Regulation of Yeast Phosphatidylserine Synthase and Phosphatidylinositol Synthase Activities by Phospholipids in Triton X-100/Phospholipid Mixed Micelles*

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The regulation of purified yeast membrane-associated phosphatidylserine synthase (CDP-diacylglycerol:serine O-phosphatidyltransferase, EC 2.7.8.8) and phosphatidylinositol synthase (CDP-diacylglycerol:myo-inositol 3-phosphatidyltransferase, EC 2.7.8.11) activities by phospholipids was examined using Triton X-100/phospholipid mixed micelles. Phosphatidate, phosphatidylethanolamine, and phosphatidylinositol stimulated phosphatidylserine synthase activity, whereas cardiolipin and the neutral lipid diacylglycerol inhibited enzyme activity. Phosphatidate was a potent activator of phosphatidylserine synthase activity with an apparent activation constant (0.033 mol %) 88-fold lower than the apparent K_m (2.9 mol %) for the surface concentration of CDP-diacylglycerol. Phosphatidate caused an increase in the apparent V_max and a decrease in the apparent K_m for the enzyme with respect to the surface concentration of CDP-diacylglycerol. Phosphatidylethanolamine and phosphatidylinositol caused an increase in the apparent V_max for phosphatidylserine synthase with respect to CDP-diacylglycerol with apparent activation constants of 3.4 and 3.2 mol %, respectively. Cardiolipin and diacylglycerol were competitive inhibitors of phosphatidylserine synthase activity with respect to CDP-diacylglycerol. The apparent K_i value for cardiolipin (0.7 mol %) was 4-fold lower than the apparent K_m for CDP-diacylglycerol, whereas the apparent K_i for diacylglycerol (7 mol %) was 2.4-fold higher than the apparent K_m for CDP-diacylglycerol. Phosphatidylethanolamine and phosphatidylylglycerol did not affect phosphatidylserine synthase activity. Phosphatidylinositol synthase activity was not significantly affected by lipids. The role of lipid activators and inhibitors on phosphatidylserine synthase activity is discussed in relation to overall lipid metabolism.

The primary and auxiliary pathways for the biosynthesis of the major phospholipids found in Saccharomyces cerevisiae are shown in Fig. 1 (1). The major phospholipid PC' is derived from PA via CDP-DG in the primary pathway, whereas PC is derived from PA via DG in the auxiliary pathway. The auxiliary pathway is used by the ethanolamine/choline-requiring mutants defective in PS synthase (2–5) and by wild-type cells when the primary pathway is repressed by inositol and other water-soluble phospholipid precursors (1). Results of biochemical and genetic studies have indicated that PC biosynthesis is coordinately regulated to PI biosynthesis (1). In the primary pathway PC biosynthesis begins with the synthesis of PS which is derived from CDP-DG. PI is also derived from CDP-DG. PS synthase (6) and PI synthase (7) catalyze the synthesis of PS and PI, respectively. Since PS synthase and PI synthase compete for a common substrate, the regulation of PS synthase and PI synthase is a logical point for the overall regulation of PC and PI biosynthesis. The partitioning of CDP-DG between PS and PI is governed by the availability of the water-soluble phospholipid precursor inositol and by cAMP-dependent protein kinase (1). Inositol supplementation to wild-type cells represses PS synthase mRNA, protein, and activity levels (8–10). In addition, inositol has a direct affect on PS synthase activity as a noncompetitive inhibitor (11). PS synthase activity is also down-regulated by phosphorylation via cAMP-dependent protein kinase (12). On the other hand, the expression of PI synthase is not affected by inositol (8, 13) nor is PI synthase activity regulated by cAMP-dependent protein kinase phosphorylation (14). The down-regulation of PS synthase by inositol and phosphorylation results in an increase in PI synthase at the expense of PS synthase (8, 11, 14, 15).

Being membrane-associated proteins, PS synthase and PI synthase have a distinct relationship with their neighboring phospholipids as precursors and products in the phospholipid biosynthetic pathway. Therefore it is possible that phospholipids may also play a role in the regulation of PS and PI synthesis. In previous studies, we reconstituted purified PS synthase (16) and PI synthase (13) into unilamellar phospholipid vesicles. We showed that the phospholipid composition of vesicles could effect the reconstituted activities of both enzymes (13, 16). However, in these experiments it is difficult to interpret whether changes in the activities of PS synthase and PI synthase are due to the physical process of reconstitution or a direct effect of a phospholipid on enzyme activity. Furthermore, it is experimentally difficult to systematically vary the concentration of CDP-DG and other phospholipids in vesicles for defined kinetic experiments. Kinetic analyses

The abbreviations used are: PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; CDP-DG, CDP-diacylglycerol; PA, phosphatidate; DG, diacylglycerol.
of enzymes using phospholipid substrates, activators, and inhibitors are best performed using detergent/phospholipid mixed micelle systems (17-21). Using Triton X-100/phospholipid mixed micelles as an experimental system, we examined the effect of phospholipids on pure PS synthase and pure PI synthase activities. We found that PA, PC, and PI were activators of PS synthase activity while cardiolipin and the lipid DG were inhibitors of PS synthase activity. In general, lipids had a small effect on PI synthase activity. The role of these lipid activators and inhibitors on PS synthase activity is discussed in relation to overall lipid metabolism.

EXPERIMENTAL PROCEDURES

Materials

All chemicals were reagent grade. L-serine, myo-inositol, PC (egg), PE (egg), PI (soybean), PS (bovine brain), phosphatidylglycerol (egg), cardiolipin (bovine heart), and bovine serum albumin were purchased from Sigma. PA (dioleoyl) and DG (dioleoyl) were from Avanti Polar Lipids. Radiochemicals were purchased from Du Pont-New England Nuclear. Scintillation counting supplies were purchased from Nuclear Methods.

Preparation of Enzymes—PS synthase (23) and PI synthase (24) were purified to near homogeneity as described previously. The specific activities of purified PS synthase and PT synthase were 4.0 and 3.3 nmol/min/mg, respectively.

Enzyme Assays—PS synthase (CDP-diacylglycerol:L-serine O-phosphatidyltransferase, EC 2.7.8.1) and PI synthase (CDP-diacylglycerol:myo-inositol 3-phosphatidyltransferase, EC 2.7.8.11) activities were measured by following the incorporation of their respective water-soluble radiolabeled substrates into their respective chloroform soluble radiolabeled products at 30 °C (25). The reaction mixture for PS synthase contained 50 mM Tris-HCl buffer (pH 8.0), 1 mM MnCl₂, 1.0 mM [3-²H]serine (10,000-30,000 cpm/nmol), 7 ng of PS synthase, and the indicated concentrations of Triton X-100 and CDP-DG in a total volume of 0.1 ml. The reaction mixture for PI synthase contained 50 mM Tris-HCl (pH 8.0), 2 mM MnCl₂, 1.0 mM [2-³H]inositol (10,000-30,000 cpm/nmol), 5 ng of PI synthase, and the indicated concentrations of Triton X-100 and CDP-DG in a total volume of 0.1 ml. All assays were conducted in triplicate with an average standard deviation of ±2%. All reactions were linear with time and protein concentration. 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 above. The specific activity was defined as units/mg of protein. Protein concentration was determined by the method of Bradford (26) using bovine serum albumin as the standard.

Analysis of Mixed Micelles—The total phospholipid concentration in the mixed micelles was less than 15 mol %. It is known that mixed micelles containing up to 15 mol % phospholipid are similar to the structure of pure Triton X-100 micelles but are slightly larger in size (27, 28). The homogeneity of Triton X-100 micelles containing CDP-DG with and without additional phospholipids was examined by gel filtration chromatography using Sephacryl S-200 as previously described (29, 30). As previously shown using Sepharose 6B chromatography (29), CDP-DG (10 mol %) co-eluted from a Sephacryl S-200 column with Triton X-100 micelles. Identical elution profiles were observed for Triton X-100 micelles containing CDP-DG (10 mol %) plus PA (5 mol %) and CDP-DG (2 mol %) plus PC (10 mol %). This indicated that these phospholipids were uniformly dispersed in mixed micelle structures with Triton X-100 (21). Previous studies have shown that Triton X-100 micelles containing PS (8 mol %) plus DG (2 mol %) are homogeneous in size (32). We have made the assumption that Triton X-100 micelles containing CDP-DG plus the other phospholipids used in this study were homogeneous in size.

RESULTS

Activities of PS Synthase and PI Synthase toward the Surface Concentration of CDP-DG in Triton X-100/CDP-DG Mixed Micelles—The nonionic detergent Triton X-100 is required for optimum in vitro activities of PS synthase (23) and PI synthase (24) from S. cerevisiae. The function of Triton X-100 in the assay systems for these enzymes is to form a mixed micelle with the lipid substrate CDP-DG (23, 24). The Triton X-100 micelle serves as a catalytically inert matrix in which CDP-DG is dispersed preventing high local concentration of substrate at the active site (29) and may provide a surface resembling the physiological surface of the membrane (18). As previously shown, PS synthase (23) and PI synthase (24) followed "surface dilution" kinetics (33) as their activities were dependent on the surface concentration of CDP-DG (Fig. 2). In addition, both enzymes exhibited typical saturation kinetics with respect to the surface concentration of CDP-DG (Fig. 2). In these kinetic experiments, the surface concentration of CDP-DG was varied at only one concentration of serine and inositol for the PS synthase and PI synthase reactions, respectively. The serine and inositol concentrations for the respective enzyme reactions have an effect on the Kₘ (concentration resulting in half-maximum activity) for CDP-DG (11). Therefore, the Kₘ values reported in kinetic experiments are apparent values obtained under standard assay conditions. The apparent Kₘ values for PS synthase and PI synthase were 2.9 and 3.1 mol %, respectively, for the surface concentration of CDP-DG. Hill plots were constructed for the data in Fig. 2 and in subsequent figures (Figs. 4 and 5) that resulted in Hill numbers close to 1. Gel filtration analysis of Triton X-100/CDP-DG mixed micelles containing phospholipids showed that the micelles were homogeneous in size. Therefore the mixed micelle system provided a homogeneous, physically defined system to investigate the effects of phospholipids on PS synthase and PI synthase activities.

FIG. 1. Phospholipid biosynthetic pathways in S. cerevisiae.

FIG. 2. Dependence of PS synthase and PI synthase activities on the surface concentration of CDP-DG in Triton X-100/CDP-DG mixed micelles. The bulk concentration of CDP-DG was held constant at 0.1 mM while the Triton X-100 concentration was varied. PS synthase (A) and PI synthase (B) activities were measured as a function of the surface concentration of CDP-DG expressed as the mol % of CDP-DG in the micelles. The data are plotted as 1/V (nmol/min/ml) versus the reciprocal of the CDP-DG concentration. The curves drawn were a result of a least squares analysis of the data.
Effect of Phospholipids on PS Synthase and PI Synthase Activities—Our rationale for choosing the phospholipids examined in this study was based on the precursor and product relationships in the yeast phospholipid biosynthetic pathway (Fig. 1). We initially examined the effect of phospholipids on PS synthase and PI synthase activities with a CDP-DG concentration (2.5 mol %) near the apparent $K_m$ value for these enzymes. In this way we could simultaneously screen lipids that were stimulatory or inhibitory to activity. The effect of various phospholipids on PS synthase activity is shown in Fig. 3A. The addition of PA to the assay system for PS synthase resulted in a dose-dependent stimulation of activity. Maximum stimulation (5.5-fold) was obtained with 2.4 mol % PA. PS synthase was also stimulated in a dose-dependent manner by PC and PI. At 11 mol %, PC and PI stimulated PS synthase activity 2.5-fold. The addition of 0.2% PA plus 5% PC or 0.2% PA plus 5% PI to the assay system resulted in a partially additive stimulation of PS synthase activity. Cardiolipin and DG inhibited PS synthase activity in a dose-dependent manner (Fig. 3A). At 11 mol %, cardiolipin and DG inhibited PS synthase activity 3.5 and 2.2-fold, respectively. The addition of 5% cardiolipin or 5% DG to the assay system reduced the stimulatory effect of 0.2% PA on PS synthase activity. PE and phosphatidylglycerol did not significantly affect PS synthase activity (Fig. 3A). PS stimulates the incorporation of labeled serine into PS by an exchange reaction catalyzed by PS synthase (23). However, PS does not stimulate PS synthase activity as determined by measuring the release of CMP from CDP-DG (23).

The effect of various phospholipids on PI synthase activity is shown in Fig. 3B. In general, the addition of phospholipids to the assay system for PI synthase resulted in a slight stimulation (1.1- to 1.4-fold) of activity. DG inhibited PI synthase activity in a dose-dependent manner with only a 1.28-fold inhibition at 11 mol %. PI stimulates the incorporation of labeled inositol into PI by an exchange reaction catalyzed by PI synthase (13). However, PI does not stimulate PI synthase activity as determined by measuring the release of CMP from CDP-DG (13). The stimulatory and inhibitory effects of lipids on PI synthase activity were less than 1.5-fold and were therefore not considered significant. Accordingly, the effect of phospholipids on PI synthase activity were not pursued further.

Effect of Phospholipid Activators on the Kinetics of PS Synthase Activity—The kinetics of PS synthase to varying concentrations of CDP-DG was examined in the presence of various concentrations of PA (Fig. 4A). The enzyme exhibited saturation kinetics with respect to the surface concentration of CDP-DG in the absence and presence of PA. The addition of PA to the assay system resulted in an increase in the apparent $V_{max}$ and a decrease in the apparent $K_m$. The data were analyzed analogous to a bisubstrate reaction (34). A
replot of the $1/V$ intercepts versus $1/PA$ concentration is shown in Fig. 4A (inset). The $1/PA$ intercept of the replot was used to calculate the PA concentration (0.033 mol %) that resulted in half-maximum activation ($K_a$) of PS synthase activity. The $1/V$ intercept of the replot was used to calculate an apparent $V_{max}$ (6.5 $\mu$mol/min/mg) for PS synthase in the presence of PA. A similar kinetic analysis was used for the activation of PS synthase activity by PC and PI (see below). Although a detailed kinetic analysis was not conducted, PA derived from egg yolk was equally potent as dioleoyl-PA as an activator of PS synthase activity.

PS synthase activity was measured as a function of the surface concentration of CDP-DG at various concentrations of PC (Fig. 4B). The enzyme showed saturation kinetics with respect to CDP-DG in the presence of PC. PC caused an increase in the apparent $V_{max}$ but had little effect on the apparent $K_m$. A replot of the $1/V$ intercepts versus the $1/PC$ concentration (Fig. 4B, inset) was used to calculate apparent $K_a$ (3.4 mol %) and apparent $V_{max}$ (8.1 $\mu$mol/min/mg) values for PC.

The enzyme also displayed saturation kinetics with respect to CDP-DG in the presence of various concentrations of PI (Fig. 4C). The addition of PI to the assay system for PS synthase resulted in an increase in the apparent $V_{max}$ with little change in the apparent $K_m$. Apparent $K_a$ (3.2 mol %) and $V_{max}$ (8.8 $\mu$mol/min/mg) values for PI were calculated from a replot of the $1/V$ intercepts versus the $1/PI$ concentrations (Fig. 4C, inset).

Effect of Lipid Inhibitors on the Kinetics of PS Synthase Activity—The kinetics of PS synthase with respect to the surface concentration of CDP-DG was examined in the presence of various concentrations of cardiolipin. Cardiolipin did not effect the apparent $V_{max}$ value for CDP-DG in these experiments but did cause an increase in the apparent $K_m$ (Fig. 5A). The pattern of lines shown in Fig. 5A is consistent with cardiolipin being a competitive inhibitor (34) of PS synthase. A replot of slopes versus the cardiolipin concentration (Fig. 5A, inset) was used to calculate an apparent $K_i$ (7 mol %) for CDP-DG expressed as the mol % of CDP-DG in the micelles. Cardiolipin (A) and DG (B) were maintained at the indicated concentrations. The data are plotted as $1/V$ (nmol/min/ml) versus the reciprocal of the CDP-DG concentration. The insets are replots of the slope versus cardiolipin and DG concentrations in panels A and B, respectively. The curves drawn were a result of a least squares analysis of the data.

![Fig. 5. Effect of cardiolipin (CL) and DG on the kinetics of PS synthase activity with respect to the surface concentration of CDP-DG. The bulk concentration of CDP-DG was held constant at 0.1 mM while the Triton X-100 concentration was varied. PS synthase activity was measured as a function of the surface concentration of CDP-DG expressed as the mol % of CDP-DG in the micelles. Cardiolipin (A) and DG (B) were maintained at the indicated concentrations. The data are plotted as $1/V$ (nmol/min/ml) versus the reciprocal of the CDP-DG concentration. The insets are replots of the slope versus cardiolipin and DG concentrations in panels A and B, respectively. The curves drawn were a result of a least squares analysis of the data.](http://www.jbc.org/)

### Table I

<table>
<thead>
<tr>
<th>Kinetic constants for PS synthase</th>
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### DISCUSSION

PS synthase is a highly regulated enzyme that is intricately involved in the control of phospholipid biosynthesis in *S. cerevisiae* (1). The enzyme is regulated at the genetic and activity levels. PS synthase expression is regulated by inositol alone in concert with serine, ethanolamine, and choline (9,
The activity of existing PS synthase is regulated by inositol (11) and by phosphorylation via cAMP-dependent protein kinase (12). In this study we have shown that PS synthase activity is effected by lipids. Thus the in vivo regulation of this membrane-associated enzyme is complex being governed by membrane associated and cytosolic associated factors. As an initial approach to dissecting the regulation of PS synthase by lipids we conducted systematic kinetic experiments using well-defined Triton X-100/phospholipid mixed micelles. We showed that PA, PC, and PI stimulated PS synthase activity, whereas cardiolipin and DG inhibited activity. What effects would the activation of PS synthase by PA, PC, and PI or the inhibition of activity by cardiolipin and DG have on lipid metabolism? PS synthase is fully derepressed (9, 10) and active (10) when wild-type cells are grown in the absence of inositol. Under this growth condition the primary pathway for PC synthesis is predominant over the auxiliary pathway (1). Since PA is a branch point intermediate in the primary and auxiliary pathways for PC biosynthesis (1), the activation of PS synthase activity by PA is consistent with PC biosynthesis through the primary pathway. The relatively low activation constant for PA is physiologically significant given the fact that PA is a minor membrane phospholipid (11). When wild-type cells are grown in the presence of inositol plus choline, PS synthase (9, 10) is repressed and PC synthesis occurs predominantly by the auxiliary pathway (1). The DG needed in the auxiliary pathway is derived from PA via PA phosphatase. Inositol induces PA phosphatase activity and causes an increase in cellular DG (36). Based on the relatively high K value for DG, the inhibition of PS synthase activity by DG would occur when DG levels were elevated. Thus, the inhibition of PS synthase by DG in cells grown in the presence of inositol is consistent with PC biosynthesis by the auxiliary pathway.

PS synthase activity in cell extracts from stationary-phase cells is 4-fold lower than the activity found in extracts from exponential-phase cells (37). The decrease in PS synthase activity in stationary-phase cells is not due to enzyme repression (37) or to cAMP-dependent protein kinase phosphorylation (12). PA phosphatase activity increases in stationary-phase cells (38) and is responsible for channeling the utilization of PA toward triacylglycerol synthesis at the expense of phospholipid synthesis (38, 39). DG levels increase in stationary-phase cells (38, 39). Therefore, the inhibition of PS synthase by DG in cells grown in the presence of inositol is consistent with PC biosynthesis by the auxiliary pathway.

Cardiolipin is derived from CDP-DG and phosphatididylycerol (1) and is only found in the mitochondria of S. cerevisiae (40-42). Cardiolipin plays a unique role in the mitochondria of S. cerevisiae in that it is required for cytochrome oxidase activity (43, 44). The inhibition of PS synthase by cardiolipin is consistent with the utilization of CDP-DG for cardiolipin synthesis over PS synthesis. Although the PS synthase used in this study was purified from microsomes, mitochondrial PS synthase and microsomal PS synthase appear to be the same enzyme (45).

Finally, the stimulation of PS synthase activity by PC and PI may be a means of maintaining the charge of the membrane. It is known that the phospholipid composition of S. cerevisiae varies considerably depending on culture conditions (1). Nevertheless, the average charge of the membrane phospholipids remains fairly constant (46). The cell adjusts for changes in phospholipids by one charge by causing parallel changes in phospholipids of another charge (1). The activation of PS synthase by PC may increase PE (neutral charge) of the membrane. On the other membrane, PI stimulation of PS synthase activity may increase PE and PC content (neutral charge) of the membrane.

The explanations given for the regulation of PS synthase activity by lipids may be an oversimplification of the complex regulation that is exerted on this enzyme in vivo. As noted above, several mechanisms exist in S. cerevisiae that control the activity of PS synthase (1). These control mechanisms must be interrelated. Accordingly, future work will involve a molecular approach toward gaining an understanding of the interrelationship between the regulation of PS synthase activity by cAMP dependent protein kinase phosphorylation, inositol, and lipids. It is clear from this study and from previous studies on the regulation of PS synthase (8-12, 14) and PI synthase (8, 11, 13, 14), that the partitioning of CDP-DG between PS and PI is primarily governed by the regulation of PS synthase.

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Regulation of PS Synthase and PI Synthase by Phospholipids

Regulation of yeast phosphatidylserine synthase and phosphatidylinositol synthase activities by phospholipids in Triton X-100/phospholipid mixed micelles.

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