Import of phosphatidylinositol and phosphatidylcholine into mitochondria of the yeast, *Saccharomyces cerevisiae*

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Abstract An in vitro assay was designed to study the import of ^{3}H-labeled phosphatidylinositol and phosphatidylcholine, respectively, from unilamellar vesicles into isolated mitochondria of the yeast, *Saccharomyces cerevisiae*. Both phospholipids reached the inner mitochondrial membrane. During import they were detected in contact sites between the outer and the inner mitochondrial membrane, supporting the notion that these zones are sites of intramitochondrial phospholipid transport. The uncoupler CCCP, the antibiotic adriamycin, and energy depletion caused by oligomycin and apyrase did not inhibit the transport of phosphatidylinositol and phosphatidylcholine into mitochondria.

Key words: Phosphatidylinositol; Phosphatidylcholine; Phospholipid transfer; Mitochondrion; Membrane contact site

1. Introduction

Mitochondrial membranes, like other subcellular membranes of eukaryotic cells, contain the whole set of glycerophospholipids, among them phosphatidylcholine and phosphatidylinositol, which are not synthesized in the mitochondrion [1]. The majority of cellular phospholipids is synthesized in the microsomal fraction (endoplasmic reticulum) [2], and phospholipids required for the biosynthesis of mitochondrial membranes have to be imported from their site of synthesis. Import of phosphatidylserine, which serves as the substrate of the mitochondrial phosphatidylserine decarboxylase [3], can be easily tested in a metabolic assay by measuring the appearance of phosphatidylethanolamine as a reporter molecule [4–10]. Assays for studying the import of phosphatidylcholine and phosphatidylinositol into mitochondria are more complicated, because these phospholipids do not undergo metabolic modification by mitochondrial enzymes. Thus, detection of phosphatidylcholine and phosphatidylinositol assembled into mitochondrial membranes necessitates subfractionation of the organelle.

Previous work from our laboratory [9–11] using yeast as an experimental system had shown, that contact sites between the outer and the inner mitochondrial membrane are most likely zones of intramitochondrial translocation of phosphatidylserine. Ardail et al. [12] demonstrated an interaction of these contact sites with a specialized microsomal fraction, which catalyzes synthesis of phospholipids destined for the import into mitochondria. An analogous microsomal fraction, which had been originally described by Vance [13,14] for mammalian cells, was recently also characterized in yeast (Gaigg et al., submitted).

Intramitochondrial translocation of phosphatidylserine was shown to be independent of a membrane potential across the inner membrane and of ATP [9]. In the present paper we address the question as to the route of import of phosphatidylinositol and phosphatidylcholine into mitochondria. These processes are characterized and compared to the import of phosphatidylserine into mitochondria.

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2. Materials and methods

2.1. Strain, culture conditions, and isolation of mitochondria

The wild-type yeast strain *Saccharomyces cerevisiae* D273-10B was cultivated under aerobic conditions at 30°C on a medium with 2% lactate as a carbon source [15]. Mitochondria were isolated from spheroplasts as described before [15].

2.2. Import of radiolabeled phosphatidylinositol and phosphatidylcholine from unilamellar vesicles into membranes of isolated mitochondria

Small unilamellar vesicles containing ^{3}H-labeled phosphatidylinositol and phosphatidylcholine, respectively, were formed by suspending appropriate amounts of the radiolabeled phospholipids and unlabeled egg phosphatidylcholine in 0.6 M mannitol, 10 mM Tris-HCl, pH 7.4, and sonicating at 75 W under cooling for 10 min. A standard mixture for one time point of an import assay contained 1.4 ml of a phospholipid vesicle suspension (approximately 10^6 dpm; 0.1 mg total phospholipid), 5 ml of a mitochondrial suspension (15 mg protein/ml), and an enriched sample of phosphatidylinositol transfer protein [16] (900 units) in a total volume of 10.5 ml 0.6 M mannitol, 10 mM Tris-HCl, pH 7.4. Samples were taken after 15, 30, and 45 min of incubation at 30°C. Mitochondria were reisolated by centrifugation at 10,000 × g for 10 min at 4°C, and subfractionated as described by Pon et al. [17].

2.3. Analytical procedures

Lipids were extracted from submitochondrial fractions with chloroform/methanol (2:1, v/v) according to the method of Folch et al. [18]. Individual phospholipids were separated by thin-layer chromatography on silica gel H60 plates (Merck), using chloroform/methanol/25% NH$_3$ (50:25:6, per vol.) as a developing solvent. Radioactive spots were scraped off, and radioactivity was measured by liquid scintillation counting using Safety Cocktail (Baker) + 5% water.

Lipid phosphorus was quantitated by the method of Broekhuyse [19]. Protein was quantitated according to Lowry et al. [20], SDS-PAGE was carried out by the method of Laemmli [21], and Western blotting using antibodies against mitochondrial porin was performed as described by Haid and Suissa [22].

3. Results

A yeast phosphatidylinositol transfer protein was used at non-limiting concentrations (11–13 units per mg mitochondrial protein) in the in vitro assay employed for this study to translocate radiolabeled phosphatidylinositol or phosphatidylcholine, respectively, from unilamellar vesicles to the surface of yeast mitochondria. It is very likely that this mechanism of lipid translocation is irrelevant in vivo, because experiments carried out with the temperature-sensitive *sec14* mutant strain, which
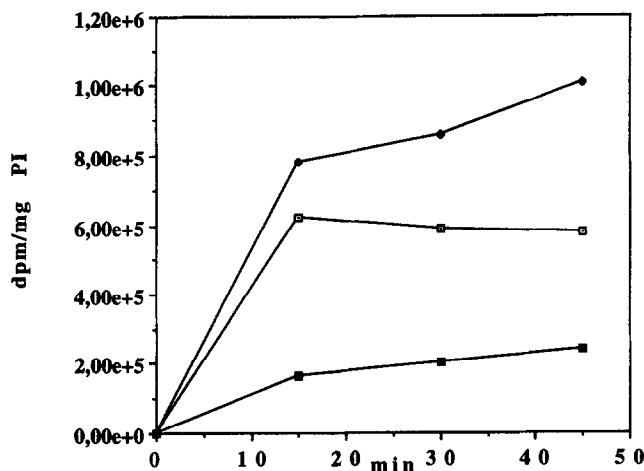
Fig. 1. Import of [^{3}H]phosphatidylinositol from unilamellar vesicles into mitochondria in vitro. Mitochondria of the yeast, *Saccharomyces cerevisiae*, were isolated and incubated with vesicles containing radiolabeled phosphatidylinositol in the presence of the yeast phosphatidylinositol transfer protein as described in section 2. Specific activities in the respective submitochondrial fractions are shown from a typical experiment, which was reproduced twice. □, outer mitochondrial membrane; ◆, contact sites; ■, inner mitochondrial membrane.

Table 1
Distribution of imported phosphatidylinositol and phosphatidylcholine in submitochondrial fractions

	Relative enrichment (-fold)		
	OM	CS	IM
[^{3}H]Phosphatidylinositol	1	1.35 ± 0.14	0.34 ± 0.02
[^{3}H]Phosphatidylcholine	1	1.05 ± 0.05	1.01 ± 0.03
Porin	1	0.29 ± 0.02	0.11 ± 0.03

OM, outer membrane; CS, contact sites; IM, inner membrane. Specific activities (dpm/mg) for radiolabeled phospholipids were calculated after import for 30 min under the conditions described in section 2. Values obtained for the outer membrane were set at 1. The amount of porin as estimated by Western blotting (peak area/mg total protein) was set at 1 for the outer membrane, and calculated for the other fractions in the same way. Mean values from 3 independent experiments are shown.

is defective in the phosphatidylinositol transfer protein [23], did not show an influence of that mutation on the transport of phospholipids to mitochondria (M. Lampl and A. Leber, unpublished observations). Nevertheless, the assay system described above enabled us to study the translocation of phosphatidylinositol and phosphatidylcholine from the outer to the inner mitochondrial membrane. In contrast to mammalian mitochondria [24], phosphatidylinositol is one of the major phospholipids of the yeast inner mitochondrial membrane [11,25].

Results demonstrating import of phosphatidylinositol and phosphatidylcholine into mitochondria are shown in Figs. 1 and 2, respectively. Equilibrium of [^{3}H]phosphatidylinositol between extramitochondrial vesicles and the outer mitochondrial membrane was rapidly established. Also the high specific radioactivity of phosphatidylinositol in the contact site fraction was rapidly reached, and remained fairly constant between 15 and 45 min of incubation. The specific activity of [^{3}H]phosphatidylinositol in the inner membrane increased moderately, but steadily, over the whole period of incubation. [^{3}H]Phosphatidylcholine was transported to the mitochondrial surface at a lower rate, which is consistent with the lower affinity of the phosphatidylinositol transfer protein for phosphatidylcholine [16]. The specific radioactivity of [^{3}H]phosphatidylcholine was very similar in the outer membrane, the inner membrane, and in contact sites at all time points.

Studies of phosphatidylinositol and phosphatidylcholine import into mitochondria rely on the subfractionation of the organelle in order to localize imported phospholipids. The crucial question has to be answered, if the amount of radiolabeled phospholipids cofractionating with the inner membrane is really indicative of a transport to this submitochondrial compartment, or if it has to be attributed to crosscontamination with the outer mitochondrial membrane. For this reason we compared the submitochondrial distribution of porin, the most prominent protein of the outer mitochondrial membrane, to

that of imported radiolabeled phospholipids (Table 1). When the relative enrichment of porin in the outer membrane fraction was set at 1, contact sites and the inner membrane fraction were shown to contain smaller amounts of the outer membrane marker. Detection of porin in contact sites was not surprising, because this fraction contains per definition components of the outer membrane [17]. A small but significant amount of porin was recovered as a contaminant in the isolated inner membrane fraction. However, the relative enrichment of [^{3}H]phosphatidylinositol and [^{3}H]phosphatidylcholine, respectively, in contact sites and the inner membrane after 30 min of incubation markedly exceeded that of the outer membrane marker porin, indicating that both phospholipids reached the inner membrane in this in vitro assay, and that contact sites between the two mitochondrial membranes are most likely zones of intramitochondrial phospholipid translocation.

In order to characterize the mechanism of import of phosphatidylinositol and phosphatidylcholine into yeast mitochondria, we tested the energy dependence of this process. Neither disruption of the electrochemical potential across the inner mitochondrial membrane by CCCP, nor energy depletion caused by apyrase and oligomycin significantly inhibited the
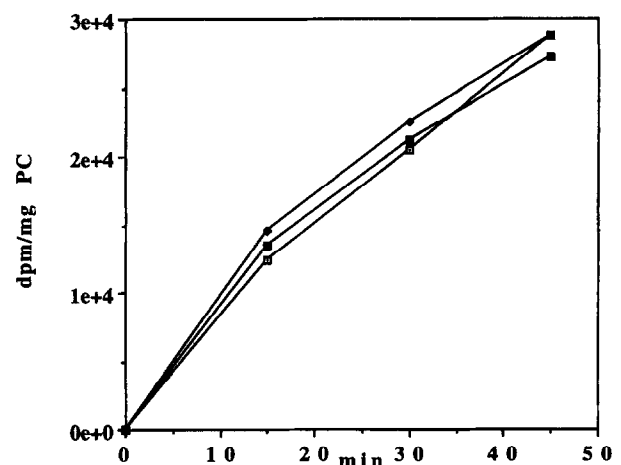

Fig. 2. Import of [^{3}H]phosphatidylcholine from unilamellar vesicles into mitochondria in vitro. Assay conditions were the same as described in the legend to Fig. 1. □, outer mitochondrial membrane; ◆, contact sites; ■, inner mitochondrial membrane.

Table 2
Characterization of phosphatidylinositol import into mitochondria

	Relative specific activity of phosphatidylinositol		
	OM	CS	IM
Control	1	1.21	0.32
+ CCCP (2 μg/mg protein)	1	1.03	0.27
+ Adriamycin (115 μg/mg protein)	1	1.53*	0.78*
+ Apyrase (2.5 U/mg protein) and oligomycin (40 μg/mg protein)	1	1.54	0.30

OM, outer membrane; CS, contact sites; IM, inner membrane. The specific activity of the respective phospholipid in the outer mitochondrial membrane was set at 1, and relative specific activities in the other fractions were calculated.
*In this assay the specific activity of phosphatidylinositol in the outer membrane was markedly lower (25%) than in the control.

Table 3
Characterization of phosphatidylcholine import into mitochondria

	Relative specific activity of phosphatidylcholine		
	OM	CS	IM
Control	1	1.00	0.98
+ CCCP (2 μg/mg protein)	1	1.18	1.13
+ Adriamycin (115 μg/mg protein)	1	1.03	0.96
+ Apyrase (2.5 U/mg protein) and oligomycin (40 μg/mg protein)	1	1.03	0.99

For abbreviations and calculation of data see Table 2.

import of phosphatidylinositol (Table 2) and phosphatidylcholine (Table 3) into mitochondria. Also the antibiotic adriamycin did not negatively influence the import of phosphatidylinositol (Table 2) and phosphatidylcholine (Table 3) into mitochondria. Adriamycin interacts with negatively charged surface phospholipids and had been shown before to inhibit the import of proteins into mitochondria [26], and the translocation of phosphatidylserine from the endoplasmic reticulum to mitochondria in a mammalian system [27]. The apparently increased relative specific activity of [³H]phosphatidylinositol in the inner membrane and, to a lesser extent, in contact sites of mitochondria treated with adriamycin as compared to the control is rather due to a decrease of the [³H]phosphatidylinositol level in the outer membrane. The reason for this effect might be the interference of adriamycin with the transfer protein-mediated transport of the negatively charged phospholipid to the mitochondrial surface.

4. Discussion

Previous work from our laboratory [9,11] and from others [28] suggested that phosphatidylserine on its way from the outer to the inner mitochondrial membrane migrates via membrane contact sites. Similarly, phosphatidylethanolamine newly synthesized in the inner mitochondrial membrane and exported to the mitochondrial surface was highly enriched in the contact site fraction [9,11]. Inefficient import of phosphatidylinositol to the inner mitochondrial membrane of mammalian cells in vitro was regarded as a possible reason for the lack of phosphatidylinositol in the mammalian inner mitochondrial membrane [24]. In contrast, the yeast inner mitochondrial membrane contains phosphatidylinositol as a major phospholipid [11,25], and

phosphatidylinositol in addition to phosphatidylcholine is readily translocated from vesicles to the inner mitochondrial membrane (see Figs. 1 and 2, and Table 1). Similar to phosphatidylserine [11], phosphatidylinositol was enriched in contact sites between the outer and the inner mitochondrial membrane during the import process. Taking into account that contact sites contain elements of the inner and the outer mitochondrial membrane at roughly equal amounts, the enrichment of phosphatidylinositol in the contact site fraction in comparison to its concentration in the outer and inner membrane is even more evident (see Table 1). Enrichment of phosphatidylinositol in contact sites may be explained by the assumption that its translocation from contacat sites to the inner mitochondrial membrane is the rate limiting step of the overall import process. In contrast to phosphatidylinositol the specific radioactivity of phosphatidylcholine was not significantly higher in contact sites as compared to other submitochondrial membrane fractions. In this case the transport of the phospholipid to the mitochondrial surface must be regarded as a limiting step. The lower affinity of the phosphatidylinositol transfer protein for phosphatidylcholine as compared to phosphatidylinositol is the obvious reason for this observation. Taken together, some indications mainly obtained with phosphatidylserine [9–11] and phosphatidylinositol (this paper) favor membrane junctions as being 'general' zones for intramitochondrial phospholipid translocation.

Experiments with energy inhibitors (Tables 2 and 3) indicate that intramitochondrial translocation of phosphatidylinositol and phosphatidylcholine requires neither an electrochemical gradient across the inner mitochondrial membrane nor ATP. Similar observations were made before for the import of phosphatidylserine [10]. A driving force of phospholipid import in the in vitro system presented in this paper is the imbalance of the phospholipid concentration in the outer mitochondrial membrane due to the action of the phospholipid transfer protein. Ongoing synthesis of phosphatidylinositol as a requirement for its import into mitochondria was shown before [9] in another type of assay. In vivo organelle contact [12–14] might be a prerequisite for the translocation of phospholipids from the endoplasmic reticulum to the outer mitochondrial membrane.

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