Import of sterols into mitochondria of the yeast *Saccharomyces cerevisiae*

Gabriele Tuller, Günther Daum*

Institut für Biochemie und Lebensmittelchemie, Technische Universität Graz, Petersgasse 12/2, A-8010 Graz, Austria

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Abstract An in vitro assay was designed to study the import of radiolabeled ergosterol and cholesterol from unilamellar vesicles into isolated mitochondria of the yeast *Saccharomyces cerevisiae*. Supply of ergosterol to the mitochondrial surface was enhanced by a cytosolic fraction containing a lipid transfer protein, whereas no such additive to the assay was required for cholesterol transport. Both sterols reached the inner mitochondrial membrane. During import, they were detected in contact sites between the outer and the inner mitochondrial membrane supporting the idea, that these zones are sites of intramitochondrial lipid translocation. Transport of ergosterol between the outer and the inner mitochondrial membrane was not affected by addition of ATP, depletion of ATP caused by treatment of mitochondria with apyrase and oligomycin, and incubation with the uncoupler CCCP, indicating that this process is energy-independent.

Key words: Ergosterol; Cholesterol; Lipid transport; Mitochondrion; Membrane contact site

1. Introduction

Sterols are in addition to phospholipids and sphingolipids integral components of eukaryotic membranes. In all types of eukaryotic cells including yeast, sterols are highly enriched in the plasma membrane [1–3]; other organelles contain sterols at lower, although significant quantities. Sterol synthesis is considered to be a microsomal process. Recent results from our laboratory obtained with the yeast *Saccharomyces cerevisiae* demonstrated the participation of so-called lipid particles [4–6] in the biosynthesis of sterols. The fact that site(s) of synthesis and subcellular destination(s) of sterols are not identical, necessitates intracellular translocation of these lipids.

The sterol concentration in mitochondria is low [7]. In higher eukaryotes and *Neurospora crassa*, most of the mitochondrial sterol is located in the outer membrane, whereas in mitochondria of the yeast *S. cerevisiae* the majority of sterols is inner membrane associated [4]. The mechanisms of import into mitochondria and intramitochondrial translocation of sterols are poorly understood. In adrenal tissue, sterol transport to this organelle is an important step in the biosynthesis of steroid hormones (for reviews see [1,2,8]). A sterol carrier protein (SCP-2) was suggested to play a role in the import of cholesterol into mitochondria during steroidogenesis, but the experimental proof for such a function in vivo is missing [1,2]. It has been hypothesized that a cycloheximide activator peptide is necessary to transport sterol to cytochrome P-450 of the inner mitochondrial membrane. It has also been suggested that cholesterol sulfate might regulate the entry of intramitochondrial cholesterol into the steriodogenic pool.

Contact sites between the outer and the inner mitochondrial membrane may be regarded as zones of intramitochondrial lipid translocation. Work from our laboratory with yeast [9–12] revealed, that phospholipids are most likely translocated within the mitochondrion across these junctions. Intramitochondrial migration of phosphatidylserine, phosphatidylinositol and phosphatidylcholine was shown to be independent of ATP and a membrane potential across the inner membrane [11,12]. Using mammalian cells, Ardail et al. [13] demonstrated an interaction of 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 [14,15] for mammalian cells, was recently also characterized in yeast [16].

In the present paper, we address the question as to the route of import of sterols into mitochondria of the yeast *S. cerevisiae*. We focused on the possible role of membrane contact sites and the energy requirement of the translocation process.

2. Materials and methods

2.1. Strain, culture conditions and isolation of mitochondria

The wild-type yeast strain *S. cerevisiae* D273-10B was cultivated under aerobic conditions at 30°C on a medium with 2% lactate as a carbon source [17]. Mitochondria were isolated from spheroplasts as described before [17].

2.2. Import of radiolabeled sterols from unilamellar vesicles into membranes of isolated mitochondria

Radiolabeled ergosterol was isolated from yeast cells (100 ml culture) grown for 48 h in the presence of [^{14}C]acetate (1 mCi, 60 mCi/mmol). Cells were harvested, washed with dist. water and disintegrated by vortexing in the presence of glass beads. Lipids were extracted according to Folch et al. [18] and saponified in the presence of 60% aqueous KOH/methanol/0.5% methanolic pyrogallic acid (2:3:2, v/v/v) for 2 h at 85°C. Sterols were extracted with light petroleum and further purified on a silica gel H 60 column (Merck) using light petroleum/diethylether (gradient from 70/30 to 0/100) as an eluent. Fractions containing [^{14}C]ergosterol were collected, taken to dryness and dissolved in a small volume of chloroform/methanol (2:1, v/v). [^{14}C]Cholesterol (60 mCi/mmol) was purchased from NEN.

Small unilamellar vesicles containing radiolabeled sterols were formed by suspending [^{14}C]labeled sterols (15–30 nmol; 10^6 dpm), [^{3}H]triacylglycerols (tracer amounts as non-transferable marker; 10^6 dpm) and unlabeled egg phosphatidylcholine (150–180 nmol) in 1 ml 0.6 M sorbitol, 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 1.5 ml of the vesicle suspension, 2 ml of a mitochondrial suspension (60 mg protein) and an enriched sample of phosphatidylserine transfer protein [19] (1.5 ml, 450 U) in a total volume of 6 ml 0.6 M sorbitol, 10 mM Tris-HCl, pH 7.4. Samples were taken after 30 and 60 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. [20].

*Corresponding author. Fax: (43) (316) 810599.
E-mail: daum@ftug01.dnet.tu-graz.ac.at

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2.3. Analytical procedures

Lipids were extracted with chloroform/methanol (2:1, v:v) according to the method of Folch et al. [18]. Sterols were separated by thin-layer chromatography on silica gel H60 plates (Merck), using light petroleum/diethylether/acetic acid (14/6/0.4, v/v/v) as a developing solvent. Radioactivity was measured by liquid scintillation counting using Safety Cocktail (Baker). Sterols [3,4] and protein [21] were quantified by published procedures. SDS-PAGE was carried out by the method of Laemmli [22] and Western blotting using antibodies against mitochondrial porin was performed as described by Haid and Suissa [23]. ATP was quantified using the firefly luciferase bioluminiscence assay as described by Lundin [24].

3. Results

An in vitro assay was established to study the import of radiolabeled sterols from unilamellar vesicles into yeast mitochondria. In order to load sterols onto the mitochondrial surface, we used a yeast phosphatidylserine transfer protein, which is known to translocate also sterols between isolated membranes. As can be seen from Fig. 1A, ergosterol translocation to mitochondria was stimulated by the presence of the transfer protein at non-limiting concentration (150 U/mg mitochondrial protein). In contrast, [^{14}C]cholesterol reached the mitochondrial surface also in the absence of the phosphatidylserine transfer protein (Fig. 2A); addition of the transfer protein to the assay mixture did not exhibit a stimulatory effect. Translocation of both sterols to mitochondria was not due to unspecific fusion of vesicles with mitochondrial membranes, because radiolabeled triacylglycerol present in trace amounts in donor vesicles as a non-transferable marker for phosphatidylserine transfer protein catalyzed transport [19], did not migrate to the acceptor membrane (Figs. 1B, 2B).

Although the role of lipid transfer proteins as vehicles for the intracellular translocation of sterols in vivo is highly hypothetical, the assay system described above enabled us to study the translocation of ergosterol from the outer to the inner mitochondrial membrane in vitro (Table 1). [^{14}C]Ergosterol was rapidly loaded onto the outer mitochondrial membrane and its intramitochondrial migration was followed by subfractionation of the organelle. A substantial percentage of [^{14}C]ergosterol reached the inner mitochondrial membrane. This amount of radioactivity could not be attributed to contamination with the outer mitochondrial membrane, because the marker of the latter subfraction, porin, was present in the isolated inner membrane only at a minor concentration. Contact sites between the outer and the inner mitochondrial membrane, which were shown before to be sites of protein import into mitochondria [20] and intramitochondrial phospholipid migration [10], contained substantial amounts of [^{14}C]ergosterol on its way to the inner mitochondrial membrane. Comparable results were obtained with [^{14}C]cholesterol, but the amount of this sterol was lower in contact sites and higher in the inner membrane of yeast mitochondria. It is noteworthy that [^{14}C]cholesterol reached the inner membrane although it is not a 'natural' component of yeast mitochondria.

One important parameter characterizing the mechanism of

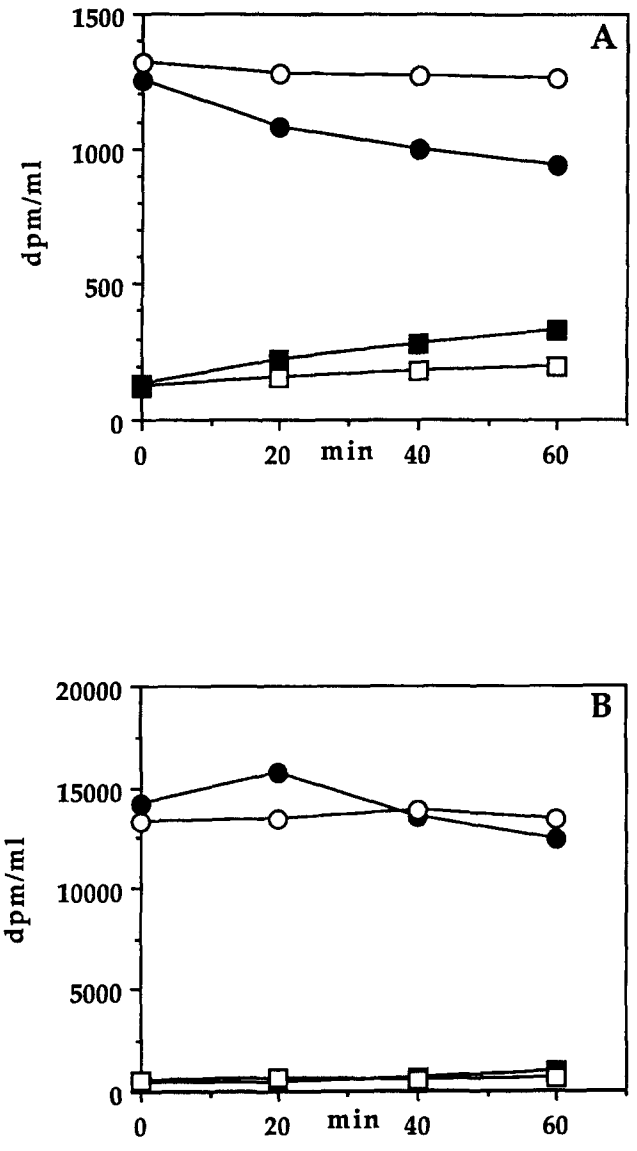

Fig. 1. Transport of [^{14}C]ergosterol and [^{3}H]triacylglycerol from unilamellar vesicles to yeast mitochondria in vitro. Mitochondria of the yeast *S. cerevisiae* were isolated and incubated with vesicles containing [^{14}C]ergosterol (A) and [^{3}H]triacylglycerol (B) in the presence (filled symbols) and absence (open symbols) of the yeast phosphatidylserine transfer protein [19] as described in Section 2. (●, ○) dpm/ml in the supernatant (vesicles); (■, □) dpm in the mitochondrial pellet (1-ml aliquot).

Table 1
Distribution of imported radiolabeled ergosterol and cholesterol in subfractions of yeast mitochondria

	Relative specific activity		
	OM	CS	IM
[^{14}C]Ergosterol[a]	1	0.51 ± 0.09	0.31 ± 0.10
[^{14}C]Cholesterol[b]	1	0.30 ± 0.12	0.56 ± 0.05
Porin	1	0.23 ± 0.08	0.08 ± 0.05

OM, outer membrane; CS, contact sites; IM, inner membrane.
Specific activities (dpm/mg protein) of radiolabeled sterols 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.
[a] Phosphatidylserine transfer protein was present during ergosterol import into mitochondria but absent[b] during cholesterol import.

the import of sterols into yeast mitochondria is the energy dependence (Table 2). When mitochondria were treated with 0.1 mM CCCP, import of ergosterol to the inner membrane was not significantly affected. This concentration of the uncoupler is sufficient to abolish the electrochemical gradient across the inner mitochondrial membrane as shown very recently by Roucou et al. [25]. Treatment of mitochondria with apyrase (0.5 U/mg protein) and oligomycin (0.07 mM), which depletes ATP practically to a zero level (as measured by the luciferin-luciferase assay), did also not inhibit the import of ergosterol to the inner mitochondrial membrane. Addition of ATP to the incubation mixture did not have a stimulatory effect. These results indicate that intramitochondrial translocation of ergosterol is an energy-independent process.

4. Discussion

Mitochondria of the yeast are unable to synthesize their own sterols, but rely on the import of these lipids. The fact that in the yeast *S. cerevisiae* mitochondrial sterols are mainly located in the inner membrane [7] necessitates their intramitochondrial translocation. We designed an in vitro assay to study the import of the yeast specific sterol, ergosterol, and for comparative reasons the mammalian specific sterol, cholesterol, from unilamellar vesicles to the inner mitochondrial membrane. Ergosterol translocation was enhanced in the presence of a lipid transfer protein (see Fig. 1A), whereas no such additive was required for loading mitochondria with cholesterol (Fig. 2A). The minor structural difference between ergosterol and cholesterol may be sufficient to affect the affinity to the transfer protein and/or the degree of spontaneous translocation between acceptor and donor membrane in the in vitro system. We do not claim, however, that protein-catalyzed transport is the mechanism of sterol import into mitochondria in vivo. A role of lipid transfer proteins (i.e. sterol carrier protein 2) as mediators of this process has been suggested, but this idea has not been sufficiently supported by experimental evidence as yet. Therefore, other possible mechanism(s) of intracellular sterol translocation, such as vesicle flow, membrane contact and local membrane fusion should be considered as well to be relevant in the living cell.

The mechanism which leads to the translocation of ergosterol and cholesterol to the inner membrane of yeast mitochondria remains still obscure. A driving force for the import of ergosterol in vitro may be the imbalance of the sterol concentration between donor vesicles and the outer mitochondrial membrane caused by the lipid transfer protein. Since the concentration of ergosterol in the inner membrane of yeast mitochondria is much higher than in the outer membrane [4,7], ergosterol has to be imported against a concentration gradient. Cholesterol is not a component of both yeast mitochondrial membranes and partitioning effects may lead to an equilibrium between donor vesicles, the outer and the inner mitochondrial membrane.

Table 2
Characterization of ergosterol import into mitochondria in vitro

	Relative specific activity of ergosterol		
	OM	CS	IM
Control	1	0.51 ± 0.09	0.31 ± 0.10
+ CCCP (2 μg/ml protein)	1	0.43 ± 0.01	0.26 ± 0.01
+ ATP (0.5 mM)	1	0.47 ± 0.01	0.26 ± 0.04
+ Apyrase (0.5 U/mg protein) and oligomycin (0.07 mM)	1	0.51 ± 0.14	0.45 ± 0.11

For abbreviation and calculation of data, see Table 1.

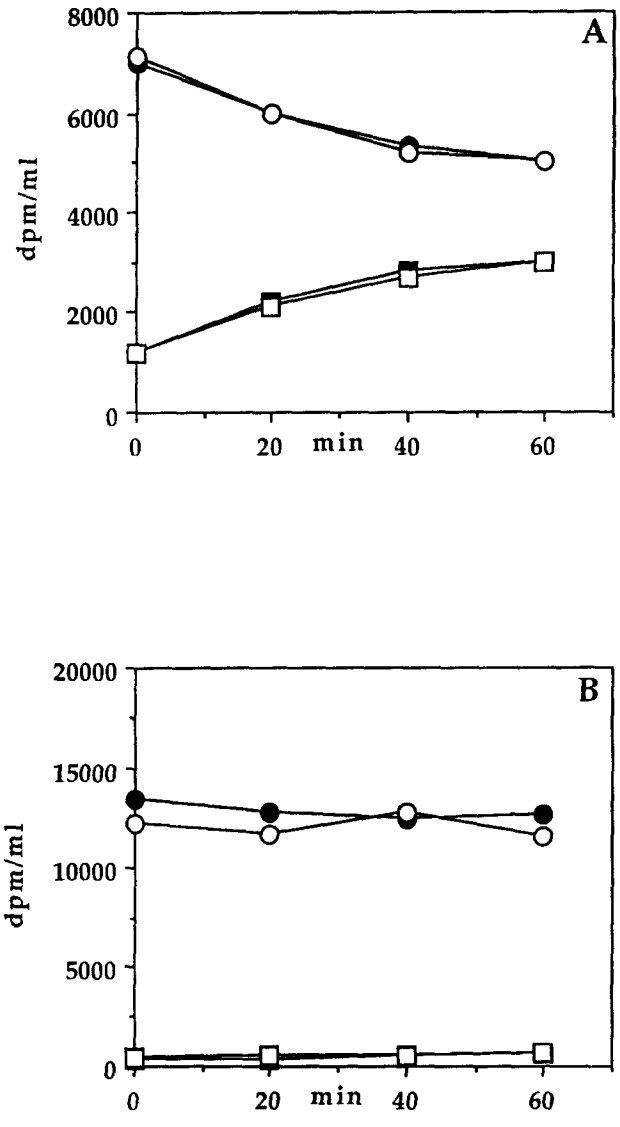

Fig. 2. Transport of [^{14}C]cholesterol and [^{3}H]triacylglycerol from unilamellar vesicles to yeast mitochondria in vitro. [^{14}C]Cholesterol (A) and [^{3}H]triacylglycerol (B) were translocated to yeast mitochondria in the presence (filled symbols) and absence (open symbols) of the yeast phosphatidylserine transfer protein as described for Fig. 1. (●, ○) dpm/ml in the supernatant (vesicles); (■, □) dpm in the mitochondrial pellet (1-ml aliquot).

Previous work from our laboratory [9–12] and from others [26] suggested that contact sites between the outer and the inner mitochondrial membrane are zones of intramitochondrial transport of phospholipids. In this communication, we demonstrate that ergosterol and cholesterol are detectable in contact sites on the way to the inner mitochondrial membrane (see Table 1). This result may serve as an indication, that also sterols migrate within the mitochondria via membrane junctions. Intramitochondrial translocation of ergosterol does not require an electrochemical gradient across the inner mitochondrial membrane or ATP (see Table 2). Similar characteristics were

found previously for the import of phosphatidylserine [11], phosphatidylinositol and phosphatidylcholine [12] into yeast mitochondria. In contrast, the import of proteins into mitochondria is strictly energy-dependent (for reviews see [27,28]). Although both types of macromolecules, proteins and lipids, seem to migrate through membrane contact sites to the inner mitochondrial membrane, the mechanisms governing these processes must be fundamentally different.

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