Regulation of Phosphatidylserine Synthase from *Saccharomyces cerevisiae* by Phospholipid Precursors†

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The addition of ethanolamine or choline to inositol-containing growth medium of *Saccharomyces cerevisiae* wild-type cells resulted in a reduction of membrane-associated phosphatidylserine synthase (CDPdiacylglycerol:L-serine *O*-phosphatidyltransferase, EC 2.7.8.8) activity in cell extracts. The reduction of activity did not occur when inositol was absent from the growth medium. Under the growth conditions where a reduction of enzyme activity occurred, there was a corresponding qualitative reduction of enzyme subunit as determined by immunoblotting with antiserum raised against purified phosphatidylserine synthase. Water-soluble phospholipid precursors did not effect purified phosphatidylserine synthase activity. Phosphatidylserine synthase (activity and enzyme subunit) was not regulated by the availability of water-soluble phospholipid precursors in *S. cerevisiae* VAL2C(YEp CHO1) and the *opil* mutant. VAL2C(YEp CHO1) is a plasmid-bearing strain that overproduces phosphatidylserine synthase activity, and the *opil* mutant is an inositol biosynthesis regulatory mutant. The results of this study suggest that the regulation of phosphatidylserine synthase by the availability of phospholipid precursors occurs at the level of enzyme formation and not at the enzyme activity level. Furthermore, the regulation of phosphatidylserine synthase is coupled to inositol synthesis.

The four major phospholipids in the yeast *Saccharomyces cerevisiae* are phosphatidylethanolamine (PE), phosphatidylserine (PS), and phosphatidylinositol (PI), and phosphatidylserine (PS) (21). Biochemical and genetic evidence suggests that the synthesis of PC is coordinately regulated to the synthesis of PI through the control of inositol synthesis (22). The biological role for this regulation may be to control the net charge of the membrane (4). PS accounts for 4 to 8% of the total membrane phospholipids in *S. cerevisiae* (21) and is important to overall phospholipid metabolism (1,2,29,30). PS is the normal precursor to PE and PC (21). However, PS synthesis-deficient (*cho1*) mutants (1) synthesize PE and PC when cells are supplemented with the water-soluble phospholipid precursors ethanolamine and choline, respectively (2). The availability of the water-soluble phospholipid precursor inositol also influences phospholipid biosynthesis. When wild-type cells are supplemented with inositol, the cellular content of PS is reduced about twofold (19,27). Under the same growth condition, there is a compensatory increase in the content of PI and minor changes in the proportions of PE and PC (19,27). The effects of inositol on phospholipid biosynthesis in wild-type cells occur in the absence or presence of ethanolamine and choline (19,27).

The enzyme responsible for the biosynthesis of PS in *S. cerevisiae* is CDP-diacylglycerol:L-serine O-phosphatidyltransferase (PS synthase; EC 2.7.8.8). PS synthase catalyzes the formation of PS and CMP from CDP-diacylglycerol (CDP-DG) and serine (25) by a sequential Bi Bi reaction mechanism (3). PS synthase, the product of the *CHO1* gene (30), is an integral membrane protein that has been purified to near homogeneity (3) and has an apparent subunit molecular weight of 23,000 (3,32).

PS synthase is one of several phospholipid biosynthetic enzymes that are regulated in response to the availability of inositol and choline (10,27). However, it is not known whether the regulation of PS synthase is the result of an alteration of PS synthase synthesis or a modulation in PS synthase activity.

In this study we examined the effect of ethanolamine, choline, and inositol on the regulation of PS synthase since these phospholipid precursors have an effect on PS synthesis and phospholipid metabolism (2,19,27,29). The regulation of enzyme formation was analyzed by using antisera directed against PS synthase, whereas the regulation of enzyme activity was studied with purified enzyme.

**MATERIALS AND METHODS**

**Materials.** All chemicals were reagent grade. L-Serine, ethanolamine, choline, myo-inositol, CDP-ethanolamine, CDP-choline, nucleotides, PS, protein A-Sepharose CL-4B, Ponceau S solution, and bovine serum albumin were purchased from Sigma Chemical Co. Radiochemicals were obtained from New England Nuclear Corp., Amersham Corp., or ICN Pharmaceuticals Inc. Triton X-100 and XAD-2 hydrophobic beads were gifts from Rohm and Haas Co. Molecular weight standards and electrophoresis and immunochemical reagents were purchased from Bio-Rad Laboratories. Unmodified nitrocellulose paper (0.2 μm) was obtained from Schleicher & Schuell Co. Phosphatidic acid (8), CDP-DG (8), and [5-3H]CDP-DG (5) were prepared as previously described.

**Yeast strains and growth conditions.** Strain VAL2C(YEp CHO1) bears a chimeric plasmid containing the *CHO1* gene, which directs the amplification of PS synthase activity (30). The wild-type *ade5 MATa* strain shows normal regulation of phospholipid biosynthesis (17,27). The mutant strain with genotype *opil-1 ade5 MATa* is defective in the regulation of inositol biosynthesis (17,27). These strains were kindly provided by Susan A. Henry (Albert Einstein College of Medicine, Bronx, N.Y.). For enzyme purification, strain

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VAL2C(YEp CHO1) was grown in complete synthetic medium (27) without leucine at 28°C to the late exponential phase, harvested by centrifugation, and stored at -80°C (15). For growth studies, the wild-type ade5 strain, VAL2C(YEp CHO1), and the opil mutant were grown at 28°C in complete synthetic medium (27) containing inositol (50 μM), ethanolamine (1 mM), and choline (1 mM) where indicated. Leucine was omitted from the growth medium for VAL2C(YEp CHO1) to maintain the YEp CHO1 plasmid in this strain by selective pressure (30). Cells were grown to the late exponential phase and harvested by centrifugation (27).

Preparation of cell extracts and purification of PS synthase. Cells from all strains were disrupted with glass beads with a Mini-Bead-Beater (Biospec Products) in 50 mM Tris hydrochloride buffer (pH 7.5) containing 1 mM disodium EDTA, 0.3 M sucrose, and 10 mM 2-mercaptoethanol (27). Glass beads and unbroken Mini-Bead-Beater (Biospec Products) tegrity beads and unbroken Mini-Bead-Beater (Biospec Products) were collected 30 min with lytic enzymes and Triton X-100 and centrifugation (27). The supernatant (cell extract) was used for enzyme assays and electroblotting. PS synthase was purified to homogeneity from VAL2C(YEp CHO1) microsomes by solubilization with Triton X-100, CDP-DG-Sepharose affinity chromatography, and DE-53 chromatography to a specific activity of 3,000 to 4,000 nmol/min per mg (3).

Preparation of antiserum. Triton X-100 was removed from pure PS synthase with XAD-2 hydrophobic beads (13). The enzyme (100 μg) was mixed (1:1, vol/vol) with Freund complete adjuvant. One half of the sample was injected intradermally and the other half injected subcutaneously into a New Zealand White rabbit. The rabbit was boosted 2 and 4 weeks after the primary immunization with enzyme (100 μg) mixed with Freund incomplete adjuvant. Serum was collected 6 weeks after the primary immunization, heated for 30 min at 56°C to inactivate complement, and stored at -80°C. The titer of antiserum was determined by measuring PS synthase activity remaining after treating cell extracts solubilized with Triton X-100 (9) with antiserum and protein A (14). Preimmune serum was used as a control for nonspecific effects during enzyme assay and immunoprecipitation.

Electrophoresis and Immunoblotting. Pure PS synthase and cell extracts were subjected to polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate with 10% slab gels (28). Proteins were then transferred electrophoretically to 0.2-μm unmodified nitrocellulose paper as described by Burnette (7) with the modifications of Haid and Suissa (20). The transfer was performed for 1 h at 100 V with a Hoefer TE 52 Transphor unit. Protein transfer was verified by staining with a 0.2% Ponceau S solution in 3% trichloroacetic acid. The dye was completely removed from the proteins by a 60-min incubation in blotting buffer (24). The nitrocellulose paper was incubated overnight at 8°C with a 1:100 dilution of PS synthase antiserum (33) with nonfat dry milk as a blocking agent (24). The paper was then incubated at room temperature for 1 h with a 1:3,000 dilution of goat anti-rabbit immunoglobulin G conjugated to horseradish peroxidase followed by development with chloronaphthol and H2O2 (33).

Electroblotting of PS synthase activity was performed as previously described (32). The cell extract was dialyzed for 3 h at 8°C against 62.5 mM Tris hydrochloride buffer (pH 6.8) containing 2% sodium dodecyl sulfate, 50% glycerol, and 5% 2-mercaptoethanol. The sample was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by electrophotoblotting to nitrocellulose. After transfer, the nitrocellulose paper was soaked for 1 h in 50 mM Tris hydrochloride buffer (pH 8.0) containing 0.6 mM MnCl2, 30 mM MgCl2, 1 mM CDP-DG, 0.5% Triton X-100, 10 mM 2-mercaptoethanol, 20% glycerol, and 3% bovine serum albumin to renature the enzyme activity. The nitrocellulose paper was then cut into 0.5-cm strips and used for the assay of PS synthase activity.

Enzyme assays. PS synthase activity was measured at 30°C by following the incorporation of L-[3H]serine (10,000 cpm/nmol) into chloroform-soluble material or the release of water-soluble [5-3H]CMP from radiolabeled CDP-DG (400 cpm/nmol) as previously described (9). The assay mixture contained 50 mM Tris hydrochloride (pH 8.0)-0.6 mM MnCl2-0.5 mM L-serine-0.25 mM CDP-DG-4 mM Triton X-100 and enzyme protein in a total volume of 0.1 ml. Activity was linear with time and protein under assay conditions when measured with either labeled substrate. PI synthase activity was measured at 30°C by following the incorporation of 0.5 mM myo-[2-3H]inositol (10,000 cpm/nmol) into PI in the presence of 50 mM Tris hydrochloride (pH 8.0)-2.0 mM MnCl2-0.2 mM CDP-DG-2.4 mM Triton X-100 and enzyme protein in a total volume of 0.1 ml (9). CDP-DG synthase activity was measured at 30°C as described previously (5) by following the incorporation of 1.0 mM [5-3H]CTP (10,000 cpm/nmol) into CDP-DG in the presence of 50 mM morpholineethanesulfonic acid hydrochloride buffer (pH 6.5)-20 mM MgCl2-0.5 mM phosphatidic acid-5 mM Triton X-100 and enzyme protein in a total volume of 0.1 ml. One unit of enzymatic activity was defined as the amount of enzyme that catalyzed the formation of 1 nmol of product per min under the assay conditions described. The specific activity was defined as the units per milligram of protein.

Protein determination. Protein was determined by the method of Bradford (6) as previously described (3) with bovine serum albumin as the standard.

RESULTS

PS synthase activity from cells grown in medium supplemented with phospholipid precursors. PS synthase activity was measured from the cell extract of the ade5 strain grown in complete synthetic medium in the absence or presence of water-soluble phospholipid precursors (Table 1). The addition of inositol to the growth medium resulted in a 38% reduction of activity. The addition of inositol plus ethanolamine and inositol plus choline to the growth medium resulted in a reduction of activity of 61% and 71%, respectively. In the absence of inositol, the addition of ethanolamine and choline did not result in reduced PS synthase activity. The effects of inositol (27) and inositol plus choline (10, 27) on the level of PS synthase activity in wild-type cells were very similar to those previously reported. The reduction of activity in cells grown in the presence of inositol plus ethanolamine have not been previously reported.

The amplification of PS synthase activity in the plasmid-bearing strain VAL2C(YEp CHO1) results in increased PS synthesis and modification of the proportions of PC, PE, and PI compared with those in wild-type cells (30). Therefore, it was of interest to examine whether PS synthase was regulated in the plasmid-bearing strain. VAL2C(YEp CHO1) was grown under the same conditions as that for the wild-type ade5 strain (Table 1). The levels of activity in VAL2C(YEp CHO1) were not significantly altered in response to inositol or in combination with ethanolamine and choline. The effects of water-soluble phospholipid precursors on two related phospholipid biosynthetic enzymes, PI synthase and CDP-DG synthase, were also examined in strain

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TABLE 1. PS synthase activity in cell extracts from the ade5 strain, VAL2C(YEp CHOI), and the opil strain grown in the presence of phospholipid precursors

<table>
<thead>
<tr>
<th>Growth medium</th>
<th>Sp act of PS synthase, U/mg ± SD (%) in the following strains:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ade5 strain</td>
</tr>
<tr>
<td>Complete synthetic</td>
<td>0.41 ± 0.02 (100)</td>
</tr>
<tr>
<td>+ Ethanolamine</td>
<td>0.41 ± 0.03 (100)</td>
</tr>
<tr>
<td>+ Choline</td>
<td>0.45 ± 0.07 (109)</td>
</tr>
<tr>
<td>+ Inositol</td>
<td>0.25 ± 0.01 (62)</td>
</tr>
<tr>
<td>+ Inositol and ethanolamine</td>
<td>0.16 ± 0.03 (39)</td>
</tr>
<tr>
<td>+ Inositol and choline</td>
<td>0.12 ± 0.04 (29)</td>
</tr>
</tbody>
</table>

* Cells were grown in medium with 50 μM inositol, 1 mM ethanolamine, and 1 mM choline where indicated. The specific activities of PS synthase were calculated from triplicate determinations from a minimum of two independent growth studies.

VAL2C(YEp CHOI). PI synthase catalyzes the formation of PI from CDP-DG and inositol (31), whereas CDP-DG synthase catalyzes the formation of CDP-DG from CTP and phosphatidic acid (12). The levels of PI synthase activity were constitutive under all growth conditions (Table 2). PI synthase is also constitutive in wild-type cells in response to phospholipid precursors (16, 27). On the other hand, CDP-DG synthase activity, which is regulated in response to inositol, inositol plus ethanolamine, and inositol plus choline in wild-type cells (23), was still regulated in strain VAL2C (YEp CHOI) (Table 2).

The opil mutant excretes inositol (18) and is constitutively elevated (17) for the regulated cytoplasmic-associated enzyme inositol-1-phosphate synthase (14). The regulation of PS synthase was examined in the opil mutant because inositol biosynthesis is coordinately regulated to overall phospholipid biosynthesis (22). The levels of PS synthase activity were elevated in the mutant compared with wild-type cells and were not significantly altered in response to inositol, ethanolamine, and choline (Table 1). The opil mutant was previously shown to be constitutively elevated for PS synthase activity when cells were grown in the presence of inositol and choline (27).

PS synthase was purified from strain VAL2C(YEp CHOI) and used for the preparation of antibodies. These antibodies immunoprecipitated PS synthase activity and could be used to identify the PS synthase subunit by Western blot analysis (Fig. 1). The cell extracts of cells grown under the various growth conditions were subjected to Western blot analysis with these antibodies. Under the growth conditions where a reduction of enzyme activity occurred in the wild-type strain, there was a corresponding qualitative reduction in the expression of the enzyme subunit (Fig. 2). We did not attempt to quantitate the levels of the enzyme subunit because of the many pitfalls of quantitative Western blotting (20). The location of PS synthase from cell extracts on the Western blots was confirmed by the immunoblotting of pure PS synthase (Fig. 2) and the electrophoretic mobility of PS synthase activity (data not shown).

When the cell extracts of the plasmid-bearing strain VAL2C(YEp CHOI) and the inositol biosynthesis regulatory mutant opil were analyzed with the anti-PS synthase antibodies, the level of enzyme subunit did not change significantly (Fig. 2) and correlated with the constitutive expression of enzyme activity (Table 1).

Effect of water-soluble phospholipid precursors on purified PS synthase activity. We examined the effect of various phospholipid precursors on purified PS synthase activity (Table 3). Inositol, ethanolamine, and choline or combinations of inositol plus ethanolamine and inositol plus choline did not significantly effect enzyme activity. PS synthase-
deficient (cho1) mutants synthesize PE and PC by the CDP-ethanolamine- and CDP-choline-based pathways, respectively (26), when supplemented with ethanolamine and choline (2). The addition of CDP-ethanolamine or CDP-choline in the reaction mixture had little effect on PS synthase activity. Since the phospholipid substrate CDP-DG for PS synthase is a liponucleotide, we examined the effects of various nucleotides on purified enzyme activity. At a final concentration of 1 mM, the mono-, di-, and triphosphorylated derivatives of adenosine, cytidine, guanosine, and uridine did not significantly affect PS synthase activity. Some of the water-soluble compounds examined in this study were also shown to have no effect on activity by using a crude preparation of PS synthase from wild-type yeast (10).

DISCUSSION

When wild-type cells are grown in the presence of choline in inositol-containing medium, the activities of the membrane-associated enzymes CDP-DG synthase (23), PS synthase (10, 27), and PS decarboxylase (11) and the phospholipid N-methyltransferases (34, 35) are reduced. Under the same growth conditions, the cytoplasm-associated enzyme inositol-1-phosphate synthase is also reduced (27). Inositol-1-phosphate synthase is involved in the synthesis of inositol, the water-soluble precursor of PI (21). PI synthase activity is not regulated in response to inositol, ethanolamine, or choline (16, 27). Immunoprecipitation studies performed with antibody raised against cytoplasmic-associated inositol-1-phosphate synthase have confirmed that the enzyme subunit is reduced in cell extracts prepared from cells grown in the presence of inositol (14) and inositol plus choline (27). Western blot analysis has shown that the levels of the PI synthase subunit are constitutive in cells grown in the presence of phospholipid precursors (16).

Western blot analysis of cell extracts of wild-type cells showed that there was a reduction in the level of the PS synthase subunit under the growth conditions (i.e., in the presence of inositol, inositol plus ethanolamine, or inositol plus choline) where enzyme activity was reduced. In the absence of inositol, ethanolamine and choline did not repress PS synthase. This suggests that inositol plays a role in PS synthase regulation. Whereas the immunoblot experiments were not quantitative, the apparent reduction of the PS synthase subunit detected by this method was consistent with a reduction in enzyme activity. The immunoblot analysis does not rule out the possibility that the reduction in the enzyme subunit in wild-type cells was due to an activation of a protease which degraded PS synthase. However, this is unlikely since no apparent reduction of activity and enzyme subunit were present in the plasmid-bearing cells and the opil mutant.

VAL2C(YEp CHO1) contains a high-copy-number plasmid which directs the amplification of PS synthase activity (30). In addition, this strain shows increased PS synthesis and altered proportions of the other major phospholipids PC, PE, and PI compared with wild-type cells (30). PS synthase was not regulated in strain VAL2C(YEp CHO1), which may provide an explanation for the overall alterations in phospholipid metabolism observed in this strain (30). These results suggest that overall phospholipid biosynthesis is affected by the regulation of PS synthase. CDP-DG synthase activity, which is regulated in response to phospholipid precursors in wild-type cells (23), was still regulated in strain VAL2C(YEp CHO1). In addition, the levels of PI synthase activity in the plasmid-bearing strain were similar to that of wild-type cells (16) and were not regulated in response to the phospholipid precursors. Therefore the loss of PS synthase regulation in strain VAL2C(YEp CHO1) is likely due to the increased dosage of the enzyme directed by the high-copy-number plasmid.

Mutant strain opil, which was isolated on the basis of an inositol excretion phenotype (18), is constitutively elevated for inositol-1-phosphate synthase (17). PS synthase was also constitutively elevated in opil cells whether or not phospholipid precursors were present. These results further support the hypothesis that the OPI1 locus participates directly in the coordinate regulation between PS synthase and inositol biosynthesis (27). Consistent with the observations in the present study, opil mutant was found to be constitutively elevated for CDP-DG synthase (23) and the phospholipid N-methyltransferase activities (27). In view of the overall elevated constitutive levels of the regulated enzyme activities in the opil mutant, the OPI1 gene product is believed to be a negative regulator of phospholipid biosynthesis (27).

TABLE 3. Effect of phospholipid precursors on PS synthase activity

<table>
<thead>
<tr>
<th>Component</th>
<th>Relative activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100</td>
</tr>
<tr>
<td>+ 1 mM inositol</td>
<td>101</td>
</tr>
<tr>
<td>+ 10 mM inositol</td>
<td>106</td>
</tr>
<tr>
<td>+ 1 mM ethanolamine</td>
<td>94</td>
</tr>
<tr>
<td>+ 10 mM ethanolamine</td>
<td>98</td>
</tr>
<tr>
<td>+ 1 mM choline</td>
<td>98</td>
</tr>
<tr>
<td>+ 10 mM choline</td>
<td>98</td>
</tr>
<tr>
<td>+ 1 mM inositol and 1 mM ethanolamine</td>
<td>114</td>
</tr>
<tr>
<td>+ 1 mM inositol and 1 mM choline</td>
<td>89</td>
</tr>
<tr>
<td>+ 0.1 mM CDP-ethanolamine</td>
<td>97</td>
</tr>
<tr>
<td>+ 1 mM CDP-ethanolamine</td>
<td>108</td>
</tr>
<tr>
<td>+ 0.1 mM CDP-choline</td>
<td>97</td>
</tr>
<tr>
<td>+ 1 mM CDP-choline</td>
<td>94</td>
</tr>
</tbody>
</table>

* PS synthase (0.14 U) was assayed under standard conditions in the presence of the indicated additions. Activity was measured by following the incorporation of radioactive serine into PS as described in the text.
The results of experiments performed with purified PS synthase indicated that the regulation of PS synthase by water-soluble phospholipid precursors does not occur as a direct result of enzyme activity modulation. It is possible, however, that changes in the phospholipid environment of the membrane due to the availability of phospholipid precursors (19, 27) could modulate phospholipid biosynthetic enzyme activities. We have developed a method to reconstitute purified PS synthase into phospholipid vesicles and are studying the effects of phospholipids on activity.

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LITERATURE CITED