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Research paper

Oxidative phosphorylation in *Debaryomyces hansenii*: Physiological uncoupling at different growth phases

Alfredo Cabrera-Orefice [a], Sergio Guerrero-Castillo [a], Rodrigo Díaz-Ruíz [b], Salvador Uribe-Carvajal [a,*]

[a] Dept of Molecular Genetics, Instituto de Fisiología Celular, Universidad Nacional Autónoma de México, Mexico City, Mexico
[b] Facultad de Medicina, Programa de Posgrado en Ciencias Médicas, Odontológicas y de la Salud, Universidad Nacional Autónoma de México, Mexico City, Mexico

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ABSTRACT

Physiological uncoupling of mitochondrial oxidative phosphorylation (OxPhos) was studied in *Debaryomyces hansenii*. In other species, such as *Yarrowia lipolytica* and *Saccharomyces cerevisiae*, OxPhos can be uncoupled through differential expression of branched respiratory chain enzymes or by opening of a mitochondrial unspecific channel ($_{Sc}$MUC), respectively. However *D. hansenii* mitochondria, which contain both a branched respiratory chain and a mitochondrial unspecific channel ($_{Dh}$MUC), selectively uncouple complex I-dependent rate of oxygen consumption in the stationary growth phase. The uncoupled complex I-dependent respiration was only 20% of the original activity. Inhibition was not due to inactivation of complex I, lack of protein expression or to differential expression of alternative oxidoreductases. Furthermore, all other respiratory chain activities were normal. Decrease of complex I-dependent respiration was due to NAD^+ loss from the matrix, probably through an open of $_{Dh}$MUC. When NAD^+ was added back, coupled complex I-activity was recovered. NAD^+ re-uptake was independent of $_{Dh}$MUC opening and seemed to be catalyzed by a NAD^+-specific transporter, which was sensitive to bathophenanthroline, bromocresol purple or pyridoxal-5′-phosphate as described for *S. cerevisiae* mitochondrial NAD^+ transporters. Loss of NAD^+ from the matrix through an open MUC is proposed as an additional mechanism to uncouple OxPhos.

1. Introduction

Some 10^9 years ago, the Great Oxidation Event (GOE) imposed a strong natural selection that led surviving organisms both to profit from the large energy released by water production and to develop protection mechanisms from toxic reactive oxygen species (ROS) [1,2]. Many animals including fish, birds and mammals developed significant barriers to exclude atmospheric oxygen (O_2). These organisms control cellular O_2 concentration tightly [3]. Less developed organisms such as amphibians, crustaceans, plants and unicellulars are permeable to atmospheric O_2 [4,5]. Thus, it is no wonder that bacteria, fungi, plants and lower animals express O_2-detoxifying mechanisms that uncouple oxidative phosphorylation (OxPhos) designed to prevent side reactions that yield ROS [6,7].

In the absence of ATP synthesis (non-phosphorylating state or state IV), mitochondrial respiratory chain activity would be slowed down by the high $\Delta\mu_H^+$ [8] increasing the likelihood that free radicals from complexes I and III react with O_2 to form ROS [6,7]. To avoid this, mitochondria accelerate the rate of oxygen consumption through different physiological mechanisms [8]. These mechanisms may (a) dissipate the proton-gradient (proton sinks) or (b) perform redox reactions without proton pumping [8,9]. Alternative, non-pumping dehydrogenases may compete or even substitute the usual respiratory complexes decreasing the efficiency of the respiratory chain [8]. Proton sinks include mitochondrial unspecific channels (MUCs) [10,11] and uncoupling proteins (UCPs) [12]. On the other hand, non-conservative redox reactions are catalyzed by peripheral alternative oxidoreductases such as mitochondrial glycerol-phosphate dehydrogenases ($_{Mit}$GPDHs), alternative type-II NADH dehydrogenases (NDH2s) and alternative oxidases (AOXs) [13,14]. NDH2s and AOXs do not exist in mammals, which contain four multi-subunit redox complexes, namely I, II, III and IV [15]. By contrast, the “branched” mitochondrial respiratory chains in plants,

* Corresponding author. Departamento de Genética Molecular, Instituto de Fisiología Celular, Universidad Nacional Autónoma de México, Ciudad Universitaria, Apdo. Postal 70-242, Mexico City, Mexico. Tel.: +52 55 5622 5632; fax: +52 55 5622 5630.
E-mail address: suribe@ifc.unam.mx (S. Uribe-Carvajal).

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fungi, yeast, protozoa and some metazoans do contain this kind of alternative redox enzymes [16–19] and thus electrons may reach O_2 through different routes [20]. In addition, "classical" respiratory complexes may decrease their H^+/e^- stoichiometry (slipping) [21]. To prevent lethal energy depletion, physiological uncoupling has to be strictly regulated [8,22,23].

Mitochondria from different yeast species may possess both, proton sinks and branched respiratory chains. For example, *Saccharomyces cerevisiae* has a mitochondrial unspecific channel ($_{Sc}$MUC) and its respiratory chain possesses multi-subunit complexes II, III and IV, two external type-II NADH dehydrogenases, one internal type-II NADH dehydrogenase and one $_{Mit}$GPDH [24,25]. *Yarrowia lipolytica* does not seem to contain a MUC [26] but instead, it expresses an UCP-like transporter [27] plus a branched respiratory chain containing the four classical complexes, an external alternative NADH dehydrogenase (NDH2e) and an AOX [28]. Interestingly, upon reaching the stationary growth phase *Y. lipolytica*, overexpresses its alternative NDH2e, which then transfers the electrons from NADH directly to quinone and to AOX and to oxygen without pumping a single proton [26] in what should be considered a futile, uncoupled respiration [26,28].

The halotolerant yeast *Debaryomyces hansenii* contains a MUC ($_{Dh}$MUC), which is sensitive to Na^+ and K^+ [29]. This species resists high monovalent cations concentrations [30]. $_{Dh}$MUC was detected in the exponential growth phase [29] but it seems to be active in the stationary phase, where less ATP is required. In addition, *D. hansenii* has a branched mitochondrial respiratory chain containing the four multi-subunit complexes, an AOX, an NDH2e and a $_{Mit}$GPDH [31,32].

While OxPhos in *D. hansenii* is probably optimal during the exponential growth phase, upon entering the stationary phase uncoupling mechanisms should be turned on to maintain a high rate of O_2 consumption and thus inhibit ROS production and regulate the redox state. Here, the expression of the non-proton pumping oxidoreductases and the state of $_{Dh}$MUC was evaluated at different growth phases in an effort to understand the physiological uncoupling mechanisms exhibited by this species. Most of the respiratory chain components in *D. hansenii* remained normal throughout growth. By contrast, the complex I-dependent rate of O_2 consumption was highly decreased and uncoupled. The mechanism for this behavior was explored.

2. Materials and methods

2.1. Chemicals

All chemicals were reagent grade. D-sorbitol, D-glucose, D-galactose, Trizma® base (Tris), malic acid, pyruvic acid, citric acid succinic acid, maleic acid, DL-α-glycerophosphate, NAD^+, NADH, ADP, rotenone, bathophenanthroline, pyridoxal-5′-phosphate, *n*-dodecyl β-D-maltoside (laurylmaltoside), Nitrotetrazolium blue chloride, safranine-O and antifoam A were from Sigma Chem Co. (St Louis, MO). Bovine serum albumin (Probulmin™) was from Millipore. Yeast extract and bacto-peptone were from BD Bioxon. DL-Lactic acid, tannic acid, H_3PO_4, NaCN, KCl and $MgCl_2$ were from J.T. Baker. Zymolyase 20 T was from Seikagaku Corp. (Tokyo, Japan). 3,3′-Diaminobenzidine tetrahydrochloride hydrate was from Fluka. Coomassie Blue G was from SERVA (Heidelberg, Germany). Coomassie® brilliant blue G-250 and electrophoresis reagents were from BIO-RAD (Richmond, CA, USA).

2.2. Biologicals

D. hansenii Y7426 strain (US Dept. of Agriculture) was used. The strain was maintained in YPGal–NaCl (1% yeast extract, 2% bacto-peptone, 2% D-galactose, 1.0 M NaCl and 2% bacto-agar) plate cultures at 4 °C.

2.3. Yeast culture and mitochondrial isolation

D. hansenii cells were grown as follows: pre-cultures were prepared inoculating 100 mL of YPLac–NaCl medium (1% yeast extract, 2% bacto-peptone, 2% lactic acid, pH 5.5 adjusted with NaOH and adding NaCl to reach 0.6 M Na^+) or YPD–NaCl (1% yeast extract, 2% bacto-peptone, 2% D-glucose and 0.6 M NaCl). Antifoam A emulsion 50 μL/L was added to all media. Pre-cultures were grown for 36 h under continuous agitation in an orbital shaker at 250 rpm at 29 °C. Then, each pre-culture was used to inoculate a 750 mL flask with the same medium. Incubation was continued for 15, 18, 24, 48, 72 and 96 h (YPLac–NaCl) or 12, 18, 22, 48, 72 and 96 h (YPD–NaCl). *D. hansenii* mitochondria were isolated as previously reported [29].

2.4. Obtention of permeabilized spheroplasts

D. hansenii spheroplasts were obtained following a method developed for *S. cerevisiae* [33] with some modifications. Briefly, yeast cells were grown in YPLac–NaCl and harvested in either exponential (24 h) or stationary phase (96 h). Cells were washed, centrifuged at 5000 rpm (5 min), resuspended in SH buffer (0.5 M β-mercaptoethanol, 0.1 M Tris, pH 9.3) and incubated at 30 °C for 10 min. Then, cells were centrifuged (5000 rpm, 5 min) and washed twice with 0.5 M KCl, 10 mM Tris, pH 7.0. To obtain spheroplasts, cells were suspended in digesting buffer (1.35 M sorbitol, 1 mM EGTA, 0.2 M phosphate buffer, pH 7.4) containing zymolyase 20 T (5 mg/g biomass dry-weight), which digested the cell wall. Progression of digestion was monitored spectrophotometrically and was stopped when turbidity of a sample suspended in water decreased to 10% of the control. Resulting spheroplasts were centrifuged (2500 rpm, 5 min) and washed three times using protoplast buffer (1.2 M sorbitol, 10 mM Tris–Maleate, pH 6.8). Samples were resuspended in spheroplast buffer (1.0 M sorbitol, 75 mM KCl, 5 mM $MgCl_2$, 0.5 mM EGTA, 20 mM Tris-phosphate, 10 mM Tris-Maleate, 0.2% bovine serum albumin, pH 6.8). Permeabilization was carried out treating spheroplasts at 1 mg protein/mL with nystatin (20 μg/mL) in the presence of constant oxygen, which was bubbled for 10 min. Complete permeabilization was confirmed by monitoring the decrease in the rate of oxygen consumption resulting from the depletion of endogenous respiratory substrates.

2.5. Protein quantification

Mitochondrial or total spheroplast protein was measured by the Biuret method [34]. Absorbance was determined at 540 nm in a Beckman DU-50 spectrophotometer. Bovine serum albumin was used as a standard.

2.6. Oxygen consumption

The rate of oxygen consumption was measured in a Strathkelvin Instruments® 782 Oxygen Meter (North Lanarkshire, Scotland, UK) interfaced to a computer. The sample was placed in a water-jacketed chamber at 30 °C. The phosphorylating state (III) was induced with 0.5 mM ADP. The reaction mixture was 1.0 M sorbitol, 10 mM maleate (pH was adjusted to 6.8 with Tris), 10 mM Tris-phosphate (Pi), 1 mM $MgCl_2$ and 75 mM KCl. Mitochondrial or total spheroplast protein (Prot) were 0.5 or 1.0 mg/mL, respectively; final volume was 1.0 mL. The concentrations of different respiratory substrates and inhibitors are indicated in the legends to the figures.

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