JOURNAL OF BACTERIOLOGY, Mar. 1986, p. 901–910
0021-9193/86/030901-10$02.00/0

Vol. 165, No. 3

Subcellular and Submitochondrial Localization of Phospholipid-Synthesizing Enzymes in *Saccharomyces cerevisiae*

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Received 5 August 1985/Accepted 9 December 1985

Using highly enriched membrane preparations from lactate-grown *Saccharomyces cerevisiae* cells, the subcellular and submitochondrial location of eight enzymes involved in the biosynthesis of phospholipids was determined. Phosphatidylserine decarboxylase and phosphatidylglycerolphosphate synthase were localized exclusively in the inner mitochondrial membrane, while phosphatidylethanolamine methyltransferase activity was confined to microsomal fractions. The other five enzymes tested in this study were common both to the outer mitochondrial membrane and to microsomes. The transmembrane orientation of the mitochondrial enzymes was investigated by protease digestion of intact mitochondria and of outside-out sealed vesicles of the outer mitochondrial membrane. Glycerolphosphate acyltransferase, phosphatidylinositol synthase, and phosphatidylserine synthase were exposed at the cytosolic surface of the outer mitochondrial membrane. Cholinephosphotransferase was apparently located at the inner aspect or within the outer mitochondrial membrane. Phosphatidate cytidylyltransferase was localized in the endoplasmic reticulum, on the cytoplasmic side of the outer mitochondrial membrane, and in the inner mitochondrial membrane. Inner membrane activity of this enzyme constituted 80% of total mitochondrial activity; inactivation by trypsin digestion was observed only after preincubation of membranes with detergent (0.1% Triton X-100). Total activity of those enzymes that are common to mitochondria and the endoplasmic reticulum was about equally distributed between the two organelles. Data concerning susceptibility to various inhibitors, heat sensitivity, and the pH optima indicate that there is a close similarity of the mitochondrial and microsomal enzymes that catalyze the same reaction.

In eucaryotic cells, phospholipids required for the biogenesis and maintenance of membranes are synthesized in distinct subcellular compartments. In mammalian cells, the bulk of cellular phospholipids is synthesized in the endoplasmic reticulum (13), with specific contributions from mitochondria (phosphatidylglycerol and cardiolipin [18]; phosphatidylethanolamine via decarboxylation of phosphatidylserine [13]) and peroxisomes (acyldihydroxyacetonephosphate [25] and its alkyl analog [15]). In contrast to mammalian cells, in *Saccharomyces cerevisiae* most of the reactions involved in phospholipid biosynthesis have been reported to occur both in mitochondria and in the endoplasmic reticulum (9).

The present study on the subcellular and submitochondrial localization of phospholipid synthesizing enzymes in *S. cerevisiae* was motivated mainly by our interest in the mechanism(s) underlying intracellular phospholipid transport. Knowledge of the origin of the various phospholipids is a prerequisite for an understanding of the process of their transfer across and between subcellular membranes.

There are only few reports dealing with the subcellular localization of phospholipid-synthesizing enzymes in *S. cerevisiae*. Some are at variance, e.g., with respect to the location of cholinephosphotransferase (9; D. Ostrow, Fed. Proc. **30**:1226, 1971), or the results obtained previously (9) are in contrast to what is well documented for higher eucaryotic cells (e.g., the location of phosphatidylserine decarboxylase). For *S. cerevisiae*, submitochondrial distribution of phospholipid-synthesizing enzymes (with the exception of cholinephosphotransferase) and their orientation within membranes have not been reported before.

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In this study, the activity of eight enzymes involved in phospholipid synthesis was examined in microsomal fractions, mitochondria, and isolated mitochondrial membranes. Accessibility to protease digestion was tested to determine transmembrane orientation of enzymes in mitochondrial membrane preparations.

In most previous studies (9, 41), incorporation of [2-^{3}H]glycerol-3-phosphate into various phospholipids was used as a measure for the phospholipid-synthesizing capacity of yeast subcellular fractions, whereas in the present study individual enzymes were measured to define more precisely the contribution of the respective organelle to total cellular phospholipid production. The effect of glucose repression on one key enzyme of phospholipid synthesis that is located exclusively in mitochondria, namely, phosphatidylserine decarboxylase, was also examined.

The fact that the majority of phospholipids can be synthesized both in mitochondria and in the endoplasmic reticulum poses the question as to the molecular identity of the enzymes involved. In the case of *sn*-glycerol-3-phosphate acyltransferase from mammalian liver, circumstantial evidence obtained from comparison of kinetic data and susceptibility to inhibitors, etc., supports the notion that the mitochondrial and microsomal enzymes are distinct (6). Similar studies are reported here for this and other phospholipid-synthesizing enzymes of *S. cerevisiae*.

MATERIALS AND METHODS

Reagents. Sorbitol, mannitol, bovine serum albumin, dithiothreitol, *sn*-glycerol-3-phosphate, palmitoyl-coenzyme A (CoA), hydroxylamine, L-serine, *myo*-inositol, CDP-diacylglycerol, CTP, ATP, GTP, CDP-choline, diolein, phospholipids, *N*-ethylmaleimide, dithiobisnitrobenzoic

acid, iodoacetamide, Triton X-100, and *p*-chloromercuribenzoic acid were purchased from Sigma Chemie (Munich, Federal Republic of Germany). Phenylmethylsulfonyl fluoride, diisopropylfluorophosphate, silica gel PF 60, and lactic acid were obtained from E. Merck AG, Darmstadt, Federal Republic of Germany. Trypsin, papain, soybean trypsin inhibitor, and antimycin were from Boehringer GmbH, Mannheim, Federal Republic of Germany. Zymolyase 5000 and 20000 were obtained from Kirin Brewery, Tokyo, Japan. Radiochemicals were purchased from Amersham International, Amersham, United Kingdom.

Yeast strains and culture conditions. *S. cerevisiae* D273-10B (ATCC 25657) was grown aerobically in 2-liter round-bottom flasks at 30°C in a rotary shaker (New Brunswick Scientific Co., Inc., Edison, N.J.) on a medium containing the following per liter: 3 g of yeast extract (Difco Laboratories, Detroit, Mich.), 1 g of glucose, 1 g of KH_2PO_4, 1 g of NH_4Cl, 0.5 g of $CaCl_2 \cdot 2H_2O$, 0.5 g of NaCl, 0.6 g of $MgCl_2 \cdot 6H_2O$, 0.3 ml of 1% $FeCl_3$, and 22 ml of 90% lactic acid as carbon source; the final pH was adjusted to 5.5 with concentrated NaOH. Cells were grown to the exponential growth phase and harvested at an optical density of $A_{546} = 6$ to 7, corresponding to 7 to 8 g of cell wet weight per liter.

Preparation of subcellular and submitochondrial fractions. Spheroplasts, mitochondria, and submitochondrial fractions were prepared essentially as described by Daum et al. (11). Microsomal fraction I was isolated from the postmitochondrial supernatant by centrifugation at 30,000 × *g* for 20 min in a Sorvall RC-5 centrifuge (rotor SS 34). The resulting supernatant was centrifuged for 1 h at 115,000 × *g* in a Sorvall OTD2 ultracentrifuge (rotor T 865) to obtain microsomal fraction II. The microsomal fractions were washed once with ice-cold buffer (10 mM Tris hydrochloride [pH 7.4]), repelleted, and suspended in the same buffer to a protein concentration of about 4 mg/ml. To remove residual mitochondria, microsomal fraction I was further purified by density gradient centrifugation on a step gradient ranging from 25 to 50% (wt/wt) sucrose, buffered with 10 mM Tris hydrochloride (pH 7.4). After centrifugation at 80,000 × *g* for 10 h (rotor SW 28) the microsomal fraction banded at a density of 1.15, corresponding to approximately 36% sucrose. The band was collected, diluted with 10 mM Tris hydrochloride (pH 7.4), and repelleted.

Characterization of subcellular fractions. Previously published procedures were used to assay the mitochondrial (succinate dehydrogenase [1], kynurenine hydroxylase [39], cytochrome b_2 [26], fumarase [34]) and the microsomal (NADPH-cytochrome *c* reductase [37]) marker enzymes.

Incorporation of UDP-*N*-acetyl[U-^{14}C]glucosamine into lipid and protein by subcellular fractions was measured by the method of Lehle et al. (28). Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis in slab gels was carried out as described by Laemmli (27). RNA was determined by the method of Ogur and Rosen (32), and lipid phosphorus was quantitated by the method of Bartlett (3). Protein was determined by the method of Lowry et al. (30) with crystalline bovine serum albumin as a standard and in the presence of 0.5% SDS to solubilize membranes.

Preparation of radioactively labeled substrates. Phosphatidyl-L-[3-^{3}H]serine was synthesized in vitro by the following procedure. Microsomal fraction I (10 mg of protein) was incubated with 0.4 μmol CDP-diacylglycerol, 1 μmol hydroxylamine, 1.2 μmol $MnCl_2$, 0.5% (wt/vol) Triton X-100, 1 μmol L-[3-^{3}H]serine (200 μCi/μmol), and 200 μmol Tris hydrochloride (pH 8) in a total volume of 2 ml for 3 h at 30°C. The reaction was stopped by the addition of 20 ml of chloroform-methanol (2:1 [vol/vol]). The organic phase was washed five times with 2 M KCl-methanol (4:1 [vol/vol]) and dried under a stream of nitrogen, and the residue was dissolved in 3 ml of chloroform-methanol (2:1 [vol/vol]). Usually, 20% of the radioactive serine was incorporated into lipid. Because the only labeled phospholipid was phosphatidylserine, total lipids were used for the phosphatidylserine decarboxylase assay without further purification. [2-^{3}H]glycerol-3-phosphate was prepared by the method of Steiner and Lester (41).

Enzyme assays. Glycerolphosphate acyltransferase (acyl-CoA:*sn*-glycerol-3-phosphate *O*-acyltransferase; EC 2.3.1.15), phosphatidylserine synthase (CDPdiacylglycerol:L-serine *O*-phosphatidyltransferase; EC 2.7.8.8), phosphatidylinositol synthase (CDPdiacylglycerol:*myo*-inositol 3-phosphatidyltransferase; EC 2.7.8.11) were assayed as described previously (5, 38).

Phosphatidate cytidylyltransferase (CTPphosphatidate cytidylyltransferase; EC 2.7.7.41) was assayed by the method of Belendiuk et al. (4) with the following modifications. The reaction mixture contained, in a total volume of 0.2 ml, 15 μmol cacodylic acid (pH 6.5), 1% (wt/vol) Triton X-100, 4 μmol KCl, 4 μmol $MgCl_2$, 0.2 μmol dioleoylphosphatidic acid, enzyme (0 to 300 μg of protein), and 0.2 μmol [5-^{3}H]CTP (10,000 dpm/nmol).

Cholinephosphotransferase (CDPcholine:1,2-diacylglycerol cholinephosphotransferase; EC 2.7.8.2) was assayed by the method of Hosaka and Yamashita (17) with the following modifications. The reaction mixture contained in a total volume of 0.2 ml 20 μmol diolein suspended in 0.02% (wt/vol) Triton X-100, enzyme (0 to 300 μg of protein) and 0.1 μmol CDP-[^{14}C]choline (2500 dpm/nmol).

Phosphatidylethanolamine methyltransferase (*S*-adenosyl-L-methionine:phosphatidylethanolamine *N*-methyltransferase; EC 2.1.1.17) was assayed as described by Daum et al. (12) with the following modifications. Phosphatidylethanolamine liposomes in 10% (vol/vol) ethyl alcohol were prepared by sonication of 5 mg of phosphatidylethanolamine per ml with a Braunsonic 300 S sonicator (Braun, Melsungen, Federal Republic of Germany). Enzyme (0 to 300 μg of protein) was preincubated for 10 min with 2 mg of phosphatidylethanolamine liposomes, 100 μmol Tris hydrochloride (pH 8.5), and 5 μmol $MgCl_2$; then, 0.5 μmol *S*-adenosyl [*methyl*-^{3}H]methionine (5,500 dpm/nmol) was added to a total volume of 1 ml.

Phosphatidylglycerolphosphate synthase (CDPdiacylglycerol:*sn*-glycerol-3-phosphate phosphatidyltransferase; EC 2.7.8.5) was assayed in a total volume of 0.2 ml containing 0.04 μmol CDP-diacylglycerol, 1 μmol $MgCl_2$, 15 μmol cacodylic acid (pH 6.5), enzyme (0 to 400 μg of protein), and 0.1 μmol [2-^{3}H]glycerol-3-phosphate (38,000 dpm/nmol). Phosphatidylserine decarboxylase (phosphatidylserine carboxylyase; EC 4.1.1.65) was assayed by the following procedure. A total of 5 nmol [^{3}H]phosphatidylserine (40,000 dpm/nmol) in chloroform-methanol (2:1 [vol/vol]) was dried under a stream of nitrogen in a 10-ml glass tube. The oily residue was dispersed in 200 μl of 0.2 M Tris hydrochloride (pH 7.2) by brief sonication. Enzyme (0 to 1 mg of protein) was added to a total volume of 0.4 ml.

Enzyme assays were started either by the addition of enzyme or by the addition of the radioactively labeled substrates and incubated at 30°C in a water bath. All enzyme activities were measured in a range linear with the amount of enzyme and the time of incubation. Controls were obtained by ommission of enzyme in the assay or by stopping the reaction immediately after it began. After the assays were

TABLE 1. Purity of subcellular fractions

Fraction	Protein (mg)	RNA (μg/mg of protein)	Phospholipid (μg/mg of protein)	Sp act (nkat/mg of protein) of the following enzymes[a]:			Incorporation (nKat/mg of protein) of ^{14}C-labeled UDP-*N*- acetyl-glucosamine
				Succinate dehydrogenase	Kynurenine hydroxylase	NADPH-cytochrome *c* reductase	
Homogenate	100	ND[b]	ND	29.0 (1)	ND	112 (1)	ND
Mitochondria	4.0	18.2	147	67.8 (2.33)	49.0	40.8 (0.36)	0.07
Inner mitochondrial membrane	1.72	ND	ND	129 (4.44)	34.8	ND	ND
Outer mitochondrial membrane	0.18	ND	610	10.8 (0.37)	265	65.4 (0.58)	0.12
Microsomal fraction I	1.0	42.6	142	16.8 (0.58)	12.7	319.2 (2.84)	1.1
Microsomal fraction II	7.0	75.0	67	0.6 (0.02)	ND	654 (5.8)	0.12

[a] Numbers in parentheses give the enrichment of specific activities in the respective fractions relative to that of the total homogenate.
[b] ND, Not determined.

stopped with 3 to 5 ml of chloroform-methanol-36% HCl (20:10:0.3 [by volume]) the reaction mixtures were vigorously mixed on a Vortex mixer and allowed to stand for 10 min at room temperature. Phases were separated by brief centrifugation, and the lower organic phase was washed 5 times with 5 ml of 2 M KCl-methanol (4:1 [vol/vol]). Radioactivity of fractions of the extracts was determined by liquid scintillation counting in Lipoluma (J. T. Baker Chemical Co., Phillipsburg, N.J.). Reproducibility was better than ±10%.

Identification of reaction products. Fractions of the extracts were separated by two-dimensional thin-layer chromatography on glass plates (20 by 20 cm), coated with 0.5 mm of silica gel PF 60 (first dimension, chloroform-methanol-25% ammonia (65:35:5 [by volume]); second dimension, chloroform-acetone-methanol-glacial acetic acid-water (50:20:10:10:5 [by volume]). Spots were visualized by exposure to iodine vapor and identified by the use of standard phospholipids. After the iodine was evaporated, spots were scraped off the plates, and the radioactivity was determined by liquid scintillation counting, using 10% BBS-3 (Beckman Instruments, Inc., Fullerton, Calif.) and 5% water in Lipoluma (Baker).

Protease treatment of mitochondria, mitoplasts, and vesicles of the outer mitochondrial membrane. Mitoplasts were isolated by the method of Daum et al. (11). Isolated intact mitochondria and mitoplasts were suspended in buffer (0.6 M mannitol, 10 mM Tris hydrochloride [pH 7.4]) to a protein concentration of 1.5 mg/ml. Mitochondria and mitoplasts were then treated with various amounts of trypsin (final concentration, 0 to 40 μg/ml) in the presence and absence of 0.1% (wt/vol) Triton X-100 for 10 min at 23°C. The reaction was stopped by the addition of a 20-fold excess of soybean trypsin inhibitor, and enzyme assays were performed as described above. Controls were obtained either by adding trypsin inhibitor before the addition of trypsin or by stopping protease treatment immediately after the start of the reaction. Leakiness of mitochondria, caused by protease digestion, was assessed by measuring the release of cytochrome b_2 from the intermembrane space. Usually 10 to 20% of total cytochrome b_2 was released. Protease treatment of vesicles of the outer mitochondrial membrane was carried out with the following modifications. Isolated vesicles of the outer membrane were suspended in 10 mM Tris hydrochloride (pH 7.4) to a protein concentration of 1 mg/ml, and the detergent concentration was lowered to 0.05% (wt/vol) Triton X-100.

Immunological methods. Antibodies against porin were raised in Chinchilla rabbits, and antisera were prepared as described previously (11). Immunological titration tests for purity of subcellular and submitochondrial fractions were carried out by previously published procedures (11, 14).

RESULTS

Characterization of subcellular fractions. Mitochondria, submitochondrial membranes, and microsomal fractions were characterized by assaying specific marker enzymes (Tables 1 and 2) and by comparing the respective protein patterns by SDS-polyacrylamide gel electrophoresis (Fig. 1) and by immunochemical quantitation of porin that is specific for the outer mitochondrial membrane (35). Cross-contamination between inner and outer mitochondrial membranes, based on specific activities of the marker enzymes succinate dehydrogenase and kynurenine hydroxylase and on the porin content, was on the order of 10 to 15%.

Microsomal marker enzymes were unevenly distributed over microsomal fraction I (obtained after centrifugation of a postmitochondrial supernatant at 30,000 × *g* for 20 min) and microsomal fraction II (sedimented at 115,000 × *g* for 1 h). Specific activity of NADPH-cytochrome *c* reductase was higher in microsomal fraction II, whereas utilization of ^{14}C-labeled UDP-*N*-acetylglucosamine was distinctly higher

TABLE 2. Purity of subcellular fractions

Fraction	Sp act ratio for the following enzymes[a]:				Porin content[b]
	Succinate dehydro-genase	Kynurenine hydrox-ylase	NADPH-cyto-chrome *c* reductase	Incorporation of ^{14}C-labeled UDP-*N*-acetylglucos-amine	
Mitochondria	0.53	0.19	0.06	0.07	0.28
Inner mitochondrial membrane	1	0.13			0.16
Outer mitochondrial membrane	0.08	1	0.1	0.12	1
Microsomal fraction I	0.13	0.05	0.49	1	0.16
Microsomal fraction II			1	0.11	0.01

[a] Ratio of specific activity in the membrane fraction to that in the reference fraction.
[b] Porin content was determined by immune titration, using a specific antiserum and ^{125}I-labeled protein A. Values are the fraction of radioactivity relative to the radioactivity obtained with the outer mitochondrial membrane.

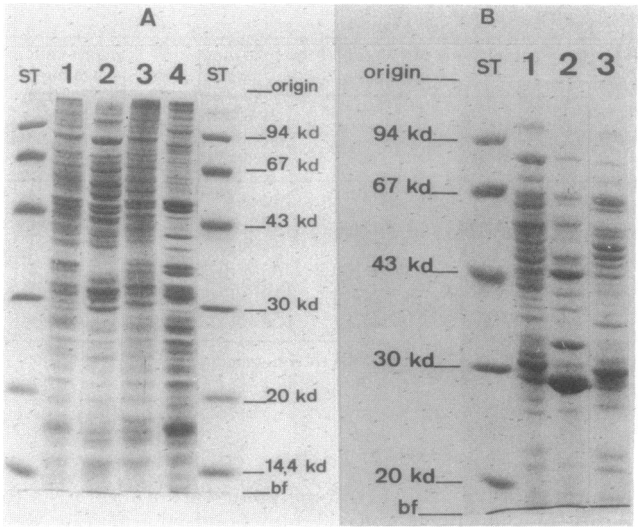

FIG. 1. SDS-polyacrylamide gel electrophoresis of subcellular (A) and submitochondrial (B) fractions. (A) Lane 1, total cell homogenate; lane 2, mitochondria; lane 3, microsomal fraction I; lane 4, microsomal fraction II. Fractions equivalent to 40 μg of protein were applied. (B) Lane 1, mitochondria; lane 2, outer mitochondrial membrane; lane 3, inner mitochondrial membrane. Fractions equivalent to 35 μg of protein were applied. Lanes ST, standard proteins (15 μg; Pharmacia Fine Chemicals, Piscataway, N.J.) with molecular masses given in kilodaltons (kd) in the figure: phosphorylase b (94,000), bovine serum albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), soybean trypsin inhibitor (20,000), lactalbumin (14,400); kd, kilodaltons; bf, bromophenol blue zone.

in microsomal fraction I. The latter fraction contained many fewer ribosomes, as shown by the lower RNA to protein ratio and by the SDS-polyacrylamide gel electrophoresis pattern (42) (Fig. 1).

Contamination of mitochondria with microsomal fraction I was approximately 7% (based on the utilization of ^{14}C-labeled UDP-*N*-acetylglucosamine and NADPH-cytochrome *c* reductase, respectively). Cross-contamination between the outer mitochondrial membrane and microsomal fraction I was on the order of 10 to 15%, as determined by assaying UDP-*N*-acetylglucosamine utilization and porin content. Contamination of the outer mitochondrial membrane with microsomal fraction II did not exceed 10%.

So-called lipid particles, which have been shown to be involved in the synthesis of neutral glycerophospholipids via phosphatidic acid (7) and of phosphatidylcholine and phosphatidylethanolamine via the Kennedy pathway (8) and which are recovered in the floating lipid layer during differential centrifugation, were not investigated in this study.

Subcellular localization of phospholipid-synthesizing enzymes. The results summarized in Tables 3 and 4 confirm previous reports (9) that mitochondria from fully derepressed yeast cells have the capacity for the synthesis of most of their component phospholipids. Only phosphatidylethanolamine methyltransferase activity was nearly absent from mitochondria. The low activity found in mitochondria can almost completely be accounted for by contamination of mitochondria with microsomal fraction I. Phosphatidylserine decarboxylase and phosphatidylglycerolphosphate synthase are localized exclusively in mitochondria. In a previous report (9), phosphatidylserine decarboxylase activity has been ascribed to mitochondria and the endoplasmic reticulum of yeast cells.

The other enzymes examined in this study, i.e., phosphatidylserine synthase, phosphatidylinositol synthase, glycerolphosphate acyltransferase, phosphatidate cytidylyltransferase, and cholinephosphotransferase, are present both in mitochondria and in the endoplasmic reticulum. In all cases, the highest specific activity was found in microsomal fraction I. Total enzyme activities, however, were about equal in microsomal fractions and in mitochondria (Table 5).

Phosphatidylserine decarboxylase, which is located exclusively in mitochondria, plays a key role in the synthesis of phosphatidylethanolamine and phosphatidylcholine, which together constitute the bulk (up to 70%) of total cellular phospholipids. It was therefore of interest to determine whether this enzyme, as many other mitochondrial enzymes of yeast, is subject to glucose repression. Enzyme activity assayed in promitochondria isolated from glucose-grown cells was only slightly less (28 nkat/mg of mitochondrial protein) than that in fully derepressed mitochondria (37 nkat/mg of mitochondrial protein) which were isolated from cells cultivated on lactate.

Submitochondrial localization of phospholipid-synthesizing enzymes. Using highly enriched preparations of outer and inner mitochondrial membranes (Tables 1 and 2), the submitochondrial distribution of phospholipid-synthesizing enzymes was determined (Tables 3 and 4; see Fig. 6). Two of the eight enzymes tested, namely, phosphatidylserine decarboxylase and phosphatidylglycerolphosphate synthase, were found exclusively in the inner mitochondrial membrane. The fact that the latter enzyme was not enriched in the inner membranes over whole mitochondria can probably be explained by the instability of the enzyme during submitochondrial fractionation (the procedure involved freezing and brief sonication). A 2.5-fold increase in specific activity was observed when total (but unfractionated) mitochondrial membranes were compared with intact mitochondria (data not shown).

TABLE 3. Subcellular and submitochondrial distribution of phospholipid-synthesizing enzymes

Fraction	Sp act (nkat/mg of protein) for the following enzymes[a]:							
	Glycerolphosphate acyltransferase	Phosphatidate cytidylyltransferase	Phosphatidylinositol synthase	Phosphatidylserine synthase	Phosphatidylserine decarboxylase	Phosphatidylglycerolphosphate synthase	Cholinephosphotransferase	Phosphatidylethanolamine methyltransferase
Homogenate	192 (1)	258 (1)	34 (1)	17 (1)	10 (1)	24.6 (1)	27 (1)	134 (1)
Microsomal fraction I	888 (4.63)	1,566 (6.07)	228 (6.70)	102 (6.0)	8 (0.8)	26.8 (1.09)	138 (5.10)	192 (1.43)
Microsomal fraction II	174 (0.91)	174 (0.67)	26 (0.76)	17 (1.0)	0.4 (0.04)	1.2 (0.05)	39 (1.44)	48 (0.35)
Mitochondria	384 (2)	528 (2.04)	72 (2.1)	25 (1.47)	37 (3.7)	123 (5.0)	67.8 (2.51)	30 (0.22)

[a] Numbers in parentheses give the enrichment of specific activities relative to that of the total cell homogenate.

TABLE 4. Subcellular and submitochondrial distribution of phospholipid-synthesizing enzymes

Fraction	Sp act enrichment of the following enzymes[a]:							
	Glycerolphosphate acyltransferase	Phosphatidate cytidylyltransferase	Phosphatidylinositol synthase	Phosphatidylserine synthase	Phosphatidylserine decarboxylase	Phosphatidylglycerolphosphate synthase	Cholinephosphotransferase	Phosphatidylethanolamine methyltransferase
Mitochondria	1	1	1	1	1	1	1	1
Outer mitochondrial membrane	5.2	5.7	5.6	6.4	0.4	0.35	2.4	
Inner mitochondrial membrane	0.5	2.1	0.7	0.8	1.3	0.9	0.5	
Microsomal fraction I	2.31	2.96	3.17	4.08	0.21	0.22	2.04	6.5
Microsomal fraction II	0.45	0.33	0.36	0.68	0.01	0.01	0.58	1.6

[a] Enrichment of specific activities relative to that of mitochondria.

Phosphatidate cytidylyltransferase was common to both the inner and outer mitochondrial membrane, whereas the other four enzymes tested were found in the outer mitochondrial membrane. It is noteworthy that those mitochondrial enzyme activities that were found exclusively in the outer membrane were also present in microsomal fractions. Because specific activities of distinct enzymes were, in all cases, in the same range in both fractions (outer mitochondrial membranes and microsomal fraction I), and considering the distribution of marker enzymes, cross-contamination can be ruled out as an explanation for the presence of identical enzyme activities in the two subcellular compartments. The question as to the identity of phospholipid-synthesizing enzymes located both in mitochondria and in microsomes is addressed in the following section.

To determine the transmembrane orientation of mitochondrial enzymes, their accessibility to proteolytic digestion was examined. For these experiments intact mitochondria, right-side-out-oriented outer membrane vesicles (35), and mitoplasts were used. Glycerolphosphate acyltransferase was rapidly and completely inactivated by treatment with trypsin or papain of outer mitochondrial membrane vesicles or intact mitochondria, whereas phosphatidylinositol synthase was inactivated only by trypsin digestion (Fig. 2). These two enzymes were located on the cytoplasmic side of the outer mitochondrial membrane. Phosphatidylserine synthase almost completely resisted trypsin digestion but was partially inactivated by treatment with papain (Fig. 3.); the degree of inactivation was slightly enhanced in the presence of 0.05% Triton X-100. From this result we conclude that mitochondrial phosphatidylserine synthase is also exposed to the cytoplasm.

Cholinephosphotransferase is apparently located at the inner aspect or within the outer mitochondrial membrane, as can be deduced from its resistance to proteases in whole mitochondria. In the presence of Triton X-100 trypsin digestion was almost complete.

Part of phosphatidate cytidylyltransferase which cofractionates with the outer mitochondrial membrane was inactivated to approximately 70% by external trypsin, indicating that this enzyme is exposed at the cytoplasmic side of mitochondria. The presence of Triton X-100 led to complete inactivation by trypsin. About 80% of the total mitochondrial enzyme activity was associated with the inner mitochondrial membrane. Treatment of mitoplasts with trypsin led to an appreciable inactivation, but only in the presence of detergent. Thus, the enzyme faces either the matrix side of the inner mitochondrial membrane or is an integral membrane protein.

Comparison of mitochondrial and microsomal enzymes of phospholipid synthesis. The results summarized in Table 6 and in Fig. 4 and 5 demonstrate that phospholipid-synthesizing enzymes which are common to both mitochondria and the endoplasmic reticulum are susceptible to the same inhibitors and have identical pH optima and heat sensitivity. These findings provide insufficient proof for the identity of the microsomal and mitochondrial enzymes, but, on the other hand, they do not disprove such a possibility. In contrast to those from *S. cerevisiae*, glycerolphosphate acyltransferases from rat liver mitochondria and microsomes are distinct with respect to inhibition by sulfhydryl reagents and in their chain length preference for acyl-CoA substrates (6).

DISCUSSION

Growing cells depend on a continuous supply of newly synthesized phospholipids which are required for the biogenesis of membrane structures. In cells of mammalian tissues, the enzymes involved in phospholipid synthesis are

TABLE 5. Distribution of phospholipid biosynthesis capacity in subcellular fractions

Fraction	Phospholipid biosynthesis capacity of the following enzymes[a]:							
	Glycerolphosphate acyltransferase	Phosphatidate cytidylyltransferase	Phosphatidylinositol synthase	Phosphatidylserine synthase	Phosphatidylglycerolphosphate synthase	Phosphatidylserine decarboxylase	Cholinephosphotransferase	Phosphatidylethanolamine methyltransferase
Mitochondria	1	1	1	1	1	1	1	1
Microsomal fraction I	0.57	0.73	0.72	1.02	0.07	0.06	0.5	1.6
Microsomal fraction II	0.78	0.56	0.57	1.2	<0.01	<0.005	1	2.8

[a] Phospholipid biosynthesis capacity was calculated from specific enzyme activities times the amount of protein recovered in each fraction (starting from 100 mg of total homogenate protein). Data are expressed as total capacity of the two microsomal fractions in relation to that of mitochondria.

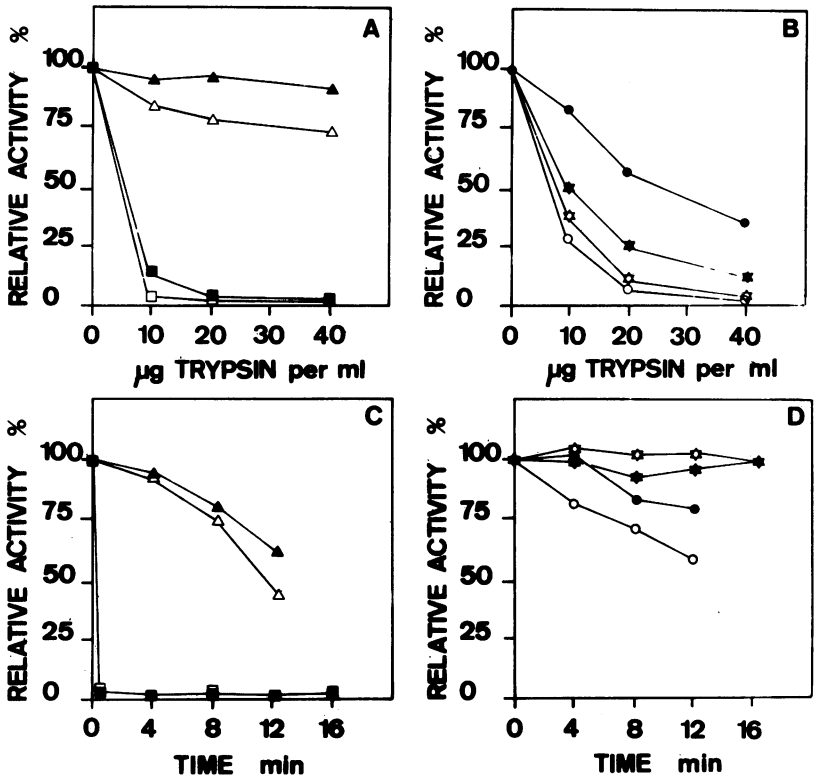

FIG. 2. Protease sensitivity of phospholipid-synthesizing enzymes in outside-out vesicles of the outer mitochondrial membrane. Outside-out vesicles of the outer mitochondrial membrane were suspended in 10 mM Tris hydrochloride (pH 7.4) to give a protein concentration of 1 mg/ml. Vesicles were treated with various amounts of trypsin for 10 min at 23°C (A and B) or with papain (40 µg/ml) for various times at 23°C (C and D). Activities of phospholipid-synthesizing enzymes were assayed as described in the text. Filled symbols refer to incubations in the absence of and open symbols refer to incubations in the presence of 0.05% (wt/vol) Triton X-100. Symbols: ■, glycerolphosphate acyltransferase; ▲, phosphatidylserine synthase; ★, phosphatidylinositol synthase; ●, phosphatidate cytidylyltransferase.

localized mainly in the endoplasmic reticulum (10; Table 7). In *S. cerevisiae*, however, mitochondria and the endoplasmic reticulum share the capacity to synthesize cellular phospholipid components.

In this study we have described the subcellular and submitochondrial distribution of eight enzymes which together catalyze the synthesis of most of the glycerophospholipids in *S. cerevisiae* cells (Tables 3 and 4).

The exclusive mitochondrial location of phosphatidylglycerolphosphate synthase in *S. cerevisiae* as reported here confirms a recent report by Carman and Matas (5) and agrees with the localization of this enzyme in mammalian (18) and plant (40) cells (Table 7).

Phosphatidylserine decarboxylase, which, according to the results of this study, is also located exclusively in mitochondria, has been found mainly in mitochondria but

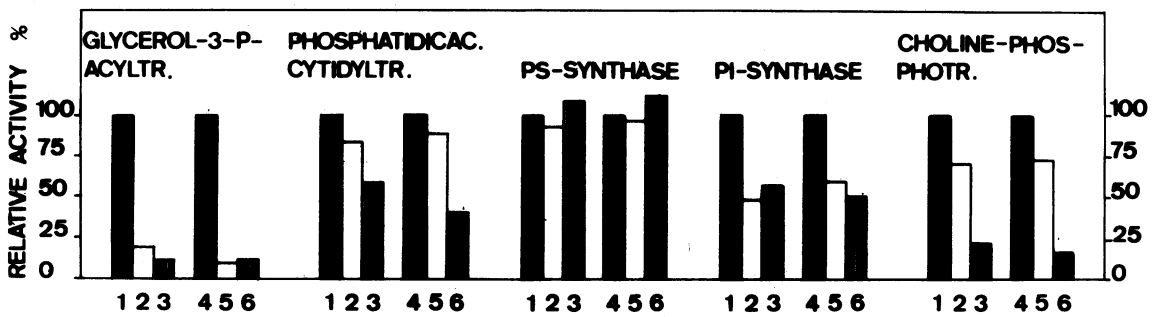

FIG. 3. Trypsin sensitivity of phospholipid-synthesizing enzymes in intact mitochondria and in mitoplasts. Mitochondria or mitoplasts (approximately 1.5 mg/ml) were treated with trypsin (40 µg/ml) for 10 min at 23°C. After the addition of trypsin inhibitor, phospholipid-synthesizing enzymes were assayed as described in the text. Where indicated, Triton X-100 was present at a concentration of 0.1% (wt/vol). Leakiness of mitochondria caused by protease digestion was assessed by measuring the efflux of cytochrome b_2 from the intermembrane space. Release of cytochrome b_2 did not exceed 10 to 20% of total activity. Bars: 1, Mitochondria; 2, mitochondria-trypsin; 3, mitochondria–trypsin–Triton X-100; 4, mitoplasts; 5, mitoplasts-trypsin; 6, mitoplasts–trypsin–Triton X-100. Abbreviations: Glycerol-3-P-acyltr, glycerolphosphate acyltransferase; phosphatidicac. cytidyltr, phosphatidate cytidylyltransferase; PS-synthase, phosphatidylserine synthase; PI-synthase, phosphatidylinositol synthase; choline-phosphotr., cholinephosphotransferase.

TABLE 6. Effect of various additions to the basal assay system on the activity of mitochondrial and microsomal phospholipid-synthesizing enzymes

Additions (mM)	Activity (% of control) of indicated enzyme in:									
	Glycerolphosphate acyltransferase		Phosphatidate cytidylyltransferase		Phosphatidylinositol synthase		Phosphatidylserine synthase		Cholinephosphotransferase	
	Mitochondria	Microsomes	Mitochondria	Microsomes	Mitochondria	Microsomes	Mitochondria	Microsomes	Mitochondria	Microsomes
None (control)	100	100	100	100	100	100	100	100	100	100
Sodium EDTA (40)	22	17	ND	ND	9	6	3	1	ND	ND
Dithiothreitol (1)	ND[a]	ND	130	110	112	100	164	121	ND	ND
N-Ethylmaleimide (1)	13	17	104	94	1	6	10	32	85	89
Iodoacetamide (2)	62	40	ND	ND	63	79	61	78	ND	ND
p-Chloromercuribenzoate (2)	4	4	8	5	19	20	23	1	ND	ND
5,5'-Dithio(2-nitrobenzoic acid) (1)	1	1	1	3	3	34	8	36	4	67
N,N,N',N'-Tetramethyl-*p*-phenylenediamine (1)	4	3	78	64	46	80	84	101	34	47
Phenylmethylsulfonyl fluoride (1)	94	104	99	103	86	84	140	129	22	45
Diisopropylfluorophosphate (1)	ND	ND	ND	ND	99	110	75	100	97	98
Triton X-100 (1% [wt/vol])	ND	ND	ND	ND	ND	ND	ND	ND	7	11

[a] ND, Not determined.

also in a microsomal fraction (corresponding to microsomal fraction I described here) from *S. cerevisiae* cells by Cobon et al. (9); in cells from rat liver (13) and calf brain (33) phosphatidylserine decarboxylase is localized exclusively in mitochondria.

The only phospholipid biosynthetic pathway of yeast which is restricted to the endoplasmic reticulum is the formation of phosphatidylcholine via methylation of phosphatidylethanolamine, which is catalyzed by phosphatidylethanolamine methyltransferase. This finding confirms previous reports on *S. cerevisiae* (9) and is paralleled by the situation in rat liver (43). In castor bean endosperm this pathway is confined to mitochondria (40).

The other five enzymes investigated in this study are common both to mitochondria and the endoplasmic reticulum. According to our results, total glycerolphosphate acyltransferase activity is higher in microsomal fractions than in mitochondria. In contrast, Cobon et al. (9) have shown that the activity of this enzyme, but also of phosphatidylinositol synthase and phosphatidylserine synthase, is significantly higher in mitochondria than in microsomes. This discrepancy can be explained either by the different assay conditions used in the two studies or, perhaps, by differences between various yeast strains. According to Yamada et al. (44), glycerolphosphate acyltransferase in *S. cerevisiae* is mainly a microsomal enzyme, with low activity in mitochondria. In rat liver apparently distinct enzymes with glycerolphosphate acyltransferase activity exist in mitochondria and in the endoplasmic reticulum (6).

Yeast phosphatidylinositol synthase is found both in microsomes and in mitochondria, while in rat liver (43) and germinating soybeans (36) the enzyme is strictly microsomal (Table 7).

In yeasts, as in bacteria, phosphatidylserine is synthesized by the reaction of CDPdiacylglycerol with serine (41). In this and previous (9) studies, corresponding enzymes were found both in the endoplasmic reticulum and in mitochondria of yeast. In mammalian (13, 23, 43) and plant (31) cells,

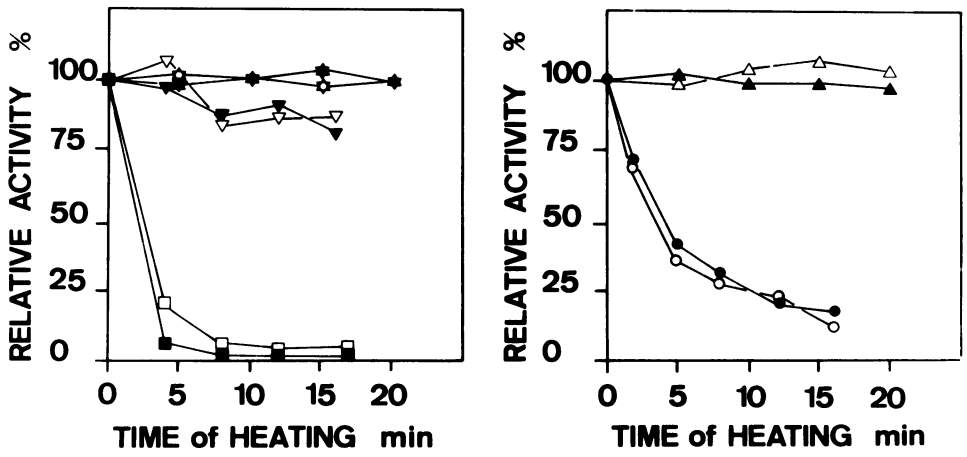

FIG. 4. Heat sensitivity of mitochondrial and microsomal phospholipid-synthesizing enzymes. Mitochondria and microsomes were incubated for various times at 50°C and cooled on ice, and enzyme activities were determined as described in the text. Filled symbols refer to mitochondrial enzymes, and open symbols refer to microsomal enzymes. Symbols: ■, glycerolphosphate acyltransferase; ★, phosphatidylinositol synthase; ▲, phosphatidylserine synthase; ▼, cholinephosphotransferase; ●, phosphatidate cytidylyltransferase.

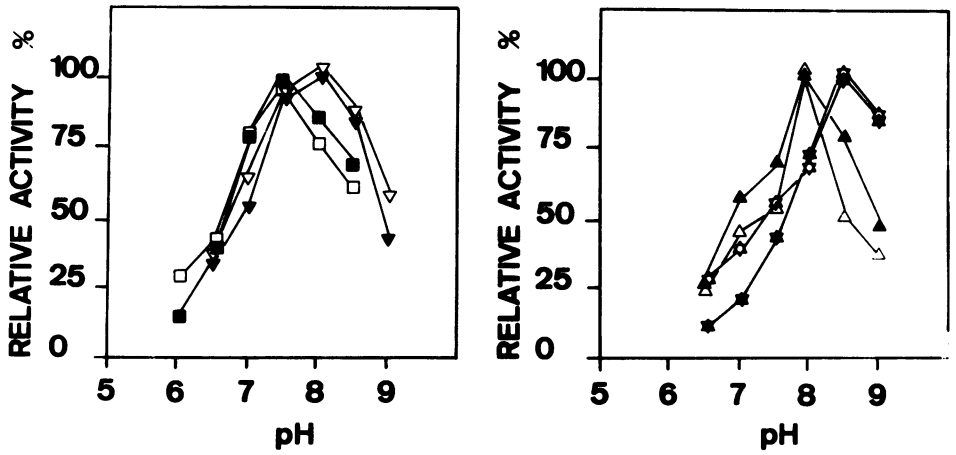

FIG. 5. pH optima of mitochondrial and microsomal phospholipid-synthesizing enzymes. Mitochondria and microsomes were incubated at the indicated pHs, and enzyme activities were assayed as described in the text. Symbols are as described in the legend to Fig. 3.

phosphatidylserine is formed by a Ca^{2+}-dependent base exchange reaction which takes place exclusively in the endoplasmic reticulum, e.g., of rat liver, rat brain, or castor bean endosperm. Interestingly, the corresponding enzymes of Ehrlich ascites tumor cells (2) or Morris hepatoma cells (20) are found mainly in mitochondria. The absence of phosphatidylserine synthesis via base exchange in *S. cerevisiae* has been confirmed in the present study (data not shown).

CDPdiacylglycerol, a key intermediate in the biosynthesis of glycerophospholipids, is formed both in the endoplasmic reticulum and in mitochondria of yeast (9, 22) (Tables 3 and 4).

According to Cobon et al. (9), the biosynthesis of phosphatidylcholine both via methylation of phosphatidylethanolamine and via the cholinephosphotransferase reaction occurs exclusively in the endoplasmic reticulum. This result contrasts with a report of Ostrow (D. Ostrow, Fed. Proc. 30:1226, 1971) who found cholinephosphotransferase in the endoplasmic reticulum and in mitochondria of *S. cerevisiae* cells. Similar results were obtained in the present study, indicating that mitochondria have the capacity to synthesize phosphatidylcholine, the major glycerophospholipid of this organelle.

The submitochondrial location of phospholipid-synthesizing enzymes in *S. cerevisiae* has not been reported before (with the exception of cholinephosphotransferase). Two enzymes which are localized exclusively in mitochondria, namely phosphatidylglycerolphosphate synthase and phosphatidylserine decarboxylase, were found to be part of the inner mitochondrial membrane. The outer mitochondrial membrane contained those enzymes which were common to mitochondria and to the endoplasmic reticulum. One of the enzyme activities tested, phosphatidate cytidylyltransferase, was present in three membrane fractions, i.e., in the endoplasmic reticulum and in the outer and the inner mitochondrial membranes.

The orientation of phospholipid-synthesizing enzymes within membranes of subcellular organelles is only scarcely documented in the literature. The available data refer to rodent liver and brain, but nothing was hitherto known about the situation in *S. cerevisiae* cells.

In rat liver mitochondria, glycerolphosphate acyltransferase is oriented toward the inner aspect of the outer membrane (6). The mitochondrial glycerolphosphate acyltransferase of *S. cerevisiae* cells faces the cytoplasmic side of the outer membrane (Fig. 2), which is similar to the case with phosphatidylinositol synthase and phosphatidylserine synthase. The transmembrane orientation of other mitochondrial enzymes investigated in this study, two of which are also present in mitochondria of higher eucaryotic cells (phosphatidate cytidylyltransferase and cholinephosphotransferase) has, to our knowledge, not been reported for animal or plant cells.

The fact that analogous enzyme activities have been found in both mitochondria and the endoplasmic reticulum poses

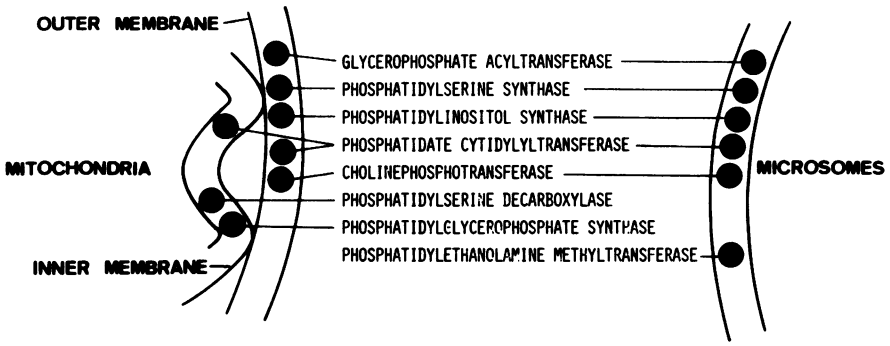

FIG. 6. Subcellular and submitochondrial location of phospholipid-synthesizing enzymes in *S. cerevisiae*.

TABLE 7. Subcellular and submitochondrial location of phospholipid-synthesizing enzymes in higher eucaryotes

Enzyme	Location in type of eucaryotic cell (reference):			
	Endoplasmic reticulum	Mitochondria	Outer mitochondrial membrane	Inner mitochondrial membrane
Glycerolphosphate acyltransferase	Rat liver (6)	Rat liver (6)	Rat liver (6, 16)	
Phosphatidylinositol synthase	Rat liver (43); germinating soybeans (36)			
Phosphatidate cytidylyltransferase	Rat liver (75%) (19)	Rat liver (25%) (19); castor bean endosperm (40); Morris hepatoma (19)	Rat liver (21)	Rat liver (18, 21); castor bean endosperm (40)
Cholinephosphotransferase	Rat liver (24); castor bean endosperm (29)	Rat liver (21)		
Phosphatidylethanolamine methyltransferase	Rat liver (43)	Castor bean endosperm (40)		
Phosphatidylglycerolphosphate synthase		Mammalian cells (18); plant cells (40)		Plant cells (40); rat liver (18, 21)
Phosphatidylserine decarboxylase		Rat liver (13); calf brain (33)		Rat liver (43); calf brain (33)

the question as to the molecular identity of the enzyme proteins involved. Data concerning pH optima, heat sensitivity, and susceptibility to various inhibitors (Table 6) indicate that the properties of mitochondrial and microsomal enzymes that catalyze the same reaction are closely related. As mentioned above and previously (6, 16), glycerolphosphate acyltransferases of rat liver mitochondria and microsomes are distinct with respect to acyl-CoA preference and inhibition by sulfhydryl reagents. We believe that neither of these experiments can prove or disprove the identity of enzymes that are common to two or more subcellular organelles. Only the results from more elaborate experiments, above all the characterization of enzymes by methods of protein chemistry, in combination with the use of mutants with defects in the respective structural genes, will provide a definite answer to this interesting problem.

Another as yet poorly understood process is the intracellular transport of phospholipids from their site of synthesis to membranes which themselves have no capacity for phospholipid synthesis. In principle, yeast mitochondria are autonomous with respect to the biosynthesis of their component glycerophospholipids. However, studies on phospholipid transfer in vivo (G. Daum, unpublished data) have shown that phosphatidylcholine synthesized via methylation in the endoplasmic reticulum is rapidly incorporated into mitochondrial membranes. On the other hand, phosphatidylethanolamine produced by the decarboxylation of phosphatidylserine in the inner mitochondrial membranes very rapidly reaches the endoplasmic reticulum, in which part of it is methylated to phosphatidylcholine. Thus, there appears to be extensive lipid traffic even between those two compartments which can synthesize most of their own phospholipids. Other subcellular membranes, such as those of vacuoles, nuclei, or the plasma membrane, are completely dependent on the delivery of lipids from the endoplasmic reticulum, the mitochondria, or both. Data presented here will be helpful for the design of further experiments aimed at understanding how phospholipids are transported across and between subcellular membranes.

ACKNOWLEDGMENT

This work was supported by Projekt 5512 from the Fonds zur Förderung der wissenschaftlichen Forschung in Österreich.

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