Synthesis and Intracellular Transport of Aminoglycerophospholipids in Permeabilized Cells of the Yeast, Saccharomyces cerevisiae*

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The sequence of biosynthetic steps from phosphatidylserine to phosphatidylethanolamine (via decarboxylation) and then phosphatidylcholine (via methylation) is linked to the intracellular transport of these aminoglycerophospholipids. Using a [3H]serine precursor and permeabilized yeast cells, it is possible to follow the synthesis of each of the aminoglycerophospholipids and examine the requirements for their interorganelle transport. This experimental approach reveals that in permeabilized cells newly synthesized phosphatidylserine is readily translocated to the locus of phosphatidylserine decarboxylase 1 in the mitochondria but not to the locus of phosphatidylserine decarboxylase 2 in the Golgi and vacuoles. Phosphatidylserine transport to the mitochondria is ATP independent and exhibits no requirements for cytosolic factors. The phosphatidylethanolamine formed in the mitochondria is exported to the locus of the methyltransferases (principally the endoplasmic reticulum) and converted to phosphatidylcholine. The export of phosphatidylethanolamine requires ATP but not any other cytosolic factors and is not obligately coupled to methyltransferase activity. The above described lipid transport reactions also occur in permeabilized cells that have been disrupted by homogenization, indicating that the processes are extremely efficient and may be dependent upon stable structural elements between organelles.

In yeast as well as in higher eukaryotes, the endoplasmic reticulum and the inner mitochondrial membrane are generally accepted as the major sites of membrane glycerophospholipid biosynthesis (Bishop and Bell, 1988; Zinser et al., 1991). Other organelles are largely devoid of phospholipid-synthesizing enzymes. This segregation of lipid-synthesizing activity necessitates efficient interorganelle transport to maintain membrane lipid composition, integrity, and function. Several mechanisms of intracellular lipid translocation (for reviews, see Bishop and Bell (1988), van Meer (1989), Trotter and Voelker (1994)) have been proposed including 1) spontaneous and protein-facilitated transport of monomeric phospholipids through the cytosol; 2) vesicle budding, translocation, and fusion; 3) regulated organelle juxtaposition and/or contact. Currently there is sufficient evidence to implicate each of the proposed mechanisms depending upon the donor and acceptor membrane and the lipid transferred.

The topological segregation of yeast enzymes involved in aminoglycerophospholipid synthesis: phosphatidylserine synthase in the endoplasmic reticulum or closely related membranes (Zinser et al., 1991; Gaigg et al., 1995); phosphatidylserine decarboxylase 1 in mitochondria and phosphatidylserine decarboxylase 2 in the Golgi and vacuole (Trotter et al., 1995); and phosphatidylethanolamine methyltransferases in the endoplasmic reticulum (Kuchler et al., 1986), enables the sequential metabolism of phosphatidylserine \( \rightarrow \) phosphatidylethanolamine \( \rightarrow \) phosphatidylcholine to be used to follow interorganelle lipid transport. This technique has been applied to intact and permeabilized mammalian cells, as well as to isolated organelles (for a review, see Trotter and Voelker (1994)). The rate of phosphatidylserine transfer to mitochondria was greatly reduced by ATP depletion in intact cells (Voelker, 1985) and stimulated by ATP addition in permeabilized cells (Voelker, 1989b, 1990, 1993). Interorganelle translocation of phosphatidylserine was shown to be largely independent of soluble cytosolic proteins in permeabilized mammalian cells (Voelker, 1989b) and in a cell free system (Voelker, 1989a). Membrane collision and/or vesicle flux have been suggested as possible mechanisms of this transport process. Membrane contact of mitochondria with a phospholipid-synthesizing microsomal fraction (Vance, 1990; Ardail et al., 1993; Gaigg et al., 1995) is probably the basis of lipid transport between these two organelles. Work with disrupted permeabilized mammalian cells provided evidence that structural elements coupling a donor membrane compartment to the mitochondria were involved in phosphatidylserine transport (Voelker, 1993).

The use of [3H]serine metabolism to [3H]phosphatidylethanolamine as a specific measure of phosphatidylserine transport to the mitochondria has come under closer scrutiny as a result of the recent finding that there are two phosphatidylserine decarboxylases, one in the mitochondria (PSD1) (Trotter et al., 1993; Clancy et al., 1993) and the other in the Golgi apparatus and the vacuole (PSD2) (Trotter and Voelker, 1995; Trotter et al., 1995). The availability of well defined psd1 and psd2 mutants (Trotter et al., 1993, 1995; Trotter and Voelker, 1995) now makes it possible to evaluate which transport pathways are being followed in the metabolism of nascent phosphatidylserine.

In this report we describe the optimization of the permeabi-
lized yeast cell technique in order to 1) define the requirements for aminoglycerophospholipid synthesis and transport, 2) discriminate between nascent phosphatidylserine transport to the mitochondria and Golgi plus vacuoles, and 3) define the energetic requirements for phosphatidylserine transport to, and phosphatidylethanolamine transport from, the mitochondria. We present evidence that the PSD1 gene product catalyzes synthesis of the vast majority of cellular phosphatidylethanolamine via decarboxylation in permeabilized cells.

**EXPERIMENTAL PROCEDURES**

**Strains and Culture Conditions**—The wild-type yeast strains Saccharomyces cerevisiae X-2180 (a SUC2 mal gal2 CUP1) and S. cerevisiae 422 (a trp1-289 leu2-3, 112 his7 lys2) (kindly provided by Dr. R. Sclafani, Denver) and the phosphatidylserine decarboxylase-deficient yeast strains PTY13 (a trp1 leu2 his2 lys2 psd1-1::TRP1), PTY22 (a trpl leu2 his ade1 lys2 psd2), and PTY18 (a trpl leu2 his ade1 psd1-1::TRP1 psd2) were grown on YPD medium (1% yeast extract, 2% peptone, 3% glucose) under aerobic conditions at 30°C to an A600 of 2-4. The PTY18 strain was additionally supplemented with 2 mM ethanolamine.

Preparation of Spheroplasts, Permeabilized Yeast Cells, and Yeast Subcellular Fractions—Yeast spheroplasts were prepared by the method of Daum et al. (1982) with the modification that YPD (0.5% glucose) was present during treatment of cells with zymolyme. Permeabilized yeast cells were prepared essentially as described by Baker et al. (1988) with minor modifications. In brief, spheroplasts regenerated for 20 min at 30°C in the presence of 0.75% yeast extract, 1.5% peptone, 1% glucose, and 0.7 M sorbitol were washed with lysis buffer consisting of 0.4 M sorbitol, 20 mM HEPES, pH 6.8, 0.05 M potassium acetate, 2 mM magnesium acetate, and 0.5 mM EGTA. Then, spheroplasts were suspended in the lysis buffer at a concentration of approximately 0.5 g wet weight per ml. The suspension was divided into 0.2–0.3-ml portions, filled in Eppendorf tubes, and frozen over liquid nitrogen. Freezing was complete within 15 min. At this stage, cells were stored at −70°C and used for 6 months. For the latter procedure, 20 μl of the suspension of permeabilized cells were diluted with 0.98 ml of 1.2 M sorbitol and centrifuged for 5 min at 4°C at 6,500 rpm in an Eppendorf tabletop centrifuge. The supernatant, which should contain only the cytosol and the pellet, which should contain permeabilized cells, were tested with antibodies against cytosolic glycerolaldehyde-3-phosphate dehydrogenase, mitochondrial porin, and 40-kDa microsomal protein, Kex2 protease, and carboxypeptidase Y. The degree of cell permeabilization was found to be approximately 85–95% as judged from the remaining cytosol in the cellular pellet. Organelles probed with antibodies mentioned above were more or less completely recovered in permeabilized cells in the pellet. Intactness of mitochondria was tested using an antibody against cytochrome b₅, an intermembrane space protein (Daum et al., 1982), and found to be unaffected by the cell permeabilization procedure.

Cell homogenates were prepared by suspending permeabilized yeast cells in 0.6 M mannitol, 10 mM Tris-Cl, pH 7.4, 1 mM phenylmethylsulfonyl fluoride, 10% glycerol, and 10 μg of leupeptin per ml. Mitochondria and microsomal fractions were prepared as described elsewhere (Daum et al., 1982; Zinser et al., 1991).

Synthesis of Aminoglycerophospholipids in Permeabilized Yeast Cells—A typical assay mixture for 8 time points to be taken contained 2 mg of cellular protein (permeabilized cells) in a total volume of 0.9 ml. The reaction mixtures consisted of 90 μl of 0.6 M mannitol, 400 μl of assay buffer (0.6 M mannitol, 50 mM Tris-Cl, pH 8.0), and 50 μl of permeabilized yeast cells (0.5 g wet weight per ml). Permeabilized cells were added last to avoid an osmotic shock. The reaction was started by the addition of 45 μl of 2 mM unlabeled serine and 30 μl of [3H]serine (specific activity 15–40 Ci/mmol; 30 μCi in 0.9-ml assay volume). The incubation temperature was 30°C, and samples of 0.1 ml were taken at the time points indicated. Under these conditions, synthesis of phosphatidylserine occurred. After 15 min of incubation, ETA (stock solution: 0.1 M in 0.6 M mannitol, 50 mM Tris-Cl, pH 8.0) was added to a final concentration of 4 mM. ETA stopped the synthesis of phosphatidylserine and stimulated the synthesis of phosphatidylethanolamine. Samples of 0.1 ml plus the aliquot volume of EDTA were taken in this second phase. After 15 min of further incubation, MgCl₂ (final concentration 8 mM) and 5-adenosylmethionine (AdoMet; final concentration 0.23 mM) were added. A stock solution was prepared of an appropriate amount of AdoMet in 0.6 M mannitol, 50 mM Tris-Cl and adjusting to pH 8.0 with Tris base. The AdoMet stock solution was freshly prepared for every experiment. MgCl₂ was prepared as a 0.1 M stock solution in 0.6 M mannitol, 50 mM Tris-Cl, pH 7.5. In the presence of MgCl₂ and AdoMet, phosphatidylethanolamine was synthesized. Samples of 0.1 ml plus the aliquot volume of added EDTA, MgCl₂, and AdoMet were taken in the third phase of the incubation.

MgATP or MnATP, an ATP-regenerating system (10 units of creatine phosphokinase per mg of protein and 5 mM creatine phosphate), N-ethylmaleimide, adriamycin, apyrase, oligomycin, and azide were added at final concentrations as indicated in Table II. Permeabilized cells were preincubated with MgATP and inhibitors for 5 min on ice and with apyrase for 30 min at 30°C. In order to remove cytosol, permeabilized cells were washed twice with 0.2 ml of lysis buffer and resolubilized by centrifugation at 1,000 × g for 30 s in an Eppendorf tabletop centrifuge. The same washing procedure was employed to remove apyrase from permeabilized cells. Subsequently, cells were carefully suspended in the respective incubation buffer.

When radiolabeled phospholipids were localized in subcellular fractions, 3.5 mg of permeabilized cells were incubated in 1.8 ml of the assay mixture as described above. After 20, 40, and 120 min of incubation under appropriate conditions, 0.4-ml samples were taken and chilled, and 5 mM azide and fluoride and 8 mM EDTA and hydroxyamine (final concentration, each) were added. Mitochondria and 40,000 × g microsomes (2 mg of protein, each) were added as carrier, and the respective fractions were isolated by standard procedures (Daum et al., 1982; Zinser et al., 1991). Azide, fluoride, EDTA, and hydroxyamine (concentrations see above) were present throughout the isolation procedure. The purity of fractions was essentially the same as described by Zinser et al. (1991).

Extraction and Analysis of Phospholipids—All aliquots (0.1–0.116 ml) of the incubation mixture (see above) were extracted with 4 ml of chloroform/methanol (2:1, v/v) for 1 h at room temperature with repeated vortexing. Prior to the extraction carrier, phospholipids (approximately 50–100 μg of soybean phospholipids, Epiuron) were added. The organic phase was washed once with 2 ml of 0.34% MgCl₂, once with 2 ml of KCl/methanol (4:1, v/v), and once with 2 ml of methanol/water/chloroform (48:47:3, v/v). 2 ml of the resulting organic phase were withdrawn and taken to dryness under a stream of nitrogen. Lipids were dissolved in 30 μl of chloroform/methanol (2:1, v/v) and subjected to thin layer chromatography on Silica Gel 60 (Merck, Darmstadt, Germany) or Silica Gel H (Analtech, Newark, DE). Chloroform, methanol, 25% ammonia, and water were used as developing solvent. Spots were visualized in iodine vapor or by spraying the thin layer plates with 0.1% aqueous 8-anilino-1-naphthalenesulfonic acid and exposure to UV light. Individual phospholipids were identified with authentic standards, scraped from thin layer plates, and counted in 8 ml of Safety Mixture (Baker) plus 5% water or 4.5 ml of Scintisol (Fisher) plus 0.3 ml water.

Miscellaneous Methods—Proteins were quantified by the method of Lowry et al. (1951), or using the BCA protein assay system (Pierce). SDS-polyacrylamide gel electrophoresis (Laemmli, 1970) and Western blotting (Hnilica and Sadowski, 1983) were carried out by published procedures. Immunoreactive proteins on nitrocellulose membranes or blots were detected by alkaline phosphatase-linked immunoglobulin G or rabbit antibodies coupled to peroxidase or alkaline phosphatase following the manufacturer’s instructions. Stained bands were quantified by densitometric scanning using a Shimadzu CS 930 chromatogram scanner.

**Cellular ATP** was quantified using the firefly luciferase bioluminescence assay as described by Lundin (1982).

**Data Analysis**—Data shown in the figures are from one representative of at least three independent experiments. Results of these experiments were essentially the same, but absolute values varied slightly due to variance in the uptake of [3H]serine, the degree of permeabilization of spheroplasts, and the amount of cells used. Permeabilized yeast cells were used for assays only when the degree of permeabilization was higher than 80%.1

1 The abbreviation used is: AdoMet, S-adenosylmethionine.
Phospholipid Synthesis and Transport in Permeabilized Yeast

Yeast Cells Can Be Efficiently Permeabilized for Studies of Synthesis and Intracellular Transport of Phospholipids—In contrast to mammalian cells, permeabilized yeast cells have not been as widely used to study problems of intracellular transport of macromolecules. Despite a low degree of permeabilization (approximately 50%), the yeast system yielded reliable results for the study of the protein secretory pathway (Baker et al., 1998), because externally supplied macromolecules were not able to enter intact spheroplasts and were functional in permeabilized cells only. By comparison, a low degree of permeabilization would make results presented in this study difficult to interpret, because the low molecular weight precursor of aminoglycerophospholipids, \[^{3}H\text{]serine, can easily cross the plasma membrane of intact spheroplasts. For this reason we optimized the permeabilization procedure of yeast cells for our purposes. In brief, rates of freezing and thawing were found to strongly influence the quality and the stability of permeabilized cells. As can be seen from Table I, the procedure outlined under “Experimental Procedures” yielded a high degree of permeabilization with minimal disruption of subcellular structures (organelles). At least 90% of the soluble enzyme glycerol-phosphate dehydrogenase was released from the cells as determined by either immunoblot analysis or enzyme activity measurement. Similar results were obtained by the activity measurement of glucose-6-phosphate dehydrogenase. Measurement of proteins associated with the mitochondrial outer membrane (porin) or the intermembrane space (cytochrome b) detected by immunoblotting demonstrated that mitochondria were at least 90% retained in the permeabilized cell pellet. These latter results also clearly demonstrate that the structural integrity of the mitochondria were not compromised by permeabilization. Additives to the permeabilized cell preparation, e.g. EDTA or AdoMet (see below), do not gain access to organelles of intact spheroplasts. The effect of EDTA and AdoMet were apparent only in permeabilized cells and served as additional independent indicators for the degree of permeabilization.

Permeabilized Yeast Synthesize Phosphatidylserine and Metabolize It to Phosphatidylethanolamine and Phosphatidylcholine—The synthesis of phosphatidylserine and its subsequent metabolism to phosphatidylethanolamine and phosphatidylcholine can be followed readily using \[^{3}H\text{]serine as a precursor. Although CDP-diaclyglycerol is also required for phosphatidylserine synthesis, we found that sufficient levels of this lipid nucleotide were present in the permeabilized yeast cells to support the first step of aminoglycerophospholipid synthesis for at least 120 min. This situation is advantageous because it avoids the addition of exogenous CDP-diaclyglycerol which has some detergent properties and can compromise organelle integrity.

The synthesis of phosphatidylserine depends on the presence of divalent cations, especially of Mn\(^{2+}\), in the incubation mixture (Bae-Lee and Carman, 1984; Sperka-Gottlieb et al., 1990). The subsequent step in the biosynthetic pathway of aminoglycerophospholipids, the decarboxylation of phosphatidylserine leading to phosphatidylethanolamine, is inhibited by divalent cations and stimulated in the presence of EDTA (Lamping et al., 1991). Finally, conversion of phosphatidylethanolamine to phosphatidylcholine needs the addition of S-adenosylmethionine (AdoMet) and the presence of Mg\(^{2+}\) (Kodaki and Yamashita, 1989). The different optima for each step in aminoglycerophospholipid synthesis necessitated the design of a three-step assay, which fulfills all the above-mentioned requirements.

In a standard experiment shown in Fig. 1 (details given under “Experimental Procedures”), permeabilized yeast cells were first incubated for 15 min with \[^{3}H\text{]serine in the presence of Mn\(^{2+}\) to form phosphatidylserine. Subsequently, EDTA was added, and the formation of phosphatidylethanolamine was followed for another 15 min. As can be seen from Fig. 1, practically no phosphatidylethanolamine was formed during the first phase of the incubation, but the addition of EDTA stopped the synthesis of phosphatidylserine and stimulated its decarboxylation to phosphatidylethanolamine. Without addition of EDTA, no phosphatidylethanolamine was formed (data not shown).

Finally, AdoMet and Mg\(^{2+}\) were added, and the formation of phosphatidylcholine was followed for another 70 min (see Fig. 1). In control experiments carried out without AdoMet and Mg\(^{2+}\), no phosphatidylcholine was synthesized (data not shown). During this phase of the assay, incorporation of \[^{3}H\text{]serine into phosphatidylserine was observed again, because phosphatidylserine synthase was reactivated in the presence of divalent cations. Phosphatidylethanolamine formation also continued under these conditions indicating that Mg\(^{2+}\) inhibition may only be relevant in assays using solubilized enzyme. Radioactivity was not only found in phosphatidylcholine, but also in phosphatidylcholine and lyso-phosphatidylethanolamine (−5 and 10% of labeled phospholipids, respectively). For the sake of clarity, only values of phosphatidylcholine, the fully methylated end product in the sequence of biosynthetic steps of aminoglycerophospholipids, are shown (see also Fig. 1).

In Permeabilized Yeast the Majority of Nascent Phosphatidylserine That Is Decarboxylated Is Transported to the Mitochondria—The occurrence of phosphatidylserine decarboxylase activity in two different compartments of the yeast (Trotter et al., 1993; Trotter and Voelker, 1995) raises the possibility that phosphatidylserine destined to be converted to phosphatidylethanolamine could be translocated to the Golgi or the vacuole in addition to being imported into mitochondria. In vivo, the activity derived from the extramitochondrial gene product is sufficient for a balanced phospholipid metabolism and cellular growth (Trotter et al., 1993). In principal, the total amount of phosphatidylethanolamine required for yeast growth can be synthesized without the participation of the mitochondrial phosphatidylserine decarboxylase. In order to clarify which pathway contributes to phosphatidylethanolamine synthesis in permeabilized cells, we conducted experiments using yeast strains with psd1 (mitochondrial) and psd2 (extramitochondrial) mutations. Permeabilized cells containing the psd1 null allele (Fig. 2B) formed \[^{3}H\text{]phosphatidylethanolamine at less
than 5% of the wild-type control (Fig. 2A), and only trace amounts of phosphatidylcholine were formed. In contrast, permeabilized cells with the psd2 mutation (Fig. 2C) exhibited significant synthesis of phosphatidylethanolamine (via decarboxylation) and phosphatidylcholine. The strain with both the psd1 and psd2 mutations, which completely lacks detectable phosphatidylserine decarboxylase activity, produced radioactive phosphatidylethanolamine and phosphatidylcholine at only background levels (Fig. 2D). The data shown in the figure are from a representative of three independent experiments.

**FIG. 1.** Aminoglycerophospholipid biosynthesis in permeabilized yeast cells. Permeabilized cells of the wild-type strain, *S. cerevisiae* X-2180, were prepared and incubated with [3H]serine as described. During the first 15 min, the incubation mixture contained 0.6 mM Mn

**FIG. 2.** Phosphatidylserine decarboxylase 1 produces the majority of phosphatidylethanolamine formed in permeabilized yeast. Permeabilized cells of the yeast strains *S. cerevisiae* 422 (wild-type) (A), PTY13 (psd1-D1::TRP1) (B) with a defect in the mitochondrial phosphatidylserine decarboxylase, PTY22 (psd2) (C) defective in a second extramitochondrial phosphatidylserine decarboxylase activity, and PTY18 (psd1-D1::TRP1psd2) (D), a double mutant lacking both phosphatidylserine decarboxylase activities, were prepared and incubated with [3H]serine as described under "Experimental Procedures" and in the legend to Fig. 1. Data shown in the figure are from a representative of three independent experiments. □, phosphatidylserine; ●, phosphatidylethanolamine; ■, phosphatidylcholine. SAM, AdoMet.
Phosphatidylserine synthesis is coupled to cellular transport. In the mammalian system, ATP is required for phosphatidylserine synthesis, which could in turn cause different requirements for intracellular ATP. The requirement of ATP for phosphatidylserine synthesis between the two types of cells may result in different physical topology of nascent phosphatidylserine transport. The discrepancies between the mammalian and the yeast system may be attributable to differences in phosphatidylserine synthesis between the two types of cells. Yeast phosphatidylserine synthesis is a CDP-diacylglycerol-dependent reaction (Paltauf et al., 1991 and Gaigg et al., 1995). Therefore, export of phosphatidylethanolamine from mitochondria is required to deliver the substrate to the methyltransferases.

Preliminary characterization of phosphatidylethanolamine export from the mitochondria as monitored by phosphatidylethanolamine formation revealed it did not require cytosol and was inhibited by the addition of apyrase plus oligomycin. Additional experiments, however, demonstrated that methyltransferase activity was also markedly inhibited by apyrase plus oligomycin treatment. This latter finding made phosphatidylethanolamine formation an unreliable indicator for phosphatidylethanolamine export from the mitochondria in the presence of apyrase and oligomycin. In order to critically test the role of ATP in phosphatidylethanolamine export from mitochondria, we carried out subcellular fractionation of permeabilized yeast cells after each stage of incubation, i.e. after 20, 40, and 120 min of incubation under the optimized conditions for phosphatidylserine, phosphatidylethanolamine, and phosphatidylcholine synthesis. Appearance of phosphatidylethanolamine in microsomes served as an indicator of its export from mitochondria. In the presence of apyrase (Fig. 3A), phosphatidylethanolamine transport from mitochondria to microsomes was dramatically decreased. As a consequence, conversion of phosphatidylethanolamine to phosphatidylcholine could not occur (Fig. 3B). In control assays without apyrase, phosphatidylethanolamine reached the microsomal fractions and was, in part, further converted to phosphatidylcholine. This result confirmed the idea that export of phosphatidylethanolamine from mitochondria is an energy-dependent step in this part of the pathway leading to phosphatidylcholine thus confirming previous findings with intact yeast cells (Daum et al., 1986; Gnamusch et al., 1992). Data shown in Fig. 3, A and B, also demonstrate that phosphatidylcholine formed in microsomes of untreated cells can be readily transported back to mitochondria.

When de-energized permeabilized yeast cells were re-energized by washing and addition of ATP, export of phosphatidylethanolamine from mitochondria and formation of phosphatidylcholine recovered (Fig. 3, C and D). Ongoing methylation of phosphatidylethanolamine was not the limiting factor of the translocation step, because in the absence of AdoMet phosphatidylethanolamine reached the endoplasmic reticulum (Fig. 3E), although further conversion to phosphatidylcholine could not occur (Fig. 3F). Thus, the methylation of

| Table II | Import of phosphatidylserine into mitochondria of permeabilized yeast cells |
|----------|-------------------------------------------------------------------------|
|          | % phosphatidylethanolamine formeda                                      |
| Controlb |                               |
| − Cytosol| 100                         |
| + Adriamycin (600 μM) | 97 ± 9                   |
| + N-Ethylmaleimide (14 mM) | 103 ± 17                 |
| + ATP + MgCl₂ (1 mM) | 87 ± 6                    |
| + ATP + MgCl₂ (0.1 mM) | 105 ± 6                   |
| + ATP-regenerating systemb | 112 ± 2                   |
| + Apyrase (10 units/mg of protein) + oligomycin (0.07 mM) | 95 ± 13                  |
| Homogenate | 94 ± 19                   |

* Relative amounts of disintegration/min in [3H]phosphatidylethanolamine after the second phase of incubation (30 min) were calculated in percent.

b Control assays contained untreated permeabilized yeast cells including the cytosol.

c 10 units of creatine phosphokinase per mg of protein and 5 mM creatine phosphate.

d The equivalent of untreated control cells was strongly homogenized and used for this assay.

Adriamycin affects an earlier step than contact site transit of phosphatidylserine, which may not be identical with that of the yeast system.

Contact between phospholipid-synthesizing membranes and mitochondria has been suggested as a possible prerequisite for the import of phospholipids into mitochondria of mammalian cells (Voelker 1989a, 1990, 1993; Vance, 1991; Ardaill et al., 1991) and yeast (Gaigg et al., 1995). In permeabilized mammalian cells that are disrupted by shearing, transport of nascent phosphatidylserine appears to be restricted by specific structural association between the mitochondria and the membrane compartment synthesizing the lipid (Voelker, 1993). As found for mammalian cells, disruption of permeabilized yeast by homogenization yields cell preparations that remain competent to synthesize, transport, and metabolize phosphatidylserine (see Table II).

**Phosphatidylethanolamine Export from Mitochondria to the Locus of the Methyltransferases:** Requires ATP—Phosphatidylethanolamine formed in mitochondria by the action of phosphatidylserine decarboxylase can be further metabolized to phosphatidylcholine by the nonmitochondrial AdoMet-dependent phosphatidylethanolamine-N-methyltransferase and phospholipid-N-methyltransferase (Kuchler et al., 1986; Gaigg et al., 1995). Therefore, export of phosphatidylethanolamine from mitochondria is required to deliver the substrate to the methyltransferases.

The import of phosphatidylserine into mitochondria of permeabilized yeast was shown to be without effect on the synthesis, transport, and decarboxylation of phosphatidylserine. Addition of MgATP to the assay mixture did not have a stimulatory effect on the formation of phosphatidylethanolamine. This latter result could be due to the high levels of ATP present in the spheroplast prior to permeabilization. However, preincubation of permeabilized cells with oligomycin and apyrase which deplete cellular ATP practically to negligible levels (as measured by the luciferin-luciferase assay) was also ineffective at altering phosphatidylserine transport.

The discrepancies between the mammalian and the yeast system may be attributable to differences in phosphatidylserine synthesis between the two types of cells. Yeast phosphatidylserine synthesis is a CDP-diacylglycerol-dependent reaction (Paitha et al., 1992), whereas the mammalian enzyme catalyzes base exchange (see Bishop and Bell, 1988). Both enzyme systems are microsomalized, located, and may result in different physical topology of nascent phosphatidylserine. As a consequence, the accessibility of phosphatidylserine to the lipid translocation machinery may be different, which could in turn cause different requirements for intracellular transport. In the mammalian system, ATP is required for both translocation and synthesis of phosphatidylserine (Voelker 1990, 1993). Phosphatidylserine synthesis is coupled to the action of the Ca²⁺-sequestering ATPase of the endoplasmic reticulum. Yeast phosphatidylserine synthesis is not Ca²⁺-dependent. The enzymatic reaction is energy-dependent due to the requirement of CDP-diacylglycerol as a co-substrate, but is not directly affected by ATP.

Permeabilized yeast cells preincubated with adriamycin exhibited a higher rate of phosphatidylserine biosynthesis than untreated cells (data not shown). However, in contrast to mammalian cells, adriamycin did not affect the import of phosphatidylserine into mitochondria of permeabilized yeast cells (see Table II). This observation is consistent with previous findings by Simbeni et al. (1993) obtained in vitro with isolated yeast mitochondria. Adriamycin binds with high affinity to cardiolipin (Goormaghtigh et al., 1984), a lipid that is present at high concentrations in contact sites between the outer and the inner mitochondrial membrane (Ardaill et al., 1990; Simbeni et al., 1991). The interaction of adriamycin with cardiolipin is one piece of evidence implicating zones of mitochondrial membrane contact as sites of protein import in yeast (Eilers et al., 1989) and phospholipid import in mammalian cells. The differential effects of adriamycin upon yeast and mammalian lipid transport remain unclear. In mammalian cells, it is possible that
phosphatidylethanolamine did not exert a feedback control on its export from mitochondria.

CONCLUSIONS

While the precise mechanism of phospholipid translocation between organelles remains uncertain, circumstantial evidence continues to accrue in favor of membrane contact as an important prerequisite for this process. Membranes with a high capacity to synthesize phospholipids were found to be associated with mitochondria of mammalian (Vance, 1990; Ardai et al., 1991, 1993) as well as of yeast cells (Gaigg et al., 1995). In addition, Voelker (1993) demonstrated that transport and decarboxylation of phosphatidylserine in disrupted mammalian cells is restricted to those mitochondria associated with the phosphatidylserine-synthesizing membrane. The observation that homogenates obtained from permeabilized spheroplasts readily translocated phosphatidylserine and phosphatidylethanolamine between endoplasmic reticulum-related membranes and mitochondria indicates the process is very efficient in a cell-free system. One explanation of these results is that stable organelle contact might be the basis of lipid translocation. Alternatively, vesicle transport along structural corridors that tether different organelles can also be envisaged as a possible mechanism of phospholipid translocation. The role of cytosolic lipid transfer proteins (Wirtz, 1991), which catalyze lipid migration in vitro, as mediators of lipid transport in living cells still remains obscure despite extensive genetic and molecular biological studies (Bankaitis et al., 1989; 1990; Cleves et al., 1991a; 1991b) with the yeast phosphatidylinositol transfer protein. Another cytosolic yeast lipid transfer protein, which catalyzes phosphatidylserine and phosphatidylethanolamine transfer in vitro (Lafer et al., 1991), was originally regarded as a candidate to facilitate the transport of phosphatidylserine into, and of phosphatidylethanolamine out of, mitochondria. The fact that removal of cytosol from permeabilized yeast cells affects neither phosphatidylserine nor phosphatidylethanolamine translocation between the endoplasmic reticulum and mitochondria argues against such a function of the phosphatidylserine transfer protein. This result is in agreement with the observation that translocation of phosphatidylserine between the endoplasmic reticulum and mitochondria of mammalian cells in vitro is not stimulated by cytosolic proteins (Voelker, 1989a).

Finally, it should be considered that all membranes of a cell, which are not able to synthesize their own lipids, rely on the supply of lipids from synthesizing organelles. Most membranes of eukaryotic organelles contain the whole set of phospholipids, although at different amounts and proportions. If lipid-synthesizing membranes, e.g., the endoplasmic reticulum, associate with other cellular membranes in order to translocate lipids, contact between organelles could predetermine the subcellular distribution of lipids. In this case, physical factors governing membrane contact could regulate the assembly of lipids into membranes.

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