Membrane-associated phosphatidate phosphatase (EC 3.1.3.4) was purified 9833-fold from the yeast *Saccharomyces cerevisiae*. The purification procedure included sodium cholate solubilization of total membranes followed by chromatography with DE53, Affi-Gel Blue, hydroxylapatite, Mono Q, and Superose 12. The procedure resulted in the isolation of a protein with a subunit molecular weight of 91,000 that was apparently homogeneous as evidenced by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Phosphatidate phosphatase activity was associated with the purified 91,000 subunit. The molecular weight of the native enzyme was estimated to be 93,000 by gel filtration chromatography with Superose 12. Maximum phosphatidate phosphatase activity was dependent on magnesium ions and Triton X-100 at pH 7. The *Km* value for phosphatidate was 50 µM, and the *Vmax* was 30 µmol/min/mg. The turnover number (molecular activity) for the enzyme was 2.7 × 10³ min⁻¹ at pH 7 and 30 °C. The activation energy for the reaction was 11.9 kcal/mol, and the enzyme was labile above 30 °C. Phosphatidate phosphatase activity was sensitive to thioreactive agents. Activity was inhibited by the phospholipid intermediate CDP-diacylglycerol and the neutral lipids diacylglycerol and triacylglycerol.

PA₁ is an important intermediate of lipid metabolism in the unicellular eucaryote *Saccharomyces cerevisiae*. The major phospholipids are derived from PA by the reaction sequence PA → CDP-diacylglycerol → phosphatidylethanolamine → phosphatidylinositol. Triacylglycerols are derived from PA by the reaction sequence PA → diacylglycerol → triacylglycerol (1). An auxiliary pathway exists in *S. cerevisiae* for phosphatidylethanolamine and phosphatidylinositol biosynthesis which is used by the ethanolamine- and choline-requiring mutants defective in phosphatidylinositol synthesis (2–4). These mutants synthesize phosphatidylethanolamine and phosphatidylinositol by the CDP-ethanolamine- and CDP-choline-based pathways (5, 6) by the reaction sequences PA → diacylglycerol → phosphatidylethanolamine and PA → diacylglycerol → phosphatidylcholine. PA phosphatase catalyzes the formation of the diacylglycerol needed for the above reactions (5, 7).

The addition of inositol to the growth medium of wild-type *S. cerevisiae* cells results in an increase in PA phosphatase activity (8). PA phosphatase activity also increases when wild-type cells enter the stationary phase of growth (8, 9). PA phosphatase activity is associated with the membrane and cytosolic fractions of *S. cerevisiae* (8, 9). The PA phosphatase activity associated with each of these cellular fractions is regulated by inositol (8) and the growth phase (8, 9) in a similar manner. The increase in PA phosphatase activity in response to inositol supplementation correlates with an increase in phospholipid content at the expense of triacylglycerol (8). On the other hand, the increase in PA phosphatase activity in the stationary phase of growth correlates with an increase in triacylglycerol content at the expense of phospholipid (9, 10). The regulation of PA phosphatase is likely to influence the proportional synthesis of phospholipids and triacylglycerols as well as the primary and auxiliary pathways for the synthesis of phosphatidylethanolamine and phosphatidylcholine in *S. cerevisiae*.

A purified preparation of PA phosphatase is required for defined studies on the mechanism and regulation of this important enzyme of lipid metabolism in *S. cerevisiae*. In this communication, we report on the purification of the membrane-associated PA phosphatase 9833-fold to apparent homogeneity. This is the first report of the purification of any form of PA phosphatase (cytosolic or membrane-associated) from any organism. We also report on the enzymological properties of the pure enzyme.

**EXPERIMENTAL PROCEDURES AND RESULTS**

**DISCUSSION**

PA phosphatase is an important enzyme of lipid metabolism. The regulation of this enzyme is likely to influence phospholipid and triacylglycerol biosynthesis in *S. cerevisiae* as well as in higher eucaryotic organisms (27). Membrane and cytosolic forms of PA phosphatase exist in animals, plants, and bacteria (28, 29). Unsuccessful attempts have been made to purify PA phosphatase from rat liver, rat lung, pig brain, adipose tissue, mung bean, and yeast (28–30). In this communication, we describe the purification and characterization of membrane-associated PA phosphatase from *S. cerevisiae*. This is the first report of the successful purification of PA phosphatase.

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*The abbreviations used are: PA, phosphatidate; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid.*

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* Portions of this paper (including "Experimental Procedures," "Results," Tables I and II, and Figs. 1–10) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.
phosphatase from any organism. The purification of PA phosphatase required the solubilization of the enzyme from membranes with sodium cholate followed by several classical protein purification techniques. The eight-step purification scheme reported here resulted in a PA phosphatase preparation that was apparently homogeneous as evidenced by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The enzyme was purified 9833-fold relative to the activity in the cell extract with a final specific activity of 30 μmol/min/mg. The fold purification for PA phosphatase was in the same range of other phospholipid biosynthetic enzymes that have been purified from S. cerevisiae (13, 21, 23, 26). However, the turnover number for PA phosphatase was about 5- to 20-fold higher than other yeast phospholipid biosynthetic enzymes (13, 21, 23, 26). Analysis of pure PA phosphatase by sodium dodecyl sulfate-polyacrylamide gel electrophoresis indicated an apparent subunit molecular weight of 91,000. The native molecular weight of the pure enzyme was estimated to be 93,000 by gel filtration chromatography with Superose 12 in the presence of sodium cholate. Since, the molecular weight of sodium cholate ranges from 900-1800 (31), the estimated molecular weight of PA phosphatase by gel filtration chromatography was in close agreement with the molecular weight estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. It appears that PA phosphatase exists as a monomer.

The cytosolic-associated PA phosphatase from S. cerevisiae has been partially purified 600-fold, and its basic properties have been studied (30). The molecular weight of the cytosolic form of the enzyme is 75,000 as determined by gel filtration chromatography with Sephadex G-100 (30). The basic enzymological properties (pH optimum, magnesium dependence, and Km for PA) of the pure membrane-associated PA phosphatase were similar to those of the partially pure cytosolic associated enzyme (30).

PA phosphatase activity was inhibited by CDP-diacylglycerol. CDP-diacylglycerol is the source of the phosphatidyl moiety in the primary route of synthesis of the major phospholipids in yeast (1). It might be expected that the partitioning of PA between CDP-diacylglycerol and diacylglycerol would favor CDP-diacylglycerol. Therefore, the regulation of PA phosphatase by CDP-diacylglycerol might be expected. The pure enzyme was also inhibited by diacylglycerol and triacylglycerol. The inhibition of PA phosphatase by these lipids may be evidence of regulation of triacylglycerol synthesis by feedback inhibition. Future studies from this laboratory will be directed toward gaining a better understanding of the regulation of PA phosphatase and its relationship to overall lipid metabolism in S. cerevisiae.

In animal cells, PA phosphatase is believed to play a major role in the regulation of lipid synthesis (32). A number of studies have shown that the cytosolic form of PA phosphatase represents an inactive reserve of enzyme which is activated upon its translocation to the endoplasmic reticulum (32). The translocation of the enzyme occurs in response to increases in the intracellular concentrations of fatty acids and acyl-CoA esters (32). There is no evidence for the translocation of PA phosphatase from the cytosol to membranes in yeast. The availability of antibodies to both the membrane and cytosolic forms of the enzymes would facilitate translocation studies in yeast.

In summary, we have purified and characterized membrane-associated PA phosphatase from S. cerevisiae. The availability of purified PA phosphatase will permit further studies on the mode of action and regulation of this important enzyme of lipid metabolism.

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Phosphatidate Phosphatase from S. cerevisiae

Yiping Lin and George M. Carman

EXPERIMENTAL PROCEDURES

Materials

All chemicals were reagent grade. Growth media supplied were purchased from Difco Laboratories. Hamilton syringes, needles, neutral washers, nuclease, bovine serum albumin, and molecular weight standards for gel filtration were purchased from Sigma Chemical Co. Disodium hydrogen phosphate and diisopropylidithioacetic acid were purchased from Bio-Rad Laboratories, and diisopropylidithioacetic acid was purchased from Fluka Chemicals Ltd. Inorganic phosphates were purchased from ICN Pharmaceuticals, Inc. Phosphatidylcholine was purchased from Avanti Polar Lipids, Inc. Phosphatidylserine and phosphatidylethanolamine were purchased from Avanti Polar Lipids, Inc. Phospholipase C was purchased from Calbiochem-Behring. All other chemicals were from commercial suppliers.

Methods

Growth Conditions: S. cerevisiae strain BBa 697 (10) was used as a representative wild-type strain for enzyme purification. The organism was maintained on YEPD (1% yeast extract, 2% peptone, 2% dextrose) media plates stored at -80°C as described previously (13). The organism was streaked over YEPD plates containing 100 mg/ml G418, and colonies were grown at 30°C for 2 days. Overnight cultures were grown in SHY-medium (1% yeast extract, 2% peptone, 2% dextrose, 100 mg/ml G418) at 30°C to mid-log phase, harvested and stored at -80°C as described previously.

Preparation of Substrate: [32P]-Phosphatidate (200 Ci/mmol) was synthesized enzymatically from [32P]-Diacylglycerol using S. cerevisiae phosphatidate phosphatase (5). Diacylglycerol was synthesized enzymatically from [32P]-Diacylglycerol using S. cerevisiae phosphatidate phosphatase (5). Diacylglycerol was synthesized enzymatically from [32P]-Diacylglycerol using S. cerevisiae phosphatidate phosphatase (5). Diacylglycerol was synthesized enzymatically from [32P]-Diacylglycerol using S. cerevisiae phosphatidate phosphatase (5). Diacylglycerol was synthesized enzymatically from [32P]-Diacylglycerol using S. cerevisiae phosphatidate phosphatase (5).

Enzyme assay: Phosphatidate phosphatase activity was assayed spectrophotometrically by monitoring the disappearance of [32P]-phosphatidate at 25°C. The standard reaction mixture contained 0.2 mg/ml S. cerevisiae total membrane fraction, 100 mL 100 mM NaCl, 100 mM Tris-maleate buffer (pH 7.0), 100 uM [32P]-Phosphatidate, 50 uM NADPH, and 10 uM diisopropylidithioacetic acid. The reaction was initiated by the addition of the enzyme and stopped after 1 h with 1 mL 5% TCA. The reaction was terminated by heating at 90°C for 5 min. To the mixture acetone was added to precipitate proteins. Vials were centrifuged at 10,000 g for 10 min, and the supernatant was counted for radioactivity. The concentration of [32P]-Phosphatidate was determined at 0.17 PM by equilibration buffer. The enzyme was then eluted from the column by 100 mM sodium cholate, followed by elution with buffer containing 0.15 M sodium cholate, and then eluted from the column with 0.2 M potassium phosphate buffer (pH 7.0) containing 0.15 M sodium cholate. The fraction was then applied to the assay mixture containing 0.17 PM [32P]-Phosphatidate. The enzyme activity was assayed as described previously.

Determination of Substrate: Phosphatidate phosphatase activity was determined by measuring the formation of Diacylglycerol in the reaction mixture containing [32P]-Phosphatidate and S. cerevisiae total membrane fraction. The reaction was initiated by the addition of the enzyme and stopped after 1 h with 1 mL 5% TCA. The reaction was terminated by heating at 90°C for 5 min. To the mixture acetone was added to precipitate proteins. Vials were centrifuged at 10,000 g for 10 min, and the supernatant was counted for radioactivity.

Enzyme Purification: A two-step process was used to purify S. cerevisiae phosphatidate phosphatase. Each step in the purification was performed with a protein concentration of 20 mg/ml. The enzyme was then eluted from the column by 100 mM sodium cholate, followed by elution with buffer containing 0.15 M sodium cholate, and then eluted from the column with 0.2 M potassium phosphate buffer (pH 7.0) containing 0.15 M sodium cholate. The fraction was then applied to the assay mixture containing 0.17 PM [32P]-Phosphatidate. The enzyme activity was assayed as described previously.

Identification of Enzyme: To identify the phosphatidate phosphatase activity, the purified enzyme was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and the protein bands were stained with Coomassie blue. The molecular masses of the purified enzyme were determined by scanning densitometer.

Biochemical Properties of Phosphatidate Phosphatase

Identification of Substrate: S. cerevisiae phosphatidate phosphatase activity was assayed by measuring the formation of [32P]-Diacylglycerol from [32P]-Phosphatidate in the reaction mixture containing S. cerevisiae total membrane fraction. The reaction was initiated by the addition of the enzyme and stopped after 1 h with 1 mL 5% TCA. The reaction was terminated by heating at 90°C for 5 min. To the mixture acetone was added to precipitate proteins. Vials were centrifuged at 10,000 g for 10 min, and the supernatant was counted for radioactivity.

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Effect of lipids, lipid precursors, and nucleotides on PA phosphatase activity. The effect of various phospholipids and neutral lipids on PA phosphatase activity was examined (Table I). The water-soluble phospholipids, phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, and phosphatidylinositol did not affect the activity. However, the phospholipid extract of the yeast cytoplasmic membrane showed a high activity (7.5). The enzyme was also inhibited by dithiothreitol and cysteine, respectively. The addition of 0.5 mM of the phospholipid precursors choline, ethanolamine, inositol, and sodium ATP, CTP, and GTP all had no effect on PA phosphatase. The purified enzyme was measured under standard assay conditions using 0.05 or 0.5 mM PA as substrate.

### Table I

| Purification step | Total units | Protein (mg) | Specific activity (units/mg) | Fold |
|------------------|-------------|--------------|-----------------------------|------|
| 1. Cell Extract  | 14          | 4060         | 0.0003                      | 100  |
| 2. Total Membranes | 9.9        | 1115         | 0.0009                      | 71   |
| 3. Sodium Cholate Extract | 7.5 | 333          | 0.024                       | 54   |
| 4. DE-53 | 4.6 | 49 | 0.006 | 68 |
| 5. Affi-Gel Blue | 2.4 | 68 | 0.035 | 17 |
| 6. Hydroxylapatite | 1.5 | 14 | 0.11 | 116 |
| 7. Mono Q | 0.72 | 0.26 | 12.0 | 56 |
| 8. Superose 12 | 0.59 | 0.29 | 25.5 | 993 |

Table II

Effect of lipids on PA phosphatase activity. PA phosphatase activity was assayed under standard conditions in the presence of 0.1 mM of the indicated additives using 0.05 mM PA as substrate. Lipids were added to the assay mix as a solution in a molar ratio of Triton X-100 to total lipid (PA plus lipid addition) was 6:1.

| Component | Relative activity |
|-----------|------------------|
| Control   | 100              |
| Phosphatidylcholine | 94 |
| Phosphatidylinositol | 80 |
| Phosphatidylethanolamine | 101 |
| Phosphatidylserine | 16 |
| Cardiolipin | 10 |
| Diacylglycerol | 43 |
| Phosphatidylglycerol | 47 |

### Fig. 1

Figure 1. Elution profile of PA phosphatase activity from DE-53. Fractions (1 ml) were collected and assayed for PA phosphatase activity (a) and protein (b) as described in the text. The native gradient profile is indicated by the dashed line.

### Fig. 2

Figure 2. Elution profile of PA phosphatase activity from DE-53. Fractions (1 ml) were collected and assayed for PA phosphatase activity (a) and protein (b) as described in the text. The native gradient profile is indicated by the dashed line.

### Fig. 3

Figure 3. Elution profile of PA phosphatase activity from Mono Q. Fractions (1 ml) were collected and assayed for PA phosphatase activity (a) and protein (b) as described in the text. The native gradient profile is indicated by the dashed line.

### Fig. 4

Figure 4. Elution profile of PA phosphatase activity from Superose 12. Fractions (0.5 ml) were collected and assayed for PA phosphatase activity (a) and protein (b) as described in the text. The native gradient profile is indicated by the dashed line.
**Phosphatidate Phosphatase from S. cerevisiae**

**Fig. 1. Determination of PA phosphatase molecular weight by gel filtration.** The molecular weight of PA phosphatase (PAase) was determined by gel filtration on Sepharose 6B. A Superose 12 gel filtration column (1 x 30 cm) was calibrated with the following macromolecules: bovine serum albumin (BSA), 66 kDa; carbonic anhydrase, 29 kDa; ovalbumin, 45 kDa; and trypsinogen, 24 kDa. The column was run at 4 °C with 50 mM sodium acetate buffer (pH 7.0) containing 10 mM MgCl₂, 10 mM 1,2-propanediol, 200 mM glycerol, and 2 mM dithiothreitol. Purified PA phosphatase was applied and eluted from the column under the identical conditions. The data are plotted as the log molecular weight vs. Kav. The void volume was determined using Blue Dextran 2000. A plot of the log Stokes radius vs. Kav was linear and used to determine the Stokes radius of PA phosphatase. The data (n = 202,000 to 43,149) from the elution profile were used to determine the molecular weight distribution of PA phosphatase. The curve drawn was a result of a least squares analysis of the data.

**Fig. 2. Dependence of PA phosphatase activity on the concentration of PA.** PA phosphatase activity was measured with the indicated concentrations of PA. The molar ratio of Triton X-100 to PA was maintained at 10:1. The inset shows the data plotted as 1/Pa vs. activity. The curve drawn was a result of a least squares analysis of the data.

**Fig. 3. Effect of pH, magnesium, and Triton X-100 on PA phosphatase activity.** PA phosphatase was assayed at the indicated pH values with 50 mM TRIS-HCl buffer (pH 8.0) and the indicated concentrations of MgCl₂ (A); the effect of Triton X-100 (B); and the indicated concentrations of Triton X-100 (C).

**Fig. 4. Effect of temperature on PA phosphatase activity.** PA phosphatase activity was measured at the indicated temperatures under standard assay conditions in a controlled temperature water bath. The data in panel A from 0 to 30 °C were plotted as log PA phosphatase activity (units/ml) vs. the reciprocal of the absolute temperature (K). The curve drawn was a result of a least squares analysis of the data. Parallel experiments were performed with PA phosphatase incubated at the indicated temperatures for 24 h. After incubation, the enzyme samples were cooled in ice and then assayed for activity at 20 °C.