The branched mitochondrial respiratory chain from *Debaryomyces hansenii*: Components and supramolecular organization

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ABSTRACT

The branched respiratory chain in mitochondria from the halotolerant yeast *Debaryomyces hansenii* contains the classical complexes I, II, III and IV plus a cyanide-insensitive, AMP-activated, alternative-oxidase (AOX). Two additional alternative oxidoreductases were found in this organism: an alternative NADH dehydrogenase (NDH2e) and a mitochondrial isoform of glycerol-phosphate dehydrogenase ($_{Mit}$GPDH). These monomeric enzymes lack proton pump activity. They are located on the outer face of the inner mitochondrial membrane. NDH2e oxidizes exogenous NADH in a rotenone-insensitive, flavone-sensitive, process. AOX seems to be constitutive; nonetheless, most electrons are transferred to the cytochromic pathway. Respiratory supercomplexes containing complexes I, III and IV in different stoichiometries were detected. Dimeric complex V was also detected. In-gel activity of NADH dehydrogenase, mass spectrometry, and cytochrome *c* oxidase and ATPase activities led to determine the composition of the putative supercomplexes. Molecular weights were estimated by comparison with those from the yeast *Y. lipolytica* and they were IV$_2$, I–IV, III$_2$–IV$_4$, V$_2$, I–III$_2$, I–III$_2$–IV, I–III$_2$–IV$_2$, I–III$_2$–IV$_3$ and I–III$_2$–IV$_4$. Binding of the alternative enzymes to supercomplexes was not detected. This is the first report on the structure and organization of the mitochondrial respiratory chain from *D. hansenii*.

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1. Introduction

The halotolerant, non-pathogenic, oleaginous yeast *Debaryomyces hansenii* is found in the sea and other hyperosmotic habitats [1,2]. *D. hansenii* grows in various environmental conditions including different salt concentrations [3–5], low temperatures [3] and different pHs [3,6]. In addition, *D. hansenii* assimilates many different carbon sources [7–9]. The ability of this yeast to synthesize and store lipids is used in biotechnology to make products of commercial interest, such as cheese [2,10].

D. hansenii has high aerobic metabolism and low fermentative activity which are enhanced by high extracellular NaCl or KCl [11–13]. Isolated *D. hansenii* mitochondria undergo permeability transition due to the opening of a mitochondrial unspecific channel (MUC) [14]. Both, the MUCs from *D. hansenii* ($_{Dh}$MUC) and *S. cerevisiae* ($_{Sc}$MUC) are regulated by effectors such as phosphate, Mg^{2+} or Ca^{2+} [14–19]. The $_{Dh}$MUC is the only MUC reported to date that is closed by Na$^+$ or K$^+$ [14] probably accounting for the monovalent cation coupling effects observed in whole yeast [12,13].

The mammalian oxidative phosphorylation system contains the four "orthodox" respiratory complexes (I, II, III and IV) plus the F$_1$F$_O$-ATP synthase (complex V) [20]. In addition to the above, mitochondria from plants, fungi, protozoa and some animals may contain "alternative" redox enzymes that substitute or coexist with the classical complexes; e.g. alternative NADH dehydrogenases and oxidases [21–25]. In fungi a mammalian-like respiratory complex may be substituted by an alternative enzyme, e.g. in *S. cerevisiae* complex I the oxidoreductase activity was substituted by an internal alternative NADH dehydrogenase [26,27].

The fungal alternative oxidases (AOXs) are single subunit proteins bound to the matrix side of the inner mitochondrial membrane (IMM) [28–31]. The cyanide-resistant AOX transfers electrons from ubiquinol to oxygen. AOX is inhibited by hydroxamic acids and by *n*-alkyl-gallates [29,32]. The presence of AOX constitutes an uncoupled branch of the respiratory chain probably designed to prevent substrate overload and overproduction of reactive oxygen species (ROS) [25,28,33–36].

Alternative type II NADH dehydrogenases (NDH2s) transfer electrons from NADH to ubiquinone without pumping protons [37]. NDH2s are monomeric proteins bound to the inner (NDH2i) or the outer (NDH2e) face of IMM [21,37]. NDH2s are not sensitive to rotenone, but instead are specifically inhibited by flavone [38].

The mitochondrial isoform of glycerol-phosphate dehydrogenase ($_{Mit}$GPDH) is another component of branched respiratory chains [39,40]. $_{Mit}$GPDH oxidizes glycerol-phosphate to dihydroxyacetone-phosphate and reduces ubiquinone. Also, this protein is located on the outer face of the IMM [41]. The peripheral proteins NDH2s, $_{Mit}$GPDH and AOX are not proton pumps [21,29,40].

Two major models describe the structure/function relationship of the respiratory chain. The *fluid* or *random collision* model proposes that respiratory complexes float freely within the IMM and electron transport occurs through the diffusible carriers ubiquinone and cytochrome *c* [42]. On the other hand, the *solid* model proposes that respiratory complexes are organized into stable hetero-oligomers (supercomplexes or "respirasomes") that channel electrons between them [43–46]. There are data that support each model [47]. Kinetic studies show that each respiratory complex can be purified individually, retaining activity [42]. By contrast, blue native gel polyacrylamide electrophoresis (BN-PAGE) reveals the existence of supercomplexes composed of several respiratory complexes [48]. Respiratory super-complexes can be observed when solubilizing mitochondrial membranes with small amounts of mild detergents such as digitonin [44]. The presence of respiratory supercomplexes has been well documented in mammals [48,49], plants [43,46,50] and different yeast species [51–54]. Additionally, a third model has been proposed: the *plasticity* model, where respiratory complexes undergo a dynamic association-dissociation process and isolated supercomplexes transfer electrons from NADH to oxygen [55]. The plasticity model suggests that complex association/dissociation regulates oxidative phosphorylation [55,56].

Here, the mitochondrial respiratory chain of *D. hansenii*, which has been reported to contain all four mammalian-like respiratory complexes [57] plus a putative stationary-phase-inducible AOX, was characterized [58,59]. This branched respiratory chain contains all the complexes reported [59] plus an external NDH2 and a glycerol-phosphate dehydrogenase. In addition, association of these complexes in different supercomplexes was observed.

2. Materials and methods

2.1. Chemicals

All chemicals were reagent grade. D-sorbitol, D-mannitol, D-glucose, D-galactose, glycerol, Trizma® base (Tris), malic acid, pyruvic acid, citric acid, maleic acid, DL-α-glycerophosphate, NADH, ATP, ADP, rotenone, flavone, antimycin A, propyl-gallate, digitonin, *n*-dodecyl β-D-maltoside (laurylmaltoside), Nitrotetrazolium blue chloride 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, H_3PO_4, NaCN, KCl, $MgCl_2$ and ethanol were from J.T. Baker. 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).

2.2. Biologicals

D. hansenii Y7426 strain (US Dept. of Agriculture) was used throughout this work. The strain was maintained in YPGal-NaCl (1% yeast extract, 2% bacto-peptone, 2% D-galactose, 1 M NaCl and 2% bacto-agar) plate cultures. *Yarrowia lipolytica* E150 strain was also used. This strain was maintained in YD (1% yeast extract and 2% D-glucose and 2% bacto-agar) plate cultures.

2.3. Yeast culture and isolation of coupled mitochondria

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^+) containing antifoam A emulsion 50 μL/L. 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 24 h (i.e. medium to late logarithmic phase). *D. hansenii* mitochondria were isolated as reported previously [14]. Mitochondria from *Y. lipolytica* were isolated as in [51].

2.4. Protein quantification

Mitochondrial protein was measured by the Biuret method [60]. Absorbance was determined at 540 nm in a Beckman DU-50 spectrophotometer. Bovine serum albumin was used as a standard.

2.5. Oxygen consumption

The rate of oxygen consumption was measured in a YSI-5300 Oxygraph equipped with a Clark-Type electrode (Yellow Springs Instruments Inc., OH) interfaced to a chart recorder. 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 M sorbitol, 10 mM maleate (pH was adjusted to 6.8 with Tris), 10 mM Tris-phosphate (Pi), 0.5 mM $MgCl_2$ and 75 mM KCl. Mitochondrial protein (Prot) was 0.5 mg/mL; final volume was 1.5 mL. The concentrations of different respiratory substrates and inhibitors are indicated in the legends to the figures.

2.6. Blue native (BN) and 2D SDS-Tricine electrophoresis

BN-PAGE was performed as described in the literature [49]. The mitochondrial pellet was suspended in sample buffer (750 mM aminocaproic acid, 25 mM imidazole (pH 7.0)) and solubilized with 2.0 mg *n*-dodecyl-β-D-maltoside (laurylmaltoside, LM)/mg Prot, or 4.0 mg digitonin (Dig)/mg Prot at 4 °C for 1 h and centrifuged at 33,000 rpm at 4 °C for 25 min. The supernatants were loaded on 4–12% (w/v) polyacrylamide gradient gels. Protein, 0.25 or 0.5 mg per lane was added to 8.5 × 6 cm or 17 × 12 cm gel sizes, respectively. The stacking gel contained 4% (w/v) polyacrylamide. Also, 0.025% digitonin was added to the gel preparation to improve protein band definition [61]. For 2D SDS-Tricine-PAGE, complete lanes from the BN-gels were loaded on 12% polyacrylamide gels to resolve the subunits that constitute each complex. 2D-gels were subjected to Coomassie-staining [61] and silver-staining [62,63]. Apparent molecular weights were estimated using Benchmark Protein (Invitrogen, CA) and Precision Plus Protein™ (BIO-RAD, Richmond, CA) standards.

2.7. In-gel enzymatic activities

In-gel NADH/nitrotetrazolium blue chloride (NTB) oxidoreductase activity was determined incubating native gels in a mixture of 10 mM Tris (pH 7.0), 0.5 mg NTB/mL and 1 mM NADH [64]. Inhibitors such as rotenone and flavone were not able to act on their target enzymes in the gel assays, probably due to dilution into the BN-gel incubation medium, their hydrophobicity or their specific inhibition sites on the protein i.e. the indicator (NTB) seems to receive electrons from flavin prosthetic groups [65], far from the inhibitor blocking sites (near the ubiquinone site) [66,67] (result not shown). In-gel cytochrome *c* oxidase (COX) activity was determined using diaminobenzidine and cytochrome *c* [68]. Cyanide was useful to inhibit COX (Result not shown), but cannot use to unveil the alternative oxidase because there is no method available to measure AOX in-gel activity. In-gel ATPase activity was measured as in [61]. Oligomycin was not able to inhibit this activity (Result not shown) as previously reported in [68].