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Lipid analysis of mitochondrial membranes from the yeast *Pichia pastoris*

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ABSTRACT

Here we describe for the first time isolation and biochemical characterization of highly purified mitochondrial inner and outer membranes from *Pichia pastoris* and systematic lipid analysis of submitochondrial fractions. Mitochondria of this yeast are best developed during growth on glycerol or sorbitol, but also on methanol or fatty acids. To obtain organelle membranes at high quality, methods of isolation and subfractionation of mitochondria originally developed for *Saccharomyces cerevisiae* were adapted and employed. A characteristic feature of the outer mitochondrial membrane of *P. pastoris* is the higher phospholipid to protein ratio and the lower ergosterol to phospholipid ratio compared to the inner membrane. Another marked difference between the two mitochondrial membranes is the phospholipid composition. Phosphatidylcholine and phosphatidylethanolamine are major phospholipids of both membranes, but the inner membrane is enriched in cardiolipin, whereas the outer membrane contains a high amount of phosphatidylinositol. The fatty acid composition of both mitochondrial membranes is similar. Variation of the carbon source, however, leads to marked changes of the fatty acid pattern both in total and mitochondrial membranes. In summary, our data are the first step to understand the *P. pastoris* lipidome which will be prerequisite to manipulate membrane components of this yeast for biotechnological purposes.

1. Introduction

The methylotrophic yeast *Pichia pastoris* is successfully used as an expression system for the production of recombinant proteins and well characterized with respect to its use as a tool in biotechnology [1–4]. However, cell biological characterization of *P. pastoris* organelles and membranes is still in its infancy. Therefore, we started a systematic investigation of subcellular compartments from this yeast with special emphasis on the lipids of organelle membranes [5].

P. pastoris is able to grow on methanol or fatty acids which leads to the induction of peroxisomal proliferation [6,7] through activation of methanol metabolism or β-oxidation of fatty acids. Consequently, growth on both carbon sources leads to upregulation of specific and distinct sets of enzymes in peroxisomes. Beside peroxisomes, however, mitochondria play an important role under these growth conditions. After degradation of fatty acids in peroxisomes, metabolites are exported and enter the mitochondria for further oxidation and energy production via oxidative phosphorylation. Therefore, both organelles are activated in parallel and should be considered at least in part as a functional unit [8–11].

The lipid composition of yeast cells changes in response to the carbon source utilized and the growth phase of the culture. Moreover, different subcellular membranes have different lipid composition and requirements. Mitochondrial membranes from mammalian cells, plants and yeast are unique insofar as they exhibit a lower phospholipid to protein ratio and sterol to protein ratio than most other subcellular fractions. Phosphatidylcholine and phosphatidylethanolamine are the main phospholipid species of mitochondria similar to other organelles, but cardiolipin is found specifically enriched. Finally, mitochondria are devoid of sphingomyelin and glycosphingolipids. Mitochondrial inner and outer membranes can also be distinguished by their lipid patterns. The outer mitochondrial membrane (OMM) is a smooth lipid rich envelope with pore-forming proteins, whereas the highly folded inner mitochondrial membrane (IMM) is protein rich harboring mainly enzymes of the respiratory chain [12]. Phosphatidylinositol is present in the OMM at a large amount whereas cardiolipin and phosphatidylethanolamine are enriched in the IMM. Enzymes catalyzing formation of the latter two phospholipids and phosphatidylglycerol are components of the IMM [13]. The presence of cardiolipin in membranes involved in energy production suggested that this phospholipid is important for efficient oxidative phosphorylation [14].

Another characteristic feature of mitochondria is their low sterol concentration. The submitochondrial distribution of sterols varies in

Abbreviations: OMM, outer mitochondrial membrane; IMM, inner mitochondrial membrane; TLC, thin layer chromatography; GLC, gas–liquid chromatography; LP, lysophospholipid; PtdSer, phosphatidylserine; PtdIns, phosphatidylinositol; PtdOH, phosphatidic acid; PtdCho, phosphatidylcholine; PtdEtn, phosphatidylethanolamine; CL, cardiolipin; DM-PtdEtn, dimethyl phosphatidylethanolamine

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different species. In mammals, plants and *Neurospora crassa* the sterol content in the OMM is significantly higher than in the IMM, whereas in *Saccharomyces cerevisiae* the sterol concentration in the IMM exceeds that of the OMM [12]. OMM and IMM are in close vicinity at the so called mitochondrial membrane contact sites. These sites are thought to play a role in the import of proteins into mitochondria but also in the intramitochondrial translocation of lipids [15,16]. Contact sites of *S. cerevisiae* and mammalian cells were characterized by a low phosphatidylcholine level compared to OMM and IMM. The cardiolipin concentration in contact sites of *S. cerevisiae* was found to be similar to the IMM, whereas in mammalian cells it was increased [12,17].

The present paper expands our knowledge of mitochondrial lipids to the yeast *P. pastoris*. In contrast to *S. cerevisiae*, *P. pastoris* belongs to the group of Crabtree-negative yeasts, which means that it is not capable of fermentation in the presence of oxygen [18,19]. Therefore, mitochondria of *P. pastoris* are of special interest because oxidative metabolism is paramount. To characterize mitochondria from *P. pastoris* biochemically and cell biologically, we analyzed outer and inner mitochondrial membranes with special emphasis on the distribution of lipids when growth conditions are varied. We compare these findings to results obtained with other experimental systems and point out the effect of the carbon source used for cultivation of this microorganism on the lipid composition of mitochondria.

2. Materials and methods

2.1. Strains and culture conditions

The *P. pastoris* X33 (MATa, Mut$^+$) strain was used throughout this study. YPD medium containing 1% yeast extract, 2% peptone and 2% glucose was used to pre-cultivate the strains for 48 h at 30 °C in Erlenmeyer flasks with baffles at 140 rpm. Main cultures contained either 2% glucose (YPD); 0.5% methanol (YPM); 0.2% oleic acid (YPO) with 0.02% Tween 40; 2% glycerol (YPG) or 2% sorbitol (YPS) as carbon sources. Cells were cultivated aerobically in 2 l-Erlenmeyer flasks with baffles under vigorous shaking. Growth rates were determined from the midlogarithmic growth phase.

2.2. Electron microscopy

Yeast cells grown on glucose, glycerol or sorbitol media to the late logarithmic phase were sedimented by centrifugation, rinsed in 0.1 M cacodylate buffer (pH 7.4) and fixed with 3% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) containing 5 mM $CaCl_2$ and 5 mM $MgCl_2$ at 4 °C for 24 h. The cells were subsequently washed twice with H_2O_{dd} and postfixed in 4% $KMnO_4$ at room temperature for 1 h. After rinsing the pellet four times in H_2O_{dd}, cells were treated with 0.5% sodium metaperiodate at room temperature for 15 min, washed twice with H_2O_{dd} and dispersed in a 4% agarose solution. The solidified agar containing the cells was cut into small cubes of about 1 mm^3, and the blocks were dehydrated through a graded series of acetone (30%, 50%, 70%, 90%, and 100%). Samples were incubated in Spurr resin [20].

Isolated mitochondria were sedimented by centrifugation, rinsed in 0.1 M cacodylate buffer (pH 7.4) and fixed with 3% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) at 4 °C for 24 h. The samples were washed twice with 0.1 M cacodylate buffer and postfixed in 1% OsO_4 prepared in 0.1 M cacodylate buffer at room temperature for 2 h. Finally, the pellet was rinsed twice with H_2O_{dd} and dispersed in a 4% agarose solution. The solidified agar containing the mitochondria was cut into small cubes of about 1 mm^3 and the blocks were dehydrated through a graded series of ethanol (30%, 50%, 70%, 90%, and 100%) and three dehydrations in 100% propylene oxide. Samples were incubated in Epon resin [21]. Ultrathin sections (75 nm) were made on a Leica Ultracut UCT ultramicrotome using a diamond knife (Diatome) and collected onto 75 mesh copper palladium grids with a support film of pioloform. Sections were stained in 1% uranyl acetate in H_2O_{dd} for 20 min, washed and stained once more in lead citrate [22]. Images were produced on a Tecnai G2 12 (FEI Company) equipped with a CCD camera (Gatan Bioscan) at 120 kV.

2.3. Isolation of mitochondria and mitochondrial subfractions

Mitochondria and mitochondrial subfractions (IMM and OMM) were isolated from cells grown to the late logarithmic phase according to the procedure of Daum et al. [23] with some modifications. In brief, cells were harvested by centrifugation and converted to spheroplasts using Zymolyase 20T. Spheroplasts were homogenized in breaking buffer (0.6 M sorbitol, 20 mM HEPES, 20 mM Na_2EDTA, pH 7.4, 1 mM PMSF) with 15 strokes in a Dounce Homogenizer. Unbroken cells and cell debris were removed by centrifugation at 5,000 rpm for 5 min. This homogenization step was repeated twice; the resulting supernatants (homogenate) were combined and centrifuged at 10,000 rpm in a Sorvall SS-34 rotor at 4 °C for 10 min. A 1 ml sample of the resulting supernatant (cytosolic fraction with microsomes) was taken; the pellet (crude mitochondria) was suspended in breaking buffer and centrifuged at 5,000 rpm for 5 min. The pellet (debris) was discarded and mitochondria were isolated by centrifugation of the supernatant at 10,000 rpm in a Sorvall SS-34 rotor at 4 °C for 10 min. Mitochondria were suspended in breaking buffer at a final concentration of 10 mg protein/ml.

For the isolation of OMM and IMM mitochondria were subjected to a swelling-shrinking procedure followed by an ultrasonication step. For this purpose, mitochondria were stirred in ice cold "swelling buffer" (20 mM HEPES, 0.5 mM Na_2EDTA, pH 7.4) for 30 min and 10 min in "shrinking buffer" (1.8 M sucrose in swelling buffer). The suspension was exposed to ultrasonication at 0 °C for 6×15 s (Brownsonic) and then centrifuged at 16,500 rpm for 25 min (Sorvall SS 34 rotor). The supernatant was collected and ultracentrifuged at 45,000 rpm for 1 h (Sorvall T865 rotor). The pellet was resuspended in 4 ml HEPES buffer (5 mM HEPES, 10 mM KCl, pH 7.4) and loaded on top of a 30–50% linear sucrose gradient in HEPES buffer. The gradient was centrifuged at 25,000 rpm for 15 h at 4 °C in a Sorvall AH629 rotor. The OMM fraction (buoyant density of ~1.08 g/cm^3), the IMM fraction (buoyant density of ~1.18 g/cm^3) and the intermediate fraction forming a band at a buoyant density of ~1.15 g/cm^3 were collected with a syringe, diluted with 10 mM Tris/HCl, pH 7.4 and sedimented at 45,000 rpm for 45 min in a Sorvall T865 rotor. Mitochondrial subfractions were resuspended in 1 ml 10 mM Tris/HCl, pH 7.4.

2.4. Protein analysis

Protein was quantified as described previously [24] using bovine serum albumin as a standard. Proteins were precipitated with trichloroacetic acid and solubilized in 0.1% SDS, 0.1 M NaOH prior to quantification. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out by the method of Laemmli [25] using 10% SDS gels. Western blot analysis was performed according to Haid and Suissa [26]. Primary antibodies used in this work were from rabbits and directed against the *S. cerevisiae* proteins Por1p (Porin), Pma1p (plasma membrane ATPase), Aac2p (ADP/ATP carrier protein), GAPDH (glyceraldehyde-3-phosphate dehydrogenase), Wbp1p (subunit of the oligosaccharyl transferase glycoprotein complex) and against the *P. pastoris* multifunctional β-oxidation protein Fox2p, respectively. Peroxidase conjugated secondary antibody and enhanced chemiluminescent signal detection (SuperSignal™, PierceChemical Company, Rockford, IL, USA) were used to visualize immunoreactive bands.

2.5. Lipid analysis

Lipids from homogenate, mitochondria and mitochondrial membranes were extracted by the method of Folch et al. [27].