Biochimica et Biophysica Acta 1817 (2012) 353–362

Contents lists available at SciVerse ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbabio

During the stationary growth phase, *Yarrowia lipolytica* prevents the overproduction of reactive oxygen species by activating an uncoupled mitochondrial respiratory pathway

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ARTICLE INFO

Article history:
Received 19 August 2011
Received in revised form 8 November 2011
Accepted 9 November 2011
Available online 22 November 2011

Keywords:
Yarrowia lipolytica
Mitochondrion
Branched respiratory chain
Alternative NADH dehydrogenase
Physiological uncoupling
Reactive oxygen species, ROS

ABSTRACT

In the branched mitochondrial respiratory chain from *Yarrowia lipolytica* there are two alternative oxido-reductases that do not pump protons, namely an external type II NADH dehydrogenase (NDH2e) and the alternative oxidase (AOX). Direct electron transfer between these proteins is not coupled to ATP synthesis and should be avoided in most physiological conditions. However, under low energy-requiring conditions an uncoupled high rate of oxygen consumption would be beneficial, as it would prevent overproduction of reactive oxygen species (ROS). In mitochondria from high energy-requiring, logarithmic-growth phase cells, most NDH2e was associated to cytochrome *c* oxidase and electrons from NADH were channeled to the cytochromic pathway. In contrast, in the low energy requiring, late stationary-growth phase, complex IV concentration decreased, the cells overexpressed NDH2e and thus a large fraction of this enzyme was found in a non-associated form. Also, the NDH2e–AOX uncoupled pathway was activated and the state IV external NADH-dependent production of ROS decreased. Association/dissociation of NDH2e to/from complex IV is proposed to be the switch that channels electrons from external NADH to the coupled cytochrome pathway or allows them to reach an uncoupled, alternative, ΔΨ-independent pathway.

1. Introduction

Mitochondria from fungi, plants and parasites often contain branched respiratory chains, constituted by orthodox and "alternative" redox enzymes present in different stoichiometries [1–3]. In mitochondria from *Yarrowia lipolytica* the respiratory chain is composed by the four multi-subunit complexes (I to IV) found in animals and plants, plus an external type II NADH dehydrogenase (NDH2e) and an alternative oxidase (AOX) [4]. Both NDH2e and AOX are single-subunit peripheral oxido-reductases that lack proton-pumping activity [5,6].

Branched respiratory chains may include different alternative dehydrogenases that reduce ubiquinone without contributing to the proton gradient. Thus, ubiquinone may be reduced by complex I, succinate-dehydrogenase, glycerol-phosphate dehydrogenase, dihydroorotate-dehydrogenase or by internal or external NDH2s [7,8]. All these enzymes use flavin in the redox reaction [9–11]. From ubiquinol, electrons can reach either complexes III/IV (cytochromic pathway) or an AOX [12]. The many pathways available open the possibility that electrons may reach oxygen with different proton-pumping stoichiometries, even passing only through enzymes that do not translocate protons at all [13], i.e. the ADP:O ratio can vary widely in branched respiratory chains [14].

Structural models of AOX, whether monomeric or dimeric [15–17], suggest it has regulatory sites for nucleotides and/or for α-ketoacids [18,19]. Some yeast species contain only one AOX, which is activated under stress. Other species contain two isoforms of AOX, one constitutive but expressed at low levels and another one inducible under stress [16]. Remarkably, no species is known where the absence of complex-I and the presence of AOX coexist; it has been proposed that such a combination would lead to uncontrolled uncoupling [1,4].

Unless uncoupled respiration is desired, energy-requiring cells must avoid pairing non proton-pumping dehydrogenases with AOX [1]. Therefore, it is of interest to define the usefulness of the non-pumping enzymes. These enzymes might prove useful in medicine and biotechnology; alternative NDH2i (internal NDH2) has been expressed in mammalian cells to partially substitute for a non-functional complex I [20]. In addition, NDH2 has been expressed in

Abbreviations: NDH2e, alternative external NADH dehydrogenase; AOX, alternative oxidase; ΔΨ, transmembrane potential; CCCP, carbonylcyanide-3-chlorophenylhydrazone; ROS, reactive oxygen species

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doi:10.1016/j.bbabio.2011.11.007

aerobic fungi or yeast harboring complex I deficiencies with the aim to analyze mitochondrial function [21,22].

Alternative component activity must be tightly regulated in response to the energy requirements or to the redox state of the cell. In different species of yeast, AOX is over-expressed under stress [23] or in the stationary growth phase [24]. Channeling electrons between a proton-pumping complex and an alternative component would prevent excessive wastage of energy and thus functional associations such as complex I-AOX [25] or NDH2-complexes III/IV [26] have been proposed. In addition, some redox enzymes might alternate between a bound, electron-channeling state and a free, non-channeling state in order to regulate the proton pumping efficiency of the respiratory chain and thus the ADP:O.

Y. lipolytica mitochondria were isolated from cells grown to either the high-energy requiring logarithmic growth phase (log-phase) or the low-energy requiring stationary growth phase (stat-phase) [27–29]. It was observed that in the log-phase, NDH2e-derived electrons were channeled to the cytochromic pathway and NDH2e was bound to complex IV, probably in a complex III/complex IV super-complex. In contrast, in the stat-phase the NDH2e–AOX uncoupled-electron transfer pathway was activated, most likely as a result of the dissociation of NDH2e from complex IV. In these conditions ROS production was largely inhibited, suggesting a physiologic role for uncoupling.

2. Experimental procedures

2.1. Materials

NADH, Glycerol-phosphate, n-propylgallate, n-β-D-dodecylmaltoside, digitonin, mannitol, pyruvate, malate, cytochrome *c*, antimycin-A, rotenone and CCCP were from Sigma Chem. Co. (St. Louis, MO, USA). Coomassie blue G was from Serva (Heidelberg, Germany). The polyclonal antibody against *Y. lipolytica* NDH2e (anti-*Yl*NDH2e) was a kind gift from Dr. Stefan Kerscher, Zentrum der Biologischen Chemie, Frankfurt University, (Germany). Monoclonal antibodies against cytochrome *c* oxidase subunits II and III from *S. cerevisiae* were from Mitoscience (Eugene, OR, USA).

2.2. Strains, culture and isolation of yeast mitochondria

The strains used in this work were the wild type: *Y. lipolytica* E150 (*MatB his1-1 ura3-302 leu2-270 xpr2-322*) and the *Δndh2e* mutant (GB5.2) [30]. All strains were a kind gift from Prof. Ulrich Brandt, ZBC, Frankfurt University (Germany). Cells were grown in YD (Yeast extract 1%, glucose 2%) [31] at 160 rpm, 30 °C for 15 or 96 h to be harvested at the logarithmic or late stationary growth phases, respectively. Cells were washed and re-suspended in 5 mM MES, 0.6 M mannitol, 0.1% BSA (pH 6.8, triethanolamine) and disrupted using a Bead Beater cell homogenizer (Biospec Products, OK, USA) with 0.45 mm glass beads (3×20 s pulses separated by 40 s resting periods). To isolate mitochondria, the homogenate was subjected to differential centrifugation [32] and protein concentration was determined by biuret [33]. Mitochondrial intactness was evaluated by measuring the respiratory controls which were between 2.0 and 3.0 [34]. The integrity of the external mitochondrial membrane was determined by reduction of diaminobenzidine in the presence of 10 μM antimycin A and cytochrome *c* either permeabilized with 0.1 mg/mg protein n-β-D-docecylmaltoside or not. Diaminobenzidine reduction was measured by absorbance changes at 490 nm. In non-permeabilized mitochondria the reaction was 9-fold less than in the presence of detergent. In addition, in the presence of external NADH as a substrate, rotenone inhibited less than 6% of the total oxygen consumption, indicating that external NADH was oxidized mainly by NDH2e.

2.3. Oxygen consumption measurements

The rate of oxygen uptake was measured in an oxygen meter model 782 (Warner/Strathkelvin Instruments) with a Clark type electrode in a 0.1 mL water-jacketed chamber at 30 °C [35] and data were analyzed using the 782 Oxygen System software (Warner/Strathkelvin Instruments). External NADH-dependent respiration was measured in the presence of rotenone in order to inhibit reverse electron transfer from ubiquinol to complex I; rotenone binds to the ubiquinone binding site of complex I [36]. Pyruvate plus malate was used to generate internal NADH which is oxidized by complex I. Cyanide or antimycin A was used to inhibit cytochrome *c* oxidase or the Qi site of complex III, respectively. Since both cyanide and antimycin A inhibit the cytochrome pathway, electrons are diverted towards AOX. The reaction mixture contained 0.6 M mannitol, 5 mM MES (pH 6.8), 20 mM KCl, 4 mM phosphate (the Tris salt was obtained using phosphoric acid and adjusting to pH 6.8 with Tris) and 1 mM $MgCl_2$. Mitochondria were added to a final concentration of 0.5 or 1.0 mg protein/mL. Respiratory controls using either external NADH or succinate were determined in the presence of 2.5 μM rotenone.

2.4. Protein separation by native electrophoresis and in-gel activities

BN- and CN-PAGE were performed as described [37,38]. Mitochondria were solubilized with 2 g n-dodecyl-β-D-maltoside (LM)/g protein, or 4 g digitonin (Dig)/g protein at 4 °C and centrifuged at 100,000 *g* at 4 °C for 25 min. Protein concentration of the supernatants was determined and 0.4 mg protein per well was loaded on 4–12% polyacrylamide gradient gels. For both BN and CN-PAGE digitonin (0.025%) was added to the gel preparation. The cathode buffer for CN-PAGE contained 0.01% LM and 0.05% deoxycholate as described in [39]. In-gel NADH:NBT oxido-reductase activity was determined by incubating the native gels in a mixture containing 10 mM Tris (pH 7.0), 0.5 mg nitro blue tetrazolium bromide (NBT)/mL and 1 mM NADH [40]. In-gel cytochrome *c* oxidase activity was determined using diaminobenzidine and cytochrome *c* as described in [41].

2.5. Ion exchange chromatography and enzymatic activities of the fractions

Mitochondria were solubilized with 0.8 g/g prot n-β-D-dodecylmaltoside (LM) in 1 mM Mg–SO_4, 1 mM PMSF, 50 mM HEPES, pH 8.0 plus 50 μg/mL TLCK (Tosyl-lysyl-chloromethyl ketone). The solubilizate was centrifuged at 100,000×*g* for 30 min and layered on top of a previously equilibrated DEAE-Sepharose column [42]. Once loaded, the column was washed with 3 volumes of 1 mM $MgSO_4$, 50 mM Tris, pH 8.0 and proteins were eluted with a 0–400 mM NaCl gradient. Fractions of 1 mL were collected. The protein concentration in each fraction was estimated spectrophotometrically at 280 nm. For NADH dehydrogenase or cytochrome *c* oxidase activities, 20 μL of each fraction was placed in a well within a micro plate in the presence of the substrate and an electron acceptor and the reaction was followed in a multimodal micro plate reader Synergy Mix, Biotek (VT, USA). Enzyme assays: a) NADH dehydrogenase, following the reduction of nitro blue tetrazolium bromide (NBT) at 570 nm. The reaction mixture was 10 mM Tris, pH 7.0, 1 mM NADH, 0.5 mg/mL NBT; b) Cytochrome *c* oxidase was measured in 50 mM phosphate buffer (sodium) pH 7.4, 2.5 mg/mL horse heart cytochrome *c*, 1 mg/mL diaminobenzidine and cytochrome *c* reduction was followed at 490 nm.

2.6. Immunoassays

Ion exchange chromatography fractions were loaded onto a nitrocellulose membrane for dot blot immunodetection with antibodies