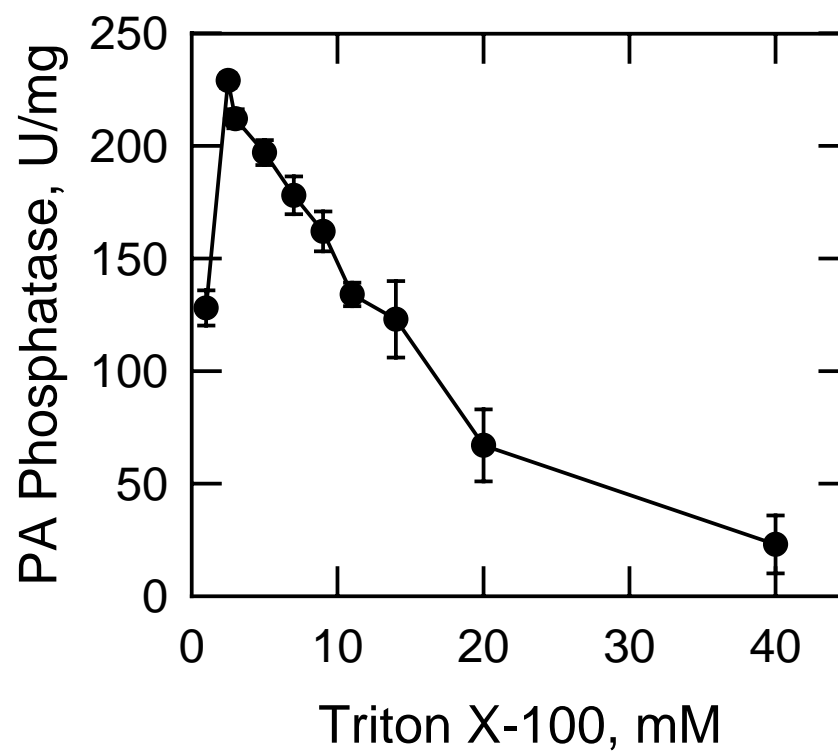
many other lipid dependent enzymes is the formation of a mixed micelle with the lipid substrate providing a surface for catalysis (55). Therefore, the concentration of PA in the mixed micelles was expressed as a surface concentration in mol % rather than molar concentration (Fig. 25). This result indicated that the  $\text{Mg}^{2+}$ -dependent PA phosphatase activity was dependent on the surface concentration of PA (at a molar PA concentration of 0.1 mM). The dependence of PA phosphatase activity on the surface concentration of PA was also examined using various set molar concentrations of PA (Fig. 25). The dependence of activity on PA surface concentration was independent of molar concentrations of PA used in these experiments (Fig. 25). The PA phosphatase activity exhibited positive cooperative kinetics with respect to the surface concentration of PA (Fig. 25). The data was analyzed according to the Hill equation using the EZ-FIT Enzyme Kinetic Model Fitting Program (153). The  $V_{\text{max}}$  was 227 U/mg, the  $K_m$  value for PA was 0.65 mol %, and the Hill number was 1.9 (Fig. 25).

#### ***Effect of Temperature on the $\text{Mg}^{2+}$ -dependent PA Phosphatase Activity***

The  $\text{Mg}^{2+}$ -dependent PA phosphatase activity was measured from 0 to 50 °C (Fig. 26A). Maximum activity was observed at 30 °C. An Arrhenius plot for the  $\text{Mg}^{2+}$ -dependent PA phosphatase was constructed (Fig. 26B) and used to calculate the activation energy for the reaction of 3.7 kcal/mol. The thermolability of PA phosphatase activity was examined in the temperature range from 30 °C to 80 °C (Fig. 27A). The  $\text{Mg}^{2+}$ -dependent PA phosphatase activity was unstable above 30 °C, and only 30 % of original enzyme activity remained at 40 °C. A  $t_{1/2}$  of 2 min for the inactivation of PA

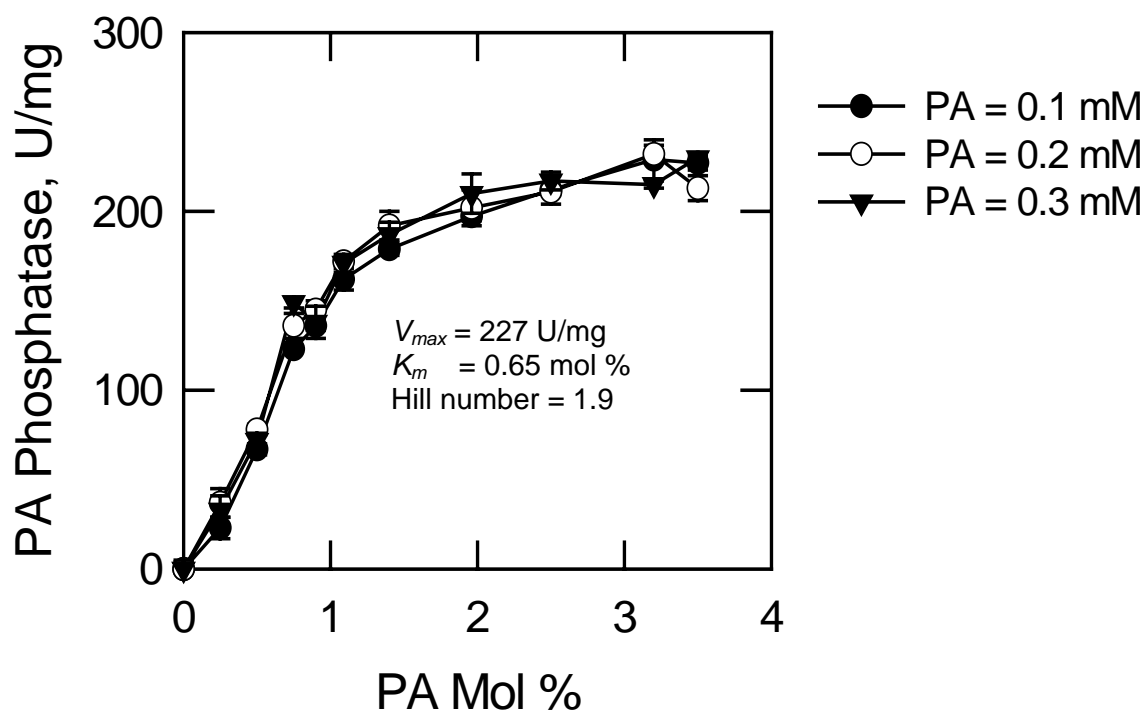
**Figure 24. Effect of Triton X-100 on the  $\text{Mg}^{2+}$ -dependent PA phosphatase activity.**

$\text{Mg}^{2+}$ -dependent PA phosphatase activity was measured in the presence of the indicated concentrations of Triton X-100. The molar concentration of PA was held constant at 0.1 mM. The data shown were determined from triplicate enzyme determinations  $\pm$  S.D



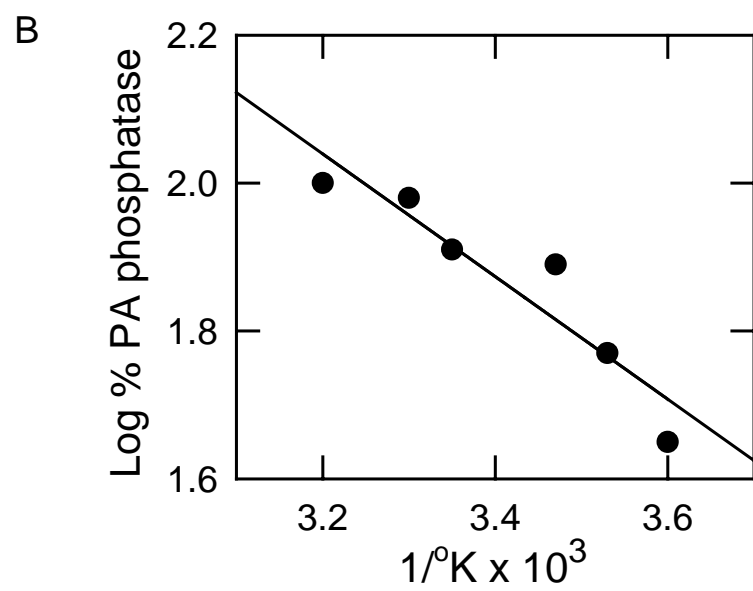
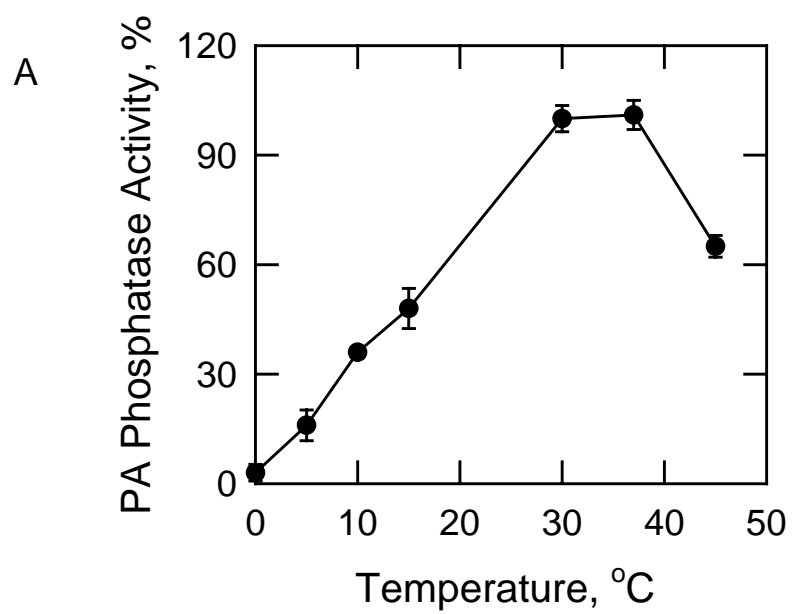
**Figure 25. Dependence of  $\text{Mg}^{2+}$ -dependent PA phosphatase activity on the surface concentration of PA.**

The  $\text{Mg}^{2+}$ -dependent PA phosphatase activity was measured as a function of the surface concentration (mol %) of PA. The molar concentration of PA was held constant at 0.1 mM, 0.2 mM and 0.3 mM while the Triton X-100 concentration was varied. Each data point represents the average of triplicate enzyme determinations from three independent experiments  $\pm$  S.D.



**Figure 26. Effect of the temperature on the  $\text{Mg}^{2+}$ -dependent PA phosphatase activity.**

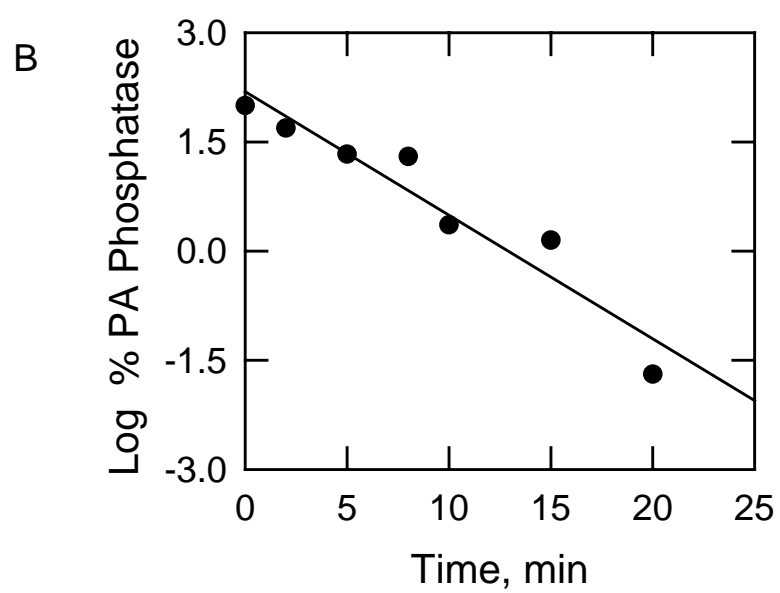
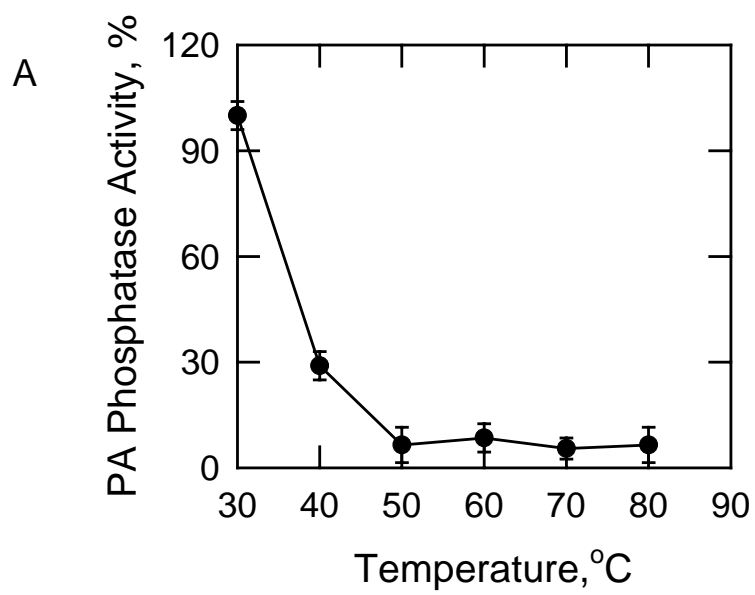
A.  $\text{Mg}^{2+}$ -dependent PA phosphatase activity was measured at the indicated temperatures under standard assay conditions in a controlled temperature water bath. B. The data in A from 0 to 40 °C were plotted as log % PA phosphatase activity *versus* the reciprocal of the absolute temperature ( $1/^{\circ}\text{K}$ ). The curves drawn were a result of a least squares analysis of the data.





**Figure 27. Thermal stability of the  $\text{Mg}^{2+}$ -dependent PA phosphatase activity.**

A.  $\text{Mg}^{2+}$ -dependent PA phosphatase was incubated for 20 min at the indicated temperatures in a controlled-temperature water bath. After incubation, the enzyme samples were cooled on ice, assay components were added, and PA phosphatase activity was measured at 30 °C. B. The enzyme was incubated at 40 °C for the indicated time intervals. After incubation, the enzyme samples were cooled on ice and PA phosphatase activity was measured at 30 °C. The curve drawn was a result of a least squares analysis of the data.



phosphatase activity was calculated from a replot of the log % activity *versus* time of incubation (Fig. 27B).

### ***Effects of NEM, Phenylglyoxal, and Propranolol on the $Mg^{2+}$ -dependent***

#### ***PA Phosphatase Activity***

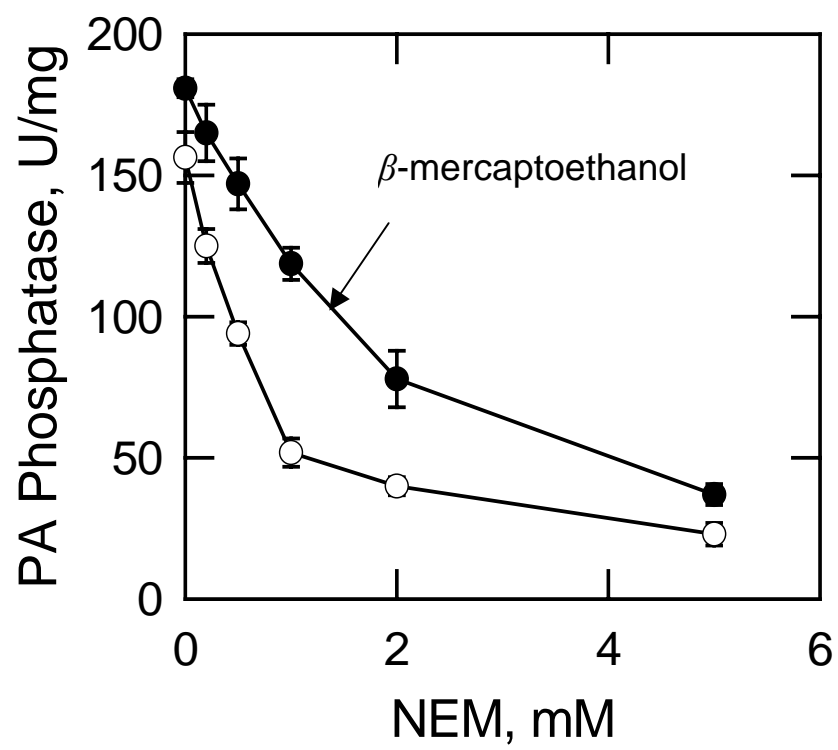
To confirm that the purified  $Mg^{2+}$ -dependent PA phosphatase is sensitive to NEM,  $Mg^{2+}$ -dependent PA phosphatase activity was measured with various concentrations of this sulfhydryl reactive reagent in the absence and presence of  $\beta$ -mercaptoethanol (Fig. 28). The enzyme was inhibited by NEM in a dose-dependent manner. The inhibitory effect of NEM was greater when the enzyme activity was measured in the absence of  $\beta$ -mercaptoethanol (Fig. 28). Phenylglyoxal and propranolol have been shown to inhibit PA phosphatase activity from animal tissues (142). Phenylglyoxal is an arginine reactive compound (155), whereas propranolol is believed to interact with the  $Mg^{2+}$  binding site (145). The effects of those inhibitors on the  $Mg^{2+}$ -dependent PA phosphatase activity were examined. Phenylglyoxal and propranolol inhibited the  $Mg^{2+}$ -dependent PA phosphatase activity in a dose-dependent manner (Figs. 29 and 30).  $IC_{50}$  values for phenylglyoxal and propranolol were calculated to be 7.5 and 0.8 mM, respectively.

### **Effectors of the $Mg^{2+}$ -dependent PA Phosphatase Activity**

The effects of various compounds on PA phosphatase activity were examined using a PA concentration (0.6 mol %) near its  $K_m$  value. In this way, the inhibitory or stimulatory effects on enzyme activity could be more readily observed.

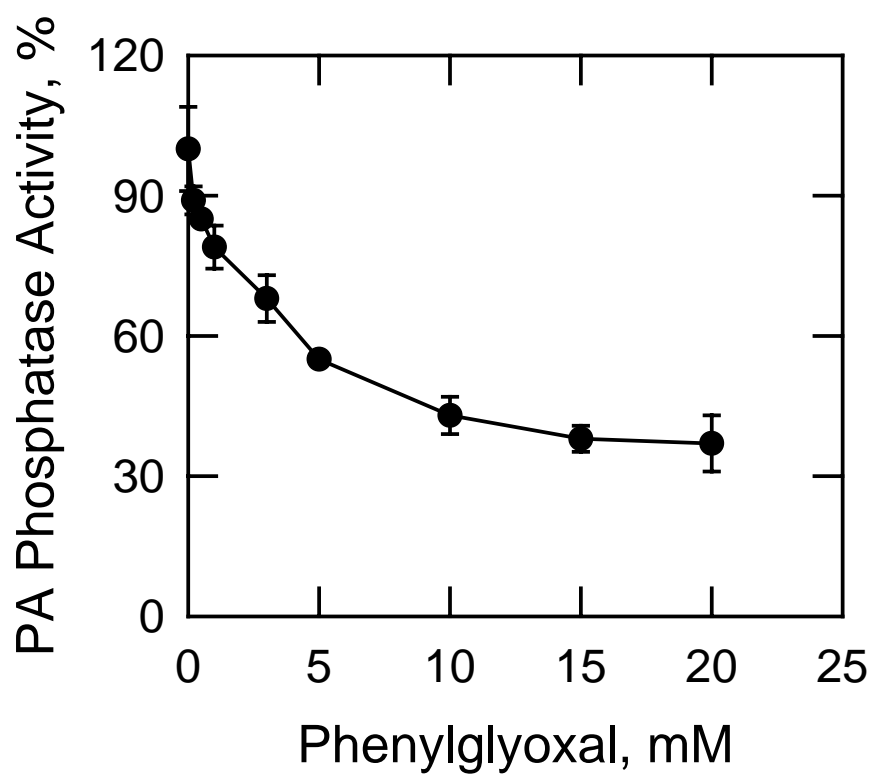
**Figure 28. Effect of NEM on the  $\text{Mg}^{2+}$ -dependent PA phosphatase activity.**

The  $\text{Mg}^{2+}$ -dependent PA phosphatase activity was measured in the absence or presence of  $\beta$ -mercaptoethanol with the indicated concentrations of NEM. The data shown were determined from triplicate enzyme determinations  $\pm$  S.D.



**Figure 29. Effect of phenylglyoxal on the  $\text{Mg}^{2+}$ -dependent PA phosphatase activity.**

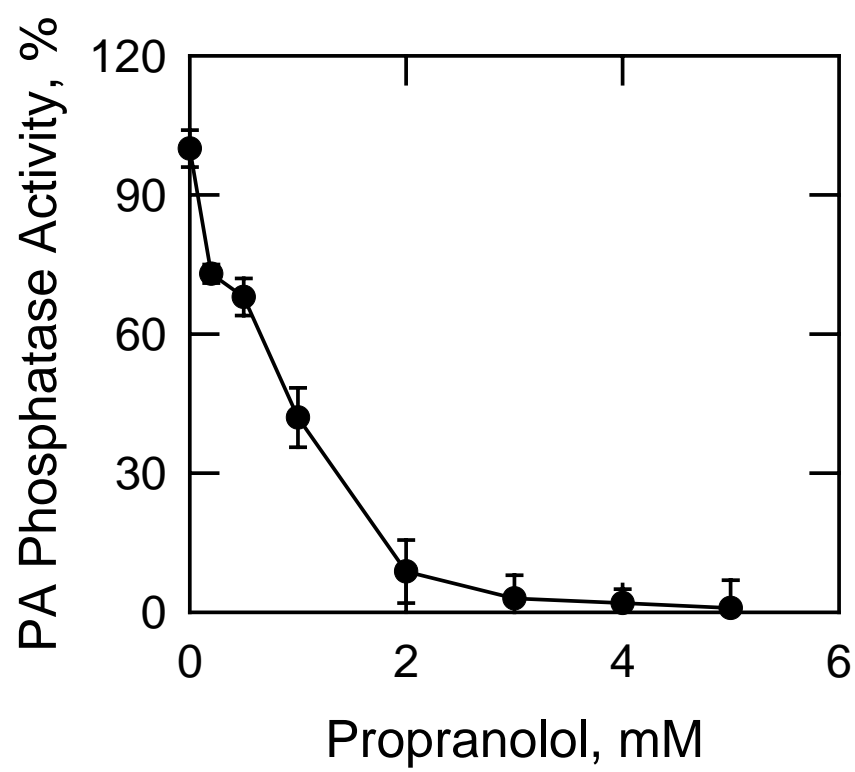
The  $\text{Mg}^{2+}$ -dependent PA phosphatase was incubated for 50 min at 30°C with the indicated concentrations of phenylglyoxal in the standard reaction mixture minus substrate. Following incubation, [ $\gamma$ - $^{32}\text{P}$ ] labeled substrate was added to the reaction mixtures and PA phosphatase activity was measured at 30°C.



**Figure 30. Effect of propranolol on the  $\text{Mg}^{2+}$ -dependent PA phosphatase activity.**

The  $\text{Mg}^{2+}$ -dependent PA phosphatase was incubated for 50 min at 30°C with the indicated concentrations of propranolol in the standard reaction mixture minus substrate. Following incubation, [ $\gamma$ - $^{32}\text{P}$ ] labeled substrate was added to the reaction mixtures and PA phosphatase activity was measured at 30°C.





### ***Effects of Nucleotides on the $Mg^{2+}$ -dependent PA Phosphatase Activity***

The substrate and product of PA phosphatase reaction are found at branch points in the pathways leading to the synthesis of phospholipids and triacylglycerols (15). Therefore, the enzyme should play a role in the proportional synthesis of these lipids (143). Nucleotides are used in reactions leading to the synthesis of phospholipids, and the substrate and product of the PA phosphatase reaction are involved in the reactions. Therefore, I questioned that nucleotides could regulate the PA phosphatase activity. To address this question, effects of nucleotides on the purified  $Mg^{2+}$ -dependent PA phosphatase activity were examined using a subsaturating concentration of PA (Fig. 31). The nucleotides ATP, UTP, CTP and TTP inhibited the  $Mg^{2+}$ -dependent PA phosphatase activity with a dose-dependent manner. TTP and UTP were the most potent inhibitors of the enzyme (Fig. 31). The  $IC_{50}$  values for TTP and UTP were 0.8 and 1.8 mM, respectively (Table IV).

### ***Effects of Sphingolipids on the $Mg^{2+}$ -dependent PA Phosphatase Activity***

To examine the regulation of the  $Mg^{2+}$ -dependent PA phosphatase activity by sphingoid bases, the activity of  $Mg^{2+}$ -dependent PA phosphatase was measured with various concentrations of sphingoid bases using a subsaturating concentration of PA (0.6 mol %). The concentrations of PA and sphingoid bases in the Triton X-100/lipid mixed micelles were expressed as surface concentrations (in mol %) as opposed to bulk concentrations (156). Sphingosine, phytosphingosine and sphingomyelin inhibited the  $Mg^{2+}$ -dependent PA phosphatase activity in dose-dependent manners (Fig. 32).

Table IV

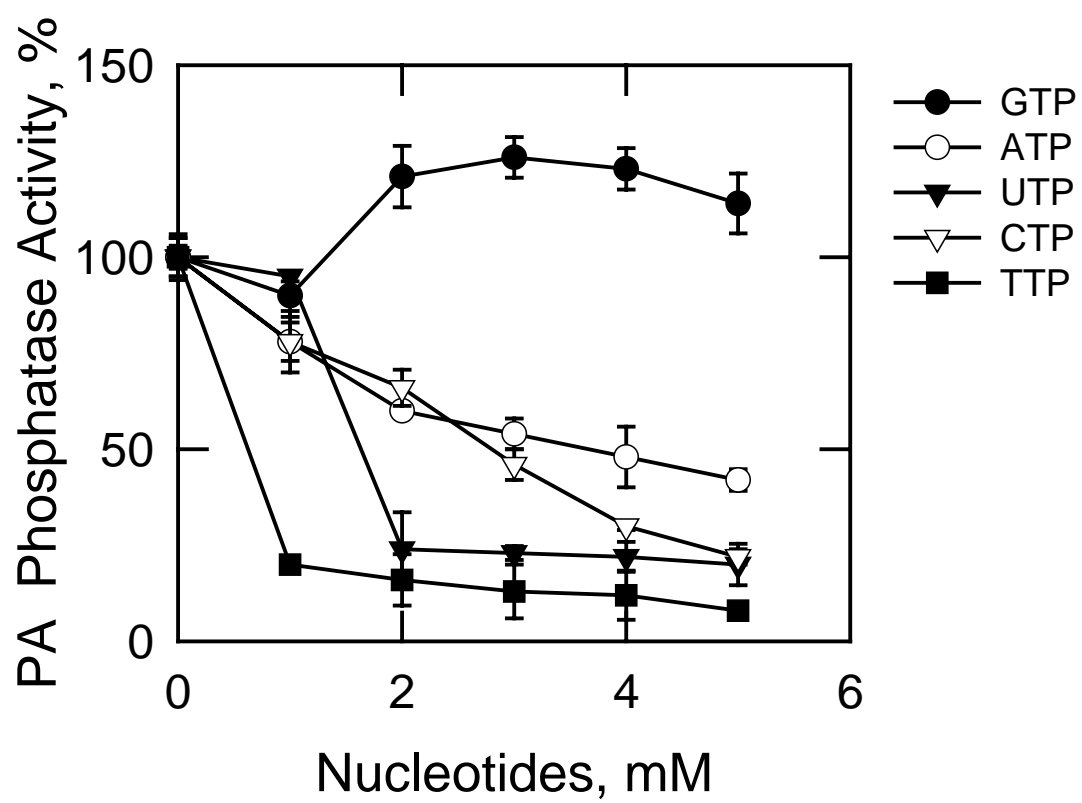
**Inhibitor Constants of Effectors**

<b>Effectors</b>	<b>IC<sub>50</sub><sup>a</sup></b>
<b>Nucleotides</b>	
ATP	3.1 mM
UTP	1.8 mM
CTP	2.9 mM
TTP	0.8 mM
<b>Sphingolipids</b>	
Sphingomyelin	3.3 mol %
Sphingosine	1.2 mol %
Phytosphingosine	4.2 mol %
<b>Phospholipids</b>	
PE	0.6 mol %
PI	4.1 mol %
CDP-DAG	0.7 mol %
PC	3.2 mol %
PS	4.0 mol %
CL	9.0 mol %
PG	11.0 mol %

<sup>a</sup>IC<sub>50</sub> values were calculated from plots of the log of the PA phosphatase activity *versus* the effector concentration.

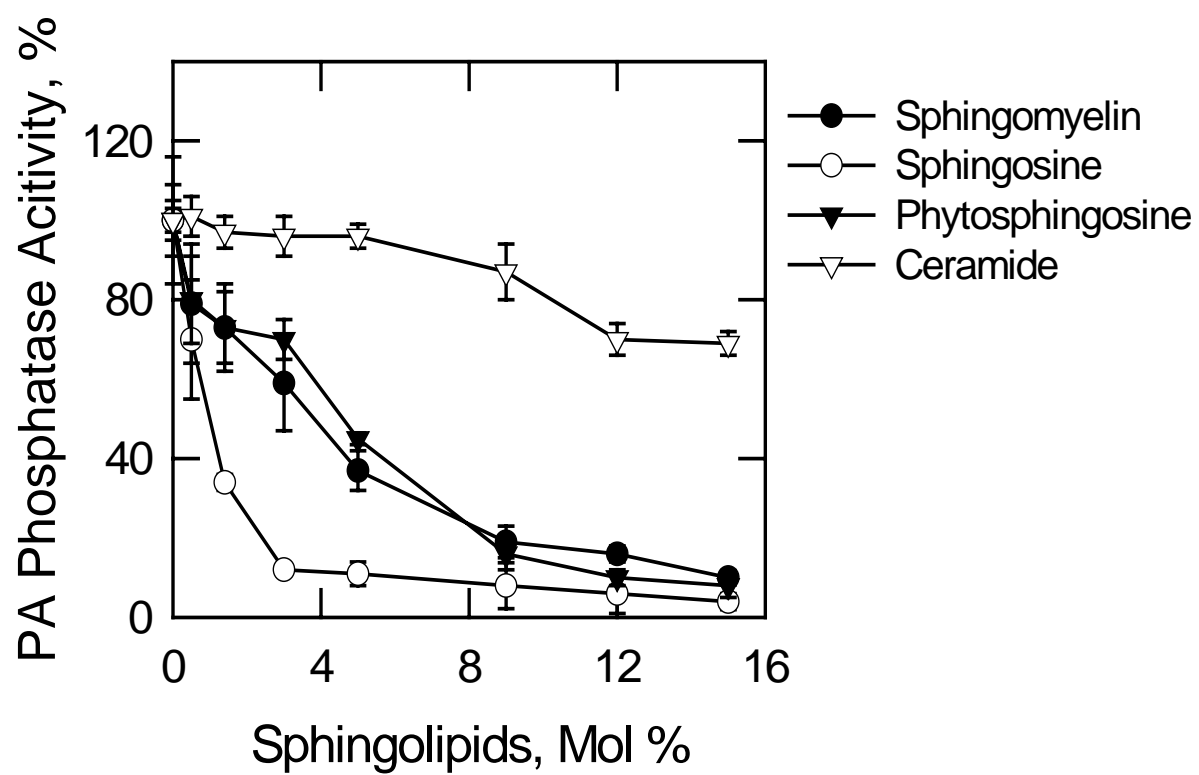
**Figure 31. Effects of nucleotides on the  $\text{Mg}^{2+}$ -dependent PA phosphatase activity.**

PA phosphatase activity was measured under standard assay conditions in the presence of the indicated concentrations of nucleotides. The surface concentration of PA was 0.6 mol% (bulk concentration of 0.1 mM). Each data point represents the average of triplicate enzyme determinations.



**Figure 32. Effects of sphingoid bases and sphingolipids on the  $\text{Mg}^{2+}$ -dependent PA phosphatase activity.**

The  $\text{Mg}^{2+}$ -dependent PA phosphatase activity was measured in the presence of the indicated surface concentrations of sphingolipids. The surface concentration of PA was 0.6 mol % (bulk concentration of 0.1 mM).



Sphingosine is the most potent inhibitor of the purified  $\text{Mg}^{2+}$ -dependent PA phosphatase (Fig. 32). The  $\text{IC}_{50}$  values for sphingosine, phytosphingosine and sphingomyelin were 1.2, 4.2 and 3.3 mol %, respectively (Table IV).

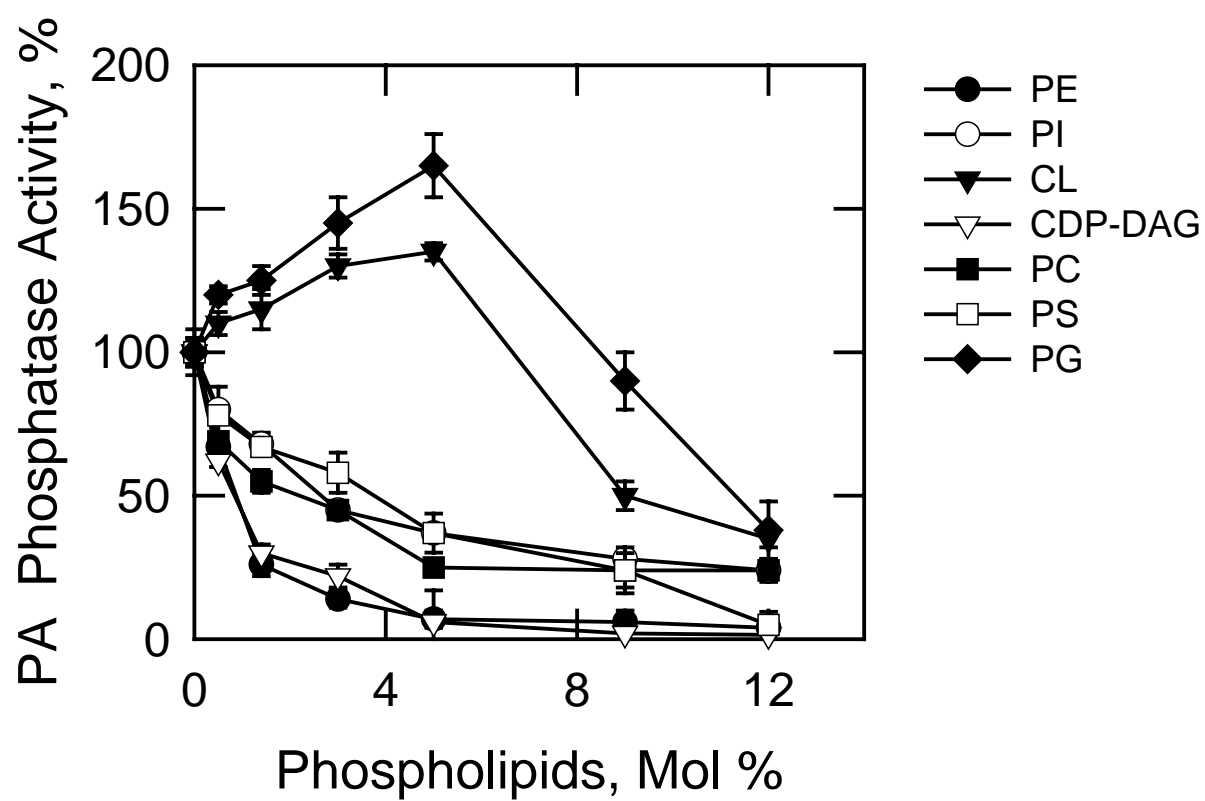
### ***Effects of Phospholipids on the $\text{Mg}^{2+}$ -dependent PA Phosphatase Activity***

Phospholipids play a role as structural components of membranes as well as activators of several membrane-associated enzymes (157). PA phosphatase has an important role in the regulation of phospholipid synthesis (15, 60). Based on this fact, I questioned whether the purified  $\text{Mg}^{2+}$ -dependent PA phosphatase activity could be regulated by phospholipids. To address this question, the  $\text{Mg}^{2+}$ -dependent PA phosphatase activity was examined with various concentrations of phospholipids using a subsaturating concentration of PA (0.6 mol %). The  $\text{Mg}^{2+}$ -dependent PA phosphatase activity was inhibited by PE, PC, CDP-DAG, PI and PS (Fig. 33). CL and PG slightly activated the  $\text{Mg}^{2+}$ -dependent PA phosphatase activity, followed by an inhibitory effect of the enzyme activity (Fig. 33). The most potent phospholipid inhibitor was PE and the most effective activator was PG (Fig. 33). The  $\text{IC}_{50}$  values for PE, PI, CDP-DAG, PC, PS, CL and PG were 0.6, 4.1, 0.7, 3.2, 4, 9 and 11 mol %, respectively (Table IV). In addition,  $\text{AC}_{50}$  (activator constant) for PG was calculated to be 3 mol %, and CL resulted in 30 % of increase in enzyme activity (Fig. 33).



**Figure 33. Effect of phospholipids on the  $\text{Mg}^{2+}$ -dependent PA phosphatase activity.**

The  $\text{Mg}^{2+}$ -dependent PA phosphatase activity was measured in the presence of the indicated surface concentrations of phospholipids. The surface concentration of PA was 0.6 mol % (bulk concentration of 0.1 mM).



## DISCUSSION

Mg<sup>2+</sup>-dependent PA phosphatase plays a role in lipid synthesis in both yeast and higher eukaryotic cells (55, 57). In yeast, at least two genes encoding Mg<sup>2+</sup>-dependent PA phosphatase are present including the *PAH1* gene (55). The *PAH1*-encoded Mg<sup>2+</sup>-dependent PA phosphatase is insensitive to NEM, and the remaining gene-encoded Mg<sup>2+</sup>-dependent PA phosphatase is sensitive to NEM (55). In this work, an attempt to purify the NEM-sensitive Mg<sup>2+</sup>-dependent PA phosphatase was carried out using a newly developed purification scheme. This included aggregation of protein, phosphocellulose, Mono Q and Superose 6 chromatography steps. The purification data showed that the enzyme was present at a very low abundance in cells. Aggregation of the protein afforded a more than 2-fold increase in specific activity over the previous step. In addition, the phosphocellulose chromatography step resulted in the greatest enrichment in specific activity over the previous step. The phosphocellulose resin has phosphate groups, which may act as an affinity resin with phosphatase enzyme. In this study, after gel filtration chromatography, the enzyme was concentrated into 1ml using Centricon® filter devices. However, the enzyme activity was reduced by 30% after being concentrated. This may be due to the instability of purified enzyme during the concentration step or to the loss of the enzyme by sticking it to the membrane filter.

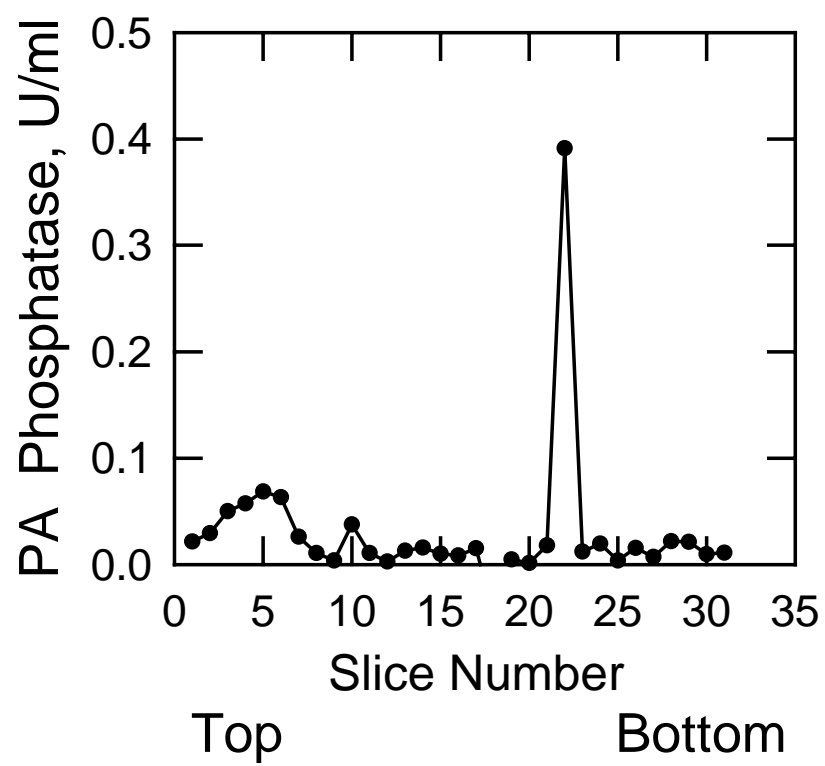
The native molecular mass of the purified protein in this study was 74.3 kDa, which is similar to the native molecular mass of the PA phosphatase enzyme purified from the cytosolic fraction by Hosaka and Yamashita (139). The purification scheme developed in this study included protein aggregation after dialysis of a salt extractable membrane fraction. This finding substantiated that the enzyme is a peripheral membrane

protein. The enzyme may be an active form when localized in the microsomal membrane and inactive when localized in the cytosol. According to Siess (158), the NEM-sensitive  $Mg^{2+}$ -dependent PA phosphatase in cytosolic fraction from rat liver is partially purified using protein precipitation with  $MgCl_2$ , extraction by KSCN, gel filtration, ion exchange and affinity chromatography. The addition of  $MgCl_2$  to dialyzed supernatant results in complete precipitation of  $Mg^{2+}$ -dependent PA phosphatase activity (158). This indicates translocation of  $Mg^{2+}$ -dependent PA from the cytosol to the microsomes (158, 159). This study also shows that  $Mg^{2+}$ -dependent PA phosphatase activity is associated with 90kDa heat shock proteins, which act as a stabilizing component to  $Mg^{2+}$ -dependent PA phosphatase (158). During the purification procedure, the separation of the stabilizing component from  $Mg^{2+}$ -dependent PA phosphatase may markedly destabilize the enzyme activity (158).

To identify the gene encoding NEM-sensitive  $Mg^{2+}$ -dependent PA phosphatase, the purified protein preparation from the Mono Q II chromatography step was subjected to SDS-PAGE as well as gel sliced assay. The purified proteins, which were separated by SDS-PAGE, were stained with Coomassie blue. As shown in Figure 19, the enzyme was not purified to homogeneity, as displayed by several bands in the gel. These proteins were identified by mass spectrometry as having known functions, but unrelated with the PA phosphatase enzyme. In addition to SDS-PAGE, the partially purified enzyme sample was also subjected to gel sliced assay using native gel. As shown in Figure 34, the PA phosphatase activity was noticeably detected at only one sliced gel fraction.

**Figure 34. Native PAGE and analysis of purified PA phosphatase activity.**

Purified enzyme from the Mono Q II chromatography step was subjected to native PAGE. Following electrophoresis, a lane containing the enzyme was cut into 0.5cm slices. Each slice was minced with a razor blade in assay buffer and homogenized at 4°C, and assayed for PA phosphatase activity.



However, the mass spectrometry analysis showed that the protein extracted from the gel slice was identified with the protein unrelated with the PA phosphatase enzyme.

Although the protein could not be purified to homogeneity, the partially purified protein was utilized to characterize the NEM-sensitive  $\text{Mg}^{2+}$ -dependent PA phosphatase enzyme. Information on the enzymological and kinetic properties of the NEM-sensitive enzyme is still lacking. Therefore, a comparison of those properties between the NEM-sensitive and NEM-insensitive enzymes is important to understanding their roles in lipid metabolism in yeast. The basic enzymological study of the *PAH1*-encoded  $\text{Mg}^{2+}$ -dependent PA phosphatase shows that optimum pH for enzyme activity is found at pH 7.5 and NEM does not affect the activity of the enzyme (55). The enzyme follows surface dilution kinetics and the analysis of kinetic data shows that a  $K_m$  value for PA is 3mol% (55). In the present study, the NEM-sensitive  $\text{Mg}^{2+}$ -dependent PA phosphatase had the optimum pH from 7 to 7.5 and the enzyme activity was inhibited by NEM in a dose-dependent manner. The enzyme also followed surface dilution kinetics and cooperative kinetics. The  $K_m$  value for PA of the NEM-sensitive  $\text{Mg}^{2+}$ -dependent PA phosphatase was 0.6mol%, which was significantly different from that of the *PAH1*-encoded NEM-sensitive  $\text{Mg}^{2+}$ -dependent PA phosphatase. These results indicate the difference in the catalytic properties of the NEM-sensitive  $\text{Mg}^{2+}$ -dependent PA phosphatase.

PA is an important molecule in the branch point between synthesis of CDP-DAG and that of DAG. CDP-DAG, which is the precursor for the synthesis of phospholipids, is synthesized by CDP-DAG synthase (Fig. 2). On the other hand, DAG, which is the substrate for the synthesis of TAG, is synthesized by PA phosphatase. The affinity of the

NEM-sensitive  $Mg^{2+}$ -dependent PA phosphatase for PA was much higher than those of the *PAHI*-encoded NEM-insensitive  $Mg^{2+}$ -dependent PA phosphatase and CDP-DAG synthase. This comparison between NEM-sensitive and NEM-insensitive enzymes led us to speculate the role of NEM-sensitive  $Mg^{2+}$ -dependent PA phosphatase in lipid metabolism. The NEM-sensitive  $Mg^{2+}$ -dependent PA phosphatase may be more intensely involved in the partition of the PA pool between the synthesis of CDP-DAG and that of DAG.

Regulation of the NEM-sensitive  $Mg^{2+}$ -dependent PA phosphatase activity by nucleotides, sphingoid bases, and phospholipids were examined. Nucleotides are substrates for enzymes that synthesize the energy-rich intermediates of phospholipid synthesis (8). Nucleotides are utilized in reactions involving the substrate and product of the PA phosphatase reaction (52). The activity of PA phosphatase purified from the membrane in yeast is inhibited by nucleotides, with ATP and CTP being the most potent inhibitors (143). ATP and CTP are the substrates for enzymes involved in the synthesis of energy-rich intermediates, such as CDP-ethanolamine or CDP-choline (15). Those intermediates are required for the synthesis of PE and PC, with DAG being the product for the PA phosphatase reaction (15). CTP is also utilized with PA for the synthesis of CDP-DAG that is a precursor for phospholipids synthesis (15). The present study showed that the synthesis of phospholipids was significantly inhibited by nucleotides, with TTP and UTP being the most potent inhibitors. The inhibition of the purified PA phosphatase activity by ATP or CTP in this study might prevent the generation of DAG, which is utilized for the synthesis of PE and PC. Additionally, the accumulation of PA due to inhibition of PA phosphatase activity by CTP may prevent the synthesis of TAG.



This might cause the synthesis of phospholipids through the CDP-DAG at the expense of TAG synthesis. UTP is a substrate for CTP synthase that is responsible for the synthesis of CTP (15). CTP is a substrate for the synthesis of intermediates required for phospholipid synthesis (15). TTP is composed of thymine that is chemically similar to uracil in RNA, by adding a methyl group onto the pyrimidine ring (1). Since both TTP and UTP have similar chemical structures and have shown to inhibit PA phosphatase activity, their mechanisms of inhibition on PA phosphatase activity may be similar. One possible mechanism of reducing the affinity of PA may be that nucleotides (triphosphates) prevent or block the phosphate group of PA from binding to their active site in the PA phosphatase enzyme. On the other hand, a previous study shows a mechanism of inhibition on the enzyme activity by ATP using a  $Mg^{2+}$  ion chelation mechanism (143). Although it is unclear what the mechanism of inhibition on PA phosphatase activity by nucleotides is, inhibitory effects on PA phosphatase activity by nucleotides in yeast may contribute to the regulation of synthesis of phospholipids in order to maintain the relative proportions of phospholipids accumulated in the membranes at the expense of TAG synthesis.

The role of sphingosine in lipid metabolism and cell signaling has been studied in mammalian cells (4, 62, 160) and has been known to regulate the PC signaling pathway since it activates phospholipase D, and inhibits both PA phosphatase and protein kinase C (4). In higher eukaryotic cells, sphingosine is produced from sphingosine-1-phosphate, which serves as a bioactive molecule in order to mediate changes in cell signaling (62), by sphingosine-1-phosphate phosphatase (62). In eukaryotic cells PAP2 enzyme is known to be involved in dephosphorylation of a variety of lipid phosphate monoesters

(PA, lyso-PA, DGPP and ceramide-1-phosphate) including sphingosine-1-phosphate. However, there is a discrepancy in true physiological substrates of PA phosphatases in mammalian cells (62). For example, the study of purified lyso-PA specific phosphatase from bovine brain cytosol shows that the enzyme does not dephosphorylate sphingosine-1-phosphate but dephosphorylates PA (62). The enzyme activity is not required for  $Mg^{2+}$ -ion and is inhibited by NEM (62). This characteristic is different from that of the PAP2 enzyme, and further studies show that the enzyme is responsible for regulation of mitochondrial lipids (62).

In this work, the inhibitory effect on the PA phosphatase activity by sphingolipids was similar to the effect of the membrane-associated PA phosphatase activity in yeast (144). The maximum inhibition on the PA phosphatase activity by sphingosine is not related in yeast because free sphingosine is not found in yeast (144). However, the study of the inhibition on the PA phosphatase activity by sphingosine in yeast could be useful for understanding the mechanism in higher eukaryotes (62). Many enzymes involved in the PC signaling pathway exist in yeast (161), therefore, even if it is not clear that a signaling mechanism by sphingosine is present, the inhibition on PA phosphatase activity by sphingosine would prevent the accumulation of DAG by the PC signaling pathway in the cell to maintain a constant level of DAG. Additionally, the inhibition on the PA phosphatase activity by sphingolipids in yeast could prevent the generation of diacylglycerol involved in the synthesis of triacylglycerols and could be involved in the proportional synthesis of phospholipids and triacylglycerols.

Phospholipids function as cofactors and activators of several membrane-associated enzymes, and PA phosphatase has an important role in the regulation of

phospholipid synthesis (157) (147). In previous studies, anionic phospholipids stimulate the activities of membrane-associated PA phosphatases, while zwitterionic phospholipids have inhibitory effects on their activities (142) (162). Phospholipids regulate the activity of several enzymes involved in lipid metabolism in yeast (163). PA phosphatase activity is regulated by biochemical and genetic mechanisms that work in a coordinated manner with the regulation of PS synthase activity (8). PS synthase is a highly regulated enzyme and it catalyzes the committed step in PC synthesis via CDP-DAG pathway (Fig. 2). Another study shows that CL activates PA phosphatase activity, whereas it inhibits PS synthase activity (164). Moreover, DAG, which is a product of PA phosphatase enzyme reactions, inhibits PS synthase activity (164). The differences in regulatory effects on the PA phosphatase activity by charges of phospholipids show that the overall charge of micelle surface contributes to the regulation of the enzyme activity (162). In this study, CL and PG (anionic phospholipids) slightly activate the purified PA phosphatase activity. Activation on the PA phosphatase activity by CL or PG could result in increased neutral charge lipids, whereas the inhibition of the PS synthase activity by CL could cause the reduction of negative charge phospholipids. Therefore, the role of activation on the PA phosphatase activity by CL might be involved in the regulatory mechanism to maintain charges of membrane in the cells.

In this work, CDP-DAG significantly inhibited the PA phosphatase activity and is the precursor for the synthesis of phospholipids from PA. PA phosphatase is responsible for the partition of PA pools between the synthesis of CDP-DAG and that of DAG (50). The inhibition of PA phosphatase activity by CDP-DAG may generate an increase in the amount of PA, and might be directly utilized for the synthesis of phospholipids at the

expense of TAG synthesis. Therefore, the inhibitory effects on the enzyme activity by phospholipids could be involved in controlling the proportional synthesis of phospholipids and triacylglycerols.

The regulation of phospholipid metabolism in yeast is complex and is governed by genetic and biochemical mechanisms (6, 15, 104, 160). PA regulates the activity of several lipid-dependent enzymes in yeast and mammalian cells (165-167). The PA phosphatase is placed on a major branch point in the phospholipids biosynthetic pathway. In summary, the partitioning of PA at this step in the pathway would influence the levels of individual phospholipid synthesis, and could alter the proportions of the phospholipid and neutral lipid (DAG and TAG) synthesis. The data for the regulation of the PA phosphatase activity in this work should support the role of the PA phosphatase in partitioning of PA between synthesis of phospholipid and that of TAG.

The regulation of PA phosphatase activity by nucleotides, sphingoid bases and phospholipids could be explained by an allosteric mechanism. Nucleotides, sphingoid bases and phospholipids could bind to a regulatory site on the enzyme other than the active site to change the shape of its active site. This conformational change may prevent PA from binding to the active site on the enzyme. It is also possible that the inhibitory effects of these compounds are synergetic or antagonistic. If those regulators separately bind to different regulatory sites of the enzyme, the inhibitory effects could be dramatically increased. On the other hand, if two or three regulators competitively bind to the same regulatory site, the inhibitory effect might be lower than that observed in the presence of those regulators.

In both yeast and higher eukaryotes, PAP1 and PAP2 enzymes have different roles in lipid metabolism (57, 60). The PAP1 enzyme has a role in lipid synthesis, while the PAP2 enzyme has a role in lipid signaling (60). Owing to the fact that PAP1 and PAP2 enzymes are structurally unrelated, PAP1 enzyme should have a different mechanism of catalysis. In the present study, the  $Mg^{2+}$ -dependent PA phosphatase activity was inhibited by propranolol, which is a compound that reacts with the  $Mg^{2+}$  binding site.  $Mg^{2+}$ -dependent enzymes require sufficient  $Mg^{2+}$  ions to activate the enzyme, and  $Mg^{2+}$  ions may bind directly to the enzyme to change its catalytic role. The significant inhibition on  $Mg^{2+}$ -dependent PA phosphatase by propranolol could suggest the presence of a  $Mg^{2+}$  binding site in the enzyme as one of the regulatory sites. In addition, the enzyme activity was inhibited by phenylglyoxal, which is an arginine amino acid reactive compound. This result indicates that the arginine amino acid residue might be involved in the catalytic activity of the enzyme although arginine has not been known to be one of the conserved amino acids in the HAD-like domain (DxDxT) that is conserved in the sequences of PAP1 enzyme catalytic motif. NEM is a sulfhydryl reactive reagent and the purified enzyme in this work was extensively inhibited by NEM, suggesting that a sulfhydryl group is important for catalytic activity. Considering the enzymological characteristics of the purified  $Mg^{2+}$ -dependent PA phosphatase observed in the present study, PAP1 enzymes would be profoundly responsible for controlling the cellular level of both PA and DAG to maintain the proportional levels of lipids between phospholipids and neutral lipids, whereas PAP2 enzymes would have a role in controlling the level of PA as well as other various lipid substrates to regulate the enzyme activity under stress conditions.

In mammalian cells, lipin proteins (lipin 1, 2 and 3) possess PAP1 enzyme activity and this enzyme activity that is NEM-sensitive (78). These proteins have the distinct tissue expression patterns, and the sequence identity of lipin 2 and lipin 3 to lipin 1 is 47 % and 46%, respectively, suggesting that these lipins might have distinct roles in lipid metabolism (78). Lipin 1, which has been shown to regulate fat metabolism in mice, shares sequence homology with the *PAH1*-encoded PA phosphatase in yeast. (55). According to the comparison of enzymological properties between the *PAH1*-encoded PA phosphatase and the purified enzyme in the present study, the NEM-sensitive  $Mg^{2+}$ -dependent PA phosphatase might have a different role of lipid metabolism in yeast. Therefore, the purified NEM-sensitive  $Mg^{2+}$ -dependent PA phosphatase in the present study could be homologue of lipin 2 or lipin 3, and the enzyme might have a specific function of lipid metabolism in yeast.

In summary, the NEM-sensitive  $Mg^{2+}$ -dependent PA phosphatase has been purified with a newly developed purification scheme from *S. cerevisiae*. The purification scheme consisted of salt extraction of membrane fraction, protein aggregation and four steps of column chromatography. The enzyme was purified with 2,250-fold increase in specific activity over the cell extract. The study of characterization of the partially purified PA phosphatase revealed that the enzyme required the  $Mg^{2+}$  ion for its activity and followed surface dilution kinetics using Triton X-100-PA mixed micelles. The  $K_m$  value for PA of the  $Mg^{2+}$ -dependent PA phosphatase was 0.6 mol %, which was notably different from that of the *PAH1*-encoded  $Mg^{2+}$ -dependent PA phosphatase. The enzyme activity was significantly inhibited by NEM, phenylglyoxal and propranolol, and was regulated by nucleotides, sphingoid bases and phospholipids. Those results indicated that

the catalytic properties of the NEM-sensitive  $\text{Mg}^{2+}$ -dependent PA phosphatase might be different from those of the *PAH1*-encoded  $\text{Mg}^{2+}$ -dependent PA phosphatase. Thus, this work established a valuable strategy for the purification of the NEM-sensitive  $\text{Mg}^{2+}$ -dependent PA phosphatase and indicated the different role of the NEM-sensitive  $\text{Mg}^{2+}$ -dependent PA phosphatase in lipid metabolism in yeast.

## FUTURE DIRECTIONS

An attempt to identify the gene(s) encoding NEM-sensitive  $Mg^{2+}$ -dependent PA phosphatase enzyme by purifying the enzyme has not been successful; however, several critical steps or conditions to purify the enzyme have been accomplished during this study. Thus, this work will provide the basic foundation to purifying the enzyme to homogeneity in the future.

In order to identify the gene encoding the enzyme, the semi-pure enzyme should be further purified based on a modification of the purification scheme developed in this study. The purification process should start with approximately 200 g (wet weight) of cells, based on the amount of protein that is required for peptide sequencing analysis by mass spectrometry. The solubilized aggregated protein, which is prepared from 200 g cells, should be divided into three batches and applied to three different phosphocellulose resins separately since the binding capacity of phosphocellulose resin for the enzyme is limited. After phosphocellulose chromatography, the purified enzymes should be combined and applied to Mono Q chromatography. In future studies, the concentration step should be removed from the purification scheme since enzyme activity is reduced due to the instability of protein after being concentrated. In this study the purified protein was shown to be heat-sensitive, therefore it is necessary to keep it cold and to purify it quickly in order to prevent the degradation of the enzyme during the purification process. Finally, the elution condition of Mono Q chromatography should be changed based on the results of this study in which the enzyme was eluted from Mono Q column using salt gradient elution from 0.1 to 1M, and the peak of activity was eluted at 0.25 or 0.3 M NaCl. For future purification, the gradient range should be narrowed down from 0.2 to



0.3 M NaCl with a fraction volume of 0.5 ml to acquire higher resolution or separation by ion exchange chromatography.

The pure enzyme should facilitate the cloning and molecular characterization of the gene encoding NEM-sensitive  $Mg^{2+}$ -dependent PA phosphatase enzyme. Furthermore, the availability of the yeast gene encoding NEM-sensitive  $Mg^{2+}$ -dependent PA phosphatase allows a combination of genetic, molecular, and biochemical approaches to achieve an understanding of the roles of PA phosphatase in lipid metabolism in yeast. In addition, the basic enzymological characteristics of the NEM-sensitive  $Mg^{2+}$ -dependent PA phosphatase enzyme in this work should help to better elucidate the mode of action of the NEM-sensitive  $Mg^{2+}$ -dependent PA phosphatase by further enzymological and biochemical studies.

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