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Import of phosphatidylserine into isolated yeast mitochondria

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A yeast phosphatidylserine transfer protein was used as a tool to transport radioactively labeled phosphatidylserine from unilamellar vesicles to isolated mitochondria of the yeast, *Saccharomyces cerevisiae*. Import of phosphatidylserine into mitochondria was monitored by the appearence of radioactively labeled phosphatidylethanolamine, which is produced from phosphatidylserine by the action of phosphatidylserine decarboxylase. This enzyme is located on the outer aspect of the inner mitochondrial membrane. Import of phosphatidylserine into mitochondria and formation of phosphatidylethanolamine does not require ATP or an electrochemical gradient, and is not affected by adriamycin. Evidence is presented that contact sites between the two mitochondrial membranes are zones of intramitochondrial translocation of phosphatidylserine and phosphatidylethanolamine. When phosphatidylserine decarboxylase is inhibited by hydroxylamine, transport of phosphatidylserine to the mitochondrial surface is unaffected. Under these conditions only a small amount of phosphatidylserine accumulates in the inner mitochondrial membrane indicating that the intramitochondrial transport of phosphatidylserine and its metabolic conversion to phosphatidylethanolamine are linked processes.

Introduction

Phospholipid synthesis in yeast mitochondria is restricted to the formation of phosphatidylethanolamine (via decarboxylation of phosphatidylserine), phosphatidylglycerol and cardiolipin [1,2]. Similar to higher eukaryotes [3] the majority of yeast cellular phospholipids is synthesized in the microsomal fraction [2]. The fact that mitochondrial membranes, like all other cellular membranes, contain the whole set of cellular phospholipids, necessitates efficient import of those phospholipids into mitochondria which are not synthesized in this organelle. One phospholipid of special interest in this respect is phosphatidylserine, which serves as a substrate for the synthesis of phosphatidylethanolamine by phosphatidylserine decarboxylase in the inner mitochondrial membrane. The precursor-product relationship between phosphatidylserine and phosphatidylethanolamine provides a convenient system to measure the import of phosphatidylserine into mitochondria. Voelker [4–7] used this assay method successfully to study phosphatidylserine traffic in mammalian cells.

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Earlier work from our laboratory [8] revealed that intramitochondrial transport of phosphatidylinositol and phosphatidylserine is a vectorial process directed towards the inner mitochondrial membrane and driven by the synthesis of these phospholipids. Contact sites between the outer and the inner mitochondrial membrane [9,10], which have been shown to be zones of protein import into mitochondria, are thought to be involved also in the intramitochondrial movement of phospholipids. This hypothesis has been discussed for many years but lacked experimental proof. Results obtained recently from experiments with isolated contact sites from yeast [11] and from mouse liver [12] provided preliminary evidence that phosphatidylserine might indeed be translocated from the outer to the inner mitochondrial membrane via membrane contact sites. Other lipids have not been studied in that respect.

In the present paper the last step of phosphatidylserine import into mitochondria, namely its translocation across the outer and to the inner mitochondrial membrane is characterized in more detail. Energy requirement and linkage between transport and metabolic conversion of phosphatidylserine to phosphatidylethanolamine have been investigated. Further evidence for the involvement of membrane contact sites in the intramitochondrial translocation of phospholipids is presented.

Materials and Methods

Strain, culture conditions and subcellular fractionation

The wild-type yeast strain *Saccharomyces cerevisiae* D273-10B was grown under aerobic conditions at 30°C on a medium containing 2% lactate as a carbon source as described before [13]. Cells were grown overnight under vigorous shaking and harvested in the logarithmic phase.

Yeast spheroplasts, mitochondria and mitoplasts were isolated by published procedures [13]. Subfractionation of mitochondria yielding outer membrane, inner membrane and membrane contact sites was carried out by the method of Pon et al. [9]. Cross-contamination between subcellular fractions was routinely tested by scanning the protein bands on SDS-polyacrylamide gels and was essentially the same as described [11].

Yeast phosphatidylserine transfer protein

An approx. 50-fold enriched fraction of yeast phosphatidylserine transfer protein [14] was isolated from yeast cytosol using ammoniumsulfate precipitation, Sephadex G-75 molecular sieve chromatography and DEAE-Sephacel ion-exchange chromatography. The transfer activity of the resulting preparations was measured employing the radioassay described by Daum and Paltauf [15]. 1 unit (U) was defined as 1% phosphatidylserine transferred within 30 min in a standard assay.

Translocation of phosphatidylserine from unilamellar vesicles to isolated mitochondria

[^{3}H]Serine-labeled phosphatidylserine was prepared biosynthetically using the 40000 × *g* microsomal pellet of yeast cell homogenate as an enzyme source. This is the subcellular fraction with the highest specific activity of phosphatidylserine synthase (approx. 20 nmol/min per mg). Hydroxylamine was added to the incubation mixture to avoid conversion of radiolabeled phosphatidylserine to phosphatidylethanolamine by contaminating mitochondria. The incubation mixture contained 20 μCi [2-^{3}H]serine (10 Ci/mmol) and 2 mg microsomal membrane protein in a total volume of 2 ml 0.1 M Tris-HCl (pH 8), 5 mM hydroxylamine, 0.2% Triton X-100, 0.6 mM $MnCl_2$, 2 mM CDP-diacylglycerol. This mixture was stirred for 2 h at 30°C; then lipids were extracted with chloroform/methanol (2:1, v/v) according to Folch et al. [16]. Phosphatidylserine was the only radiolabeled compound in this extract. The specific activity of the product was approx. 1.5 Ci/mmol. For the preparation of 2 ml donor vesicle suspension an aliquot of the extract containing 0.3 nmol phosphatidylserine (3 nmol total phospholipid; 10^6 cpm), tracer amounts of [^{14}C]triacylglycerol (10^5 cpm) used as a non-transferable marker, and 17 nmol egg phosphatidylcholine were mixed, taken to dryness under a stream of nitrogen, suspended in 0.6 M mannitol, 10 mM Tris-HCl (pH 7.4) and sonicated at 75 Watt under cooling for 10 min.

When translocation assays were carried out in the presence of inhibitors or effectors, mitochondria were preincubated with the respective substances for 5 min on ice. Pretreatment of mitochondria with proteinases (proteinase K, trypsin) was carried out for 30 min on ice. Unless otherwise indicated the ratio of proteinase to mitochondrial protein was 1:40. Proteolysis was stopped by the addition of a 20-fold excess (w/w) of proteinase inhibitors, PMSF or soybean trypsin inhibitor, respectively. After further incubation for 5 min on ice mitochondria were used for transport assays. In control assays the inhibitor was added to the incubation mixture prior to the addition of proteinase.

Aliquots of assay mixtures used for one time-point to measure the transport of phosphatidylserine from vesicles to mitochondria consisted of 50 μl of a phospholipid vesicle suspension containing [^{3}H]phosphatidylserine (approx. 20000 cpm; see above), 50 μl of mitochondrial suspension (10 mg protein/ml), and additional components (phospholipid transfer protein, inhibitors) in a total volume of 0.3 ml 0.6 M mannitol, 10 mM Tris-HCl (pH 7.4). A standard assay contained 85–90 U yeast phosphatidylserine transfer protein in a total volume of 0.3 ml. After 5, 10 and 15 min of incubation samples were extracted with 4 ml chloroform/methanol (2:1, v/v) according to the method of Folch et al. [16], and lipid extracts were used for further analyses.

Analytical procedures

Enzymatic activities of cytochrome b_2 [17], phosphatidylserine synthase [18] and phosphatidylserine decarboxylase [19] were measured following published procedures.

Individual phospholipids were separated by thin-layer chromatography using silica gel H-60 plates (Merck). Chloroform/methanol/25% NH_3 (65:35:5; per vol.) or chloroform/methanol/glacial acetic acid/water (25:15:4:2, per vol.) were used as developing solvents. Radioactive spots were scraped off, and radioactivity was counted using Ready Solv HP (Beckman) + 5% water as a scintillation cocktail. Lipid phosphorus was quantitated by the method of Broekhuyse [20], and protein was quantitated according to Lowry et al. [21].

Results

Translocation of phosphatidylserine from unilamellar phospholipid vesicles to mitochondria

The appearence of radioactive phosphatidylethanolamine can serve as a measure for the import of

[^{3}H]phosphatidylserine from unilamellar vesicles into mitochondria in vitro. As can be seen from Figs. 1 A and B the presence of a yeast phosphatidylserine transfer protein (PSTP) significantly enhances this process. The rate of import of phosphatidylserine into mitochondria and of phosphatidylethanolamine formation increased with the amount of transfer protein added and came to a maximum with 60–80 U transfer protein present in a standard assay volume of 0.3 ml. Unspecific fusion or adsorption of vesicles containing [^{3}H]phosphatidylserine with mitochondria was low; only marginal amounts of the non-transferable marker [^{14}C]triacylglycerol present in donor vesicles (^{3}H/^{14}C ratio was approx. 10) were found in mitochondrial membranes after the incubation (^{3}H/^{14}C ratio was 100–200). Under these conditions 20% of phosphatidylserine (2.5 pmol) translocated to mitochondria are converted to phosphatidylethanolamine. The large excess of remaining phosphatidylserine indicates that transport from vesicles to the mitochondrial surface is not a limiting step in the overall translocation of phosphatidylserine from vesicles to the mitochondrial surface and across the outer to the inner mitochondrial membrane.

Pretreatment of mitochondria with proteinases (proteinase K or trypsin) led to a marked reduction of the rate of phosphatidylethanolamine formation (Table I). The possibility that remaining activity of proteinase could inactivate the phosphatidylserine transfer protein added to the assay mixture was excluded. Under conditions used for this experiment (see Materials and Methods) the inactivation of the phosphatidylserine transfer protein did not exceed 5% over a period of 20 min. The presence of the proteinase itself did not influence the import of phosphatidylserine into mitochondria, which could be demonstrated by addition of the proteinase inhibitor to the assay mixture prior to the addition of the enzyme. Also the possibility of gross damage of mitochondria by proteinase treatment could be excluded, because 93% of the activity of cytochrome b_2, a soluble intermembrane space protein, and 90% of the activity of phosphatidylserine decarboxylase, the reporter enzyme of the import reaction, were recovered. It has to be mentioned at this point, however, that import competence of mitochondria decreased during prolonged storage of mitochondria prior to incubations. Controls shown in Table I which were preincubated for the same time as proteinase treated samples therefore exhibited a lower import capacity than mitochondria which were used without a delay of time (see below, Table II).

In order to test the possibility that proteinase-sensitive component(s) at the mitochondrial surface involved in the import of phosphatidylserine might be of cytosolic or microsomal origin yeast cytosol or a yeast microsomal suspension, respectively, were added to proteinase-treated mitochondria. None of these additions restored the rate of phosphatidylserine import indicating that 'true' mitochondrial component(s) were affected by proteolysis (Table I). When similar assays were carried out with mitoplasts instead of mitochondria proteinase treatment dramatically reduced the formation of radiolabeled phosphatidylethanolamine due to direct access of the proteinase to the

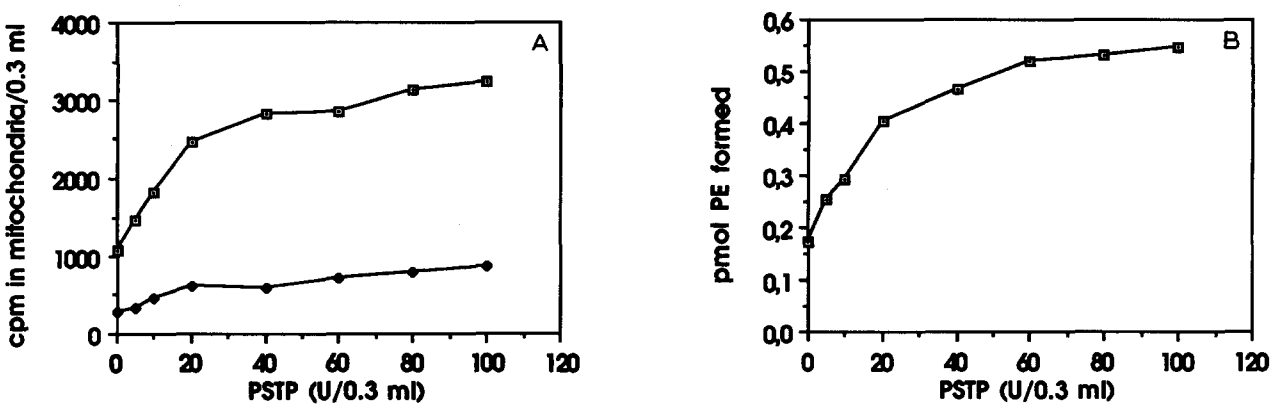

Fig. 1. The yeast phosphatidylserine transfer protein stimulates the transport of phosphatidylserine from unilamellar vesicles to mitochondria and the formation of phosphatidylethanolamine in vitro. Donor vesicles containing 7.5 pmol [^{3}H]phosphatidylserine ($2.5 \cdot 10^4$ cpm) and tracer amounts of [^{14}C]triacylglycerol ($2.5 \cdot 10^3$ cpm), and mitochondria (0.5 mg protein) were present in 0.3 ml 0.6 M mannitol, 10 mM Tris-HCl (pH 7.4). An approx. 50-fold enriched preparation of a yeast phosphatidylserine transfer protein (PSTP) was used as a tool to translocate radiolabeled phosphatidylserine from unilamellar vesicles to mitochondria. The transfer of phosphatidylserine to mitochondria was linear with respect to the amount of transfer protein within a range of 0–20 U PSTP/0.3 ml. A plateau is reached at 60–70 U PSTP/0.3 ml. The amount of phosphatidylserine transported to mitochondria under standard conditions (85 U PSTP/0.3 ml) was approximately 2.5 pmol. After 10 min of incubation lipids were extracted and analyzed by thin-layer chromatography. Experiments were carried out in duplicate with a mean deviation of ±5%. (A) Radioactivity detectable in phosphatidylserine (□) and phosphatidylethanolamine (♦) of mitochondria after 10 min of incubation with vesicles containing [^{3}H]phosphatidylserine. (B) Amount of phosphatidylethanolamine formed in mitochondria during incubation with [^{3}H]phosphatidylserine.

TABLE I

Effect of proteinase treatment of mitochondria or mitoplasts on the formation of phosphatidylethanolamine

The import of [^{3}H]phosphatidylserine from unilamellar vesicles into mitochondria was monitored by the appearence of [^{3}H]phosphatidylethanolamine as described in Materials and Methods. Mitochondria and mitoplasts were preincubated with proteinases for 30 min on ice, and for additional 5 min in the presence of the respective proteinase inhibitor (see Materials and Methods). Donor vesicles contained [^{14}C]triacylglycerol as a non-transferable marker; unspecific fusion or adsorption to acceptor membranes turned out to be marginal. Phosphatidylserine transfer protein from yeast (PSTP) was present at a final concentration of 85 U/0.3 ml (standard assay volume per time point). Incubations were carried out at 30°C. Samples were taken after 5, 10 and 15 min of incubation (linear range), and lipids from the total incubation mixture were extracted according to the method of Folch et al. [16] and analyzed by thin-layer chromatography. The percentage of radioactivity present in phosphatidylethanolamine was calculated from total radioactivity present in phosphatidylserine and phosphatidylethanolamine. Data shown in the table are from 10 min measurements and are the mean value from at least three experiments.

	% cpm in phosphatidylethanolamine
Mitochondria	
No transfer protein	2.3 ± 0.3
+ PSTP	8.7 ± 0.6
+ PSTP + proteinase K (50 μg/ml)	6.4 ± 0.5
+ PSTP + proteinase K (200 μg/ml)	4.2 ± 0.4
+ PSTP + trypsin (50 μg/ml)	5.7 ± 1.0
+ PSTP + trypsin (200 μg/ml)	4.1 ± 0.5
+ PSTP + proteinase K (200 μg/ml)	
+ yeast cytosol	5.8 ± 0.5
+ yeast microsomes	4.0 ± 1.0
Mitoplasts	
No transfer protein	3.1 ± 0.2
+ PSTP	33.9 ± 3.8
+ PSTP + proteinase K (200 μg/ml)	2.0 ± 0.4

inner mitochondrial membrane located phosphatidylserine decarboxylase (91% inactivation). Untreated mitoplasts, in contrast, showed a high rate of conversion of phosphatidylserine to phosphatidylethanolamine. The reason for this effect will be discussed below.

The fact that phosphatidylserine decarboxylase itself is not affected by mild treatment of intact mitochondria with proteinases left us with the question if proteinase treatment of mitochondria inhibited only translocation of phosphatidylserine from vesicles to mitochondria or perhaps also its intramitochondrial transport. Proteinase treatment of mitochondria reduced the translocation rate of phosphatidylserine from vesicles to mitochondria to 60–70% of the control, whereas approximately the same proportion (17–20%) of phosphatidylserine associated with mitochondria was converted to phosphatidylethanolamine in proteinase-treated and -untreated mitochondria. These results indicate that proteinase treatment reduces the rate of translocation of phosphatidylserine from vesicles to mitochondria rather than its movement between mitochondrial membranes.

Translocation of phosphatidylserine from the outer to the inner mitochondrial membrane

In order to test whether or not the outer mitochondrial membrane is a limiting barrier for phospholipid import into mitochondria we compared transport of phosphatidylserine to intact mitochondria and mitoplasts. When mitoplasts were used as acceptor, an increased rate of conversion of phosphatidylserine to phosphatidylethanolamine was observed as compared to intact mitochondria (Fig. 2). This result is not surprising, since in mitoplasts the transfer protein loaded with phosphatidylserine has direct access to the inner mitochondrial membrane, where the formation of phosphatidylethanolamine occurs. The increased rate of appearence of radiolabeled phosphatidylethanolamine in mitoplasts versus intact mitochondria also indicates that decarboxylation of phosphatidylserine is not the rate-limiting step in our assay system. This notion is confirmed by the fact that mitochondria (0.5 mg protein) added to the standard assay represent a phosphatidylserine decarboxylase activity of 0.7 nmol/min. This is a large excess of the reporter enzyme, because imported [^{3}H]phosphatidylserine is converted to [^{3}H]phosphatidylethanolamine only at a rate of 0.02 pmol/min. Due to the fact that under standard conditions 85–90 U of yeast phosphatidylserine transfer protein were added to the assay (total volume 0.3 ml) transport of phosphatidylserine from vesicles to the mitochondrial surface was not limiting (see Fig. 1 A). Therefore the limiting step in this in vitro assay is the

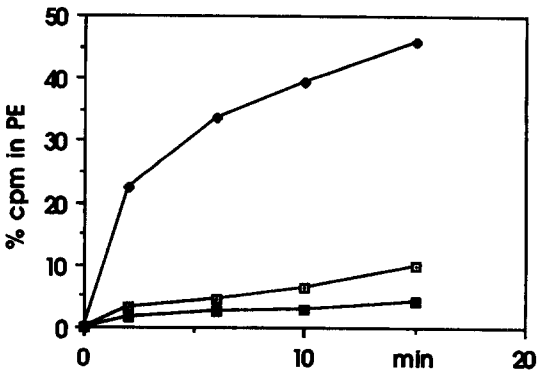

Fig. 2. Transfer of [^{3}H]phosphatidylserine from vesicles to mitochondria or mitoplasts, respectively, and its conversion to [^{3}H]phosphatidylethanolamine. Assay conditions were the same as outlined in the legend to Fig. 1. Enriched preparations of a yeast phosphatidylserine transfer protein (PSTP) were added to assay mixtures at a final concentration of 85 U/0.3 ml in standard assays. Equivalent amounts of mitochondria (0.5 mg protein/0.3 ml) and mitoplasts were used. ■, Mitochondria or mitoplasts, no transfer protein; □ mitochondria + phosphatidylserine transfer protein; ◆, mitoplasts + phosphatidylserine transfer protein.

TABLE II

Characterization of the intramitochondrial transport of phosphatidylserine

Decarboxylation of [^{3}H]phosphatidylserine to [^{3}H]phosphatidylethanolamine served as a measure for the import of phosphatidylserine into mitochondria. The experimental protocol was the same as outlined in the legend to Table I, except that preincubations with inhibitors were carried out for 5 min on ice. The additives listed had no inhibitory effect on phosphatidylserine decarboxylase. The rate of conversion of phosphatidylserine to phosphatidylethanolamine within 10 min in a standard assay was set 100%. Experiments were carried out in triplicate.

	Formation of phosphatidylethanolamine (% of control)
Control	100
No transfer protein	10± 8
+EDTA (5 mM)	70± 5
+ *o*-Phenanthroline (5 mM)	118± 5
+Adriamycin (0.16 mM)	122± 9
+Oligomycin (0.07 mM)	125±11
+Apyrase (2 U/mg)	104± 4
+Apyrase (2 U/mg)+ oligomycin (0.07 mM)	139± 9
+CCCP (0.1 mM)	101± 4
+ATP (0.5 mM)	97± 6
+GTP (0.5 mM)	112±10
+CTP (0.5 mM)	71± 8

translocation of phosphatidylserine across the outer and to the inner mitochondrial membrane.

The import assay described above enabled us to characterize the process of translocation of phosphatidylserine within the mitochondrion. Table II shows that the chelators EDTA and *o*-phenanthroline inhibited the translocation only to a minor extent, if at all. Adriamycin, which had been shown to inhibit the import of proteins into mitochondria in vitro at a concentration of 0.16 mM [22], had no inhibitory effect on the formation of phosphatidylethanolamine from extramitochondrially added phosphatidylserine. Neither depletion of ATP by the ATP-cleaving enzyme apyrase in combination with the ATPase inhibitor oligomycin nor the breakdown of the electrochemical gradient across the inner membrane by the uncoupler carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) inhibited the intramitochondrial translocation of phosphatidylserine. Addition of nucleoside triphosphates to the assay mixture had no significant stimulatory effect.

Route of phosphatidylserine transport within the mitochondrion

Earlier work from our laboratory indicated that contact sites between the inner and the outer mitochondrial membrane might be zones of intramitochondrial phospholipid translocation [8,11]. Using the assay conditions described above we were able to demonstrate upon subfractionation of mitochondria, that newly imported phosphatidylserine is found at high specific activity in the outer membrane, but also in membrane contact sites (Table III). The specific activity of phosphatidylserine in the inner mitochondrial membrane is low, because the vast majority of phosphatidylserine arriving at the inner membrane is rapidly converted to phosphatidylethanolamine. Newly synthesized phosphatidylethanolamine is preferentially transported to the outer mitochondrial membrane. In this case a large proportion of radiolabeled phosphatidylethanolamine could be detected in the contact sites fraction indicating that the export of this phospholipid from the inner mitochondrial membrane to the mitochondrial surface occurs via contact sites.

Addition of 5 mM hydroxylamine to the import assay mixture largely inhibits phosphatidylserine decarboxylase. Inhibition of metabolic conversion to phosphatidylethanolamine, however, had no inhibitory effect on the protein-catalyzed transport of phosphatidylserine from vesicles to mitochondria (data not shown). The distribution of radioactively labeled phosphatidylserine between the outer membrane and the contact site fraction is rather similar as compared to the con-

TABLE III

Submitochondrial distribution of phosphatidylserine transported from vesicles to mitochondria

Standard import assays were carried out as described in the legend to Table I. After 10 min of incubation with vesicles containing [^{3}H]phosphatidylserine, the mitochondria were isolated, subfractionated, and phospholipids of subfractions were extracted and analyzed. Hydroxylamine was present at a concentration of 5 mM. Data shown are the mean values ± S.D. of three independent experiments. Enrichment factors of submitochondrial fractions were essentially the same as described by Simbeni et al. [11]. OMM, outer mitochondrial membrane; IMM, inner mitochondrial membrane; CS, membrane contact sites; PS, phosphatidylserine; PE, phosphatidylethanolamine.

	Specific radioactivity (cpm/mg)			
	control		+NH_2OH	
	PS	PE	PS	PE
OMM	816000±120000	5580±1100	533000±94000	1360±250
CS	123000± 5000	3600± 650	97000± 6000	290± 50
IMM	2250± 450	710± 160	6250± 1600	80± 20

trol experiment without inhibitor. In the presence of hydroxylamine an increase of specific activity of phosphatidylserine in the inner membrane could be observed. Taking into account that the distribution of total phospholipids between the outer membrane, contact sites and the inner membrane is approx. 1:0.7:1.6 only a slight, but significant accumulation (6.2%) of total radiolabeled phosphatidylserine versus the control (1.5%) was found in the inner membrane. This result indicates that import of phosphatidylserine to the inner mitochondrial membrane is reduced when metabolic conversion by phosphatidylserine decarboxylase is inhibited.

Discussion

In contrast to mammalian cells in yeast phosphatidylserine is synthesized in an energy-dependent reaction using CDP-diacylglycerol and serine as the substrates [23]. Subcellular fractionation revealed that yeast phosphatidylserine synthase is located in a special subfraction of microsomes [2].

An important route of phosphatidylserine transport within the cell is translocation to mitochondria, where it is converted to phosphatidylethanolamine via decarboxylation. The enzyme catalyzing the latter step, phosphatidylserine decarboxylase, is a component of the inner mitochondrial membrane [24]. Protection against inactivation by proteinase treatment in intact mitochondria, but inactivation when mitoplasts are treated with proteinase K demonstrated that this enzyme is located at the outer aspect of the inner mitochondrial membrane. A similar localization has been demonstrated for mammalian phosphatidylserine decarboxylase [25]. The mechanism of translocation of phosphatidylserine from its site of synthesis to the site of conversion is unclear. Very recent results (Simbeni, R., unpublished data) indicate that phosphatidylserine-synthesizing microsomal particles are closely associated with the mitochondrial surface. Newly synthesized phosphatidylserine could be imported to mitochondria via membrane contact between the two organelles involved. Phospholipid synthesis in a membrane fraction associated with mammalian mitochondria has been described by Vance [26]. Membrane contact as a possible mechanism of phospholipid translocation between the endoplasmic reticulum and mitochondria was postulated [27].

In our in vitro assay translocation of phosphatidylserine from mixed phosphatidylserine/phosphatidylcholine vesicles to mitochondria was effective only in the presence of a phosphatidylserine-specific transfer protein (Fig. 1 and Table I). While it is an open question whether phosphatidylserine transfer protein-catalyzed transport of phosphatidylserine to mitochondria is of relevance in vivo the vesicle/mitochondria system allowed us to load phosphatidylserine onto mitochondria and to study its further movement across the outer and to the inner mitochondrial membrane.

The finding that import of phosphatidylserine from vesicles into mitochondria is stimulated by a proteinase-sensitive factor on the mitochondrial surface (Table I) contradicts earlier reports by Voelker [5], who found that phosphatidylserine transfer from mammalian microsomes to mitochondria was not affected by proteinase treatment of mitochondria. Different donor/acceptor systems and the use of a different transfer protein might explain these discrepancies. Addition of either cytosol or microsomes to proteinase-treated yeast mitochondria did not restore the original rate of phosphatidylserine transfer (Table I). Therefore, neither cytosolic nor microsomal protein(s) had been removed from the mitochondrial surface by proteinase treatment. As rather high concentrations of proteinases (50–200 μg/ml) were necessary to inhibit the import of phosphatidylserine into mitochondria we assume that the protein(s) involved are not fully exposed at the mitochondrial surface, but at least in part embedded into the outer membrane.

Voelker [6,7] had shown that phosphatidylserine transport to mitochondria was stimulated by ATP in permeabilized cultured cells, but not with isolated mitochondria and microsomes. The author suggested a two-step process with the first step being ATP-dependent and placing phosphatidylserine in a permissive environment for further translocation. Our results (Table II) confirm that the last step of phosphatidylserine translocation, namely transport across the outer mitochondrial membrane to the site of decarboxylation, is an energy independent process. Inhibition of phosphatidylserine import into mitochondria by adriamycin, as reported by Voelker [28] with the permeabilized cell system, could not be observed with isolated mitochondria in the present study (Table II). Thus, binding of the drug to cardiolipin and to other acidic phospholipids does not interfere with intramitochondrial phosphatidylserine transport. This result is remarkable insofar as cardiolipin, which has the tendency to adopt non-bilayer structures under appropriate conditions, was found to be essential for the import of proteins into mitochondria [22].

The fact that membrane contact sites between the outer and the inner mitochondrial membrane are zones of intramitochondrial translocation of proteins led to the hypothesis that also phospholipids might be transported into the mitochondrion via this route. We were able to demonstrate [8,11] that contact sites are indeed involved in the translocation of phosphatidylserine to the inner mitochondrial membrane. Results presented here (Table III) confirm this notion insofar as imported [^{3}H]phosphatidylserine can be found in the

mitochondrial contact site fraction. In contrast to data presented by Ardail et al. [12] who worked with mouse liver mitochondria accumulation of labeled phosphatidylserine in contact sites could not be observed, when the conversion to phosphatidylethanolamine was inhibited. In our assay system phosphatidylserine rather accumulates in the inner mitochondrial membrane under these conditions, although to a minor extent. Discrepancies between our results and those presented by Ardail et al. [12] cannot easily be explained, but different assay systems (donor membrane, supply of phospholipids to mitochondria, fractionation technique) might be some of the possible reasons. Newly synthesized phosphatidylethanolamine is preferentially translocated from the inner mitochondrial membrane, where it is synthesized, to the outer mitochondrial membrane without prior mixing with the pool of phosphatidylethanolamine of the inner membrane (Table III). This observation and the high specific activity of phosphatidylethanolamine in contact sites, which is in accordance with data reported by Ardail et al. [12], point more clearly to an involvement of this submitochondrial fraction in the intramitochondrial movement of phospholipids. The low specific activity of phosphatidylethanolamine found in the inner mitochondrial membrane supports the notion that decarboxylation of newly imported phosphatidylserine occurs already in a zone close to or within contact sites. Although the majority of phosphatidylserine decarboxylase is located in the inner mitochondrial membrane and enrichment in the contact site fraction is only marginal [11], the amount of enzyme present in contact sites seems to be sufficient to convert most of the imported phosphatidylserine to phosphatidylethanolamine. The possible role of specific components of contact sites in the intramitochondrial movement of phospholipids remains an open question. It will be a task for the future to define the route of intramitochondrial migration of phospholipids more precisely and to search for interactions of membrane components with phospholipids on their way between mitochondrial membranes.

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