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Mitochondria from *Dipodascus* (*Endomyces*) *magnusii* and *Yarrowia lipolytica* yeasts did not undergo a Ca^{2+}-dependent permeability transition even under anaerobic conditions

Tat'yana Trendeleva · Evgeniya Sukhanova · Ludmila Ural'skaya · Nils-Erik Saris · Renata Zvyagilskaya

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Abstract In this study we used tightly-coupled mitochondria from *Yarrowia lipolytica* and *Dipodascus* (*Endomyces*) *magnusii* yeasts. The two yeast strains are good alternatives to *Saccharomyces cerevisiae*, being aerobes containing well-structured mitochondria (thus ensuring less structural limitation to observe their appreciable swelling) and fully competent respiratory chain with three invariantly functioning energy conservation points, including Complex I, that can be involved in induction of the canonical Ca^{2+}/P_i-dependent mitochondrial permeability transition (mPTP pore) with an increased open probability when electron flux increases (Fontaine et al. J Biol Chem 273:25734–25740, 1998; Bernardi et al. FEBS J 273:2077–2099, 2006). High-amplitude swelling and collapse of the membrane potential were used as parameters for demonstrating pore opening. Previously (Kovaleva et al. J Bioenerg Biomembr 41:239–249, 2009; Kovaleva et al. Biochemistry (Moscow) 75:297–303, 2010) we have shown that mitochondria from *Y. lipolytica* and *D. magnusii* were very resistant to the Ca^{2+} overload combined with varying concentrations of P_i, palmitic acid, SH-reagents, carboxyatractyloside (an inhibitor of ADP/ATP translocator), as well as depletion of intra-mitochondrial adenine nucleotide pools, deenergization of mitochondria, and shifting to acidic pH values in the presence of high [P_i]. Here we subjected yeast mitochondria to other conditions known to induce an mPTP in animal and plant mitochondria, namely to Ca^{2+} overload under hypoxic conditions (anaerobiosis). We were unable to observe Ca^{2+}-induced high permeability of the inner membrane of *D. magnusii* and *Y. lipolytica* yeast mitochondria under anaerobic conditions, thus suggesting that an mPTP-like pore, if it ever occurs in yeast mitochondria, is not coupled with the Ca^{2+} uptake. The results provide the first demonstration of ATP-dependent energization of yeast mitochondria under conditions of anaerobiosis.

Keywords Yeasts · *Dipodascus* (*Endomyces*) *magnusii* · *Yarrowia lipolytica* · Anaerobiosis · Membrane potential · Swelling · Nonspecific permeability transition · Ca^{2+} · Mitochondria

Abbreviations

ΔΨ	mitochondrial transmembrane potential
Alam	alamethicin
Atr	atractyloside
ANT	adenine nucleotide translocase
BSA	bovine serum albumin
CCCP	carbonyl cyanide 3-chlorophenylhydrazone
CsA	cyclosporin A
DTT	dithiothreitol
EGTA	ethylene glycol-bis(aminoethyl ether) *N*,*N*,*N*′,*N*′-tetraacetic acid
α-GP	α-glycerol phosphate
ETH129	specific Ca^{2+} ionophore

T. Trendeleva · E. Sukhanova · L. Ural'skaya · R. Zvyagilskaya (✉)
A. N. Bach Institute of Biochemistry,
Russian Academy of Sciences,
119071 Moscow, Russia
e-mail: renata_z@inbi.ras.ru

N.-E. Saris
Department of Food and Environmental Sciences,
University of Helsinki,
Viikki Biocenter 1, POB 56, Fi-00014 Helsinki, Finland

mPTP	permeability transition pore
PMSF	phenylmethylsulfonyl fluoride
RC	respiratory control ratio
Tris	tris(hydroxymethyl)aminomethane
EDTA	ethylenediaminetetraacetic acid
BKA	bongkrekic acid
Oligo	oligomycin

Introduction

Apoptosis has been classified as a highly time-regulated form of programmed cell death that results in the removal of infected, mutated, or simply dispensable cells. According to the recommendation of the Nomenclature Committee on Cell Death, apoptosis is distinguished from other forms of cell death (necrosis, autophagy, mitotic catastrophe, and others) by characteristic morphological and biochemical markers. In vertebrates programmed cell death is controlled by a complex regulatory network that can be activated by external signals and internal defects.

Whether yeast cells undergo apoptosis has long been controversial, partly because of doubts of whether cellular suicide could constitute an evolutionary advantage for unicellular organisms. However, since the first description of apoptosis in the yeast *S. cerevisiae* (Madeo et al. 1997), accumulating evidence suggests that yeasts are capable of undergoing programmed cell death (PCD) as a response to many intra- or extracellular factors to benefit long-term survival of the species, and that yeasts and mammals may share at least partially conserved PCD pathways. Recent advances in the elucidation of the molecular pathways underlying apoptotic cell death in yeasts have made a strong case for the relevance of yeast models of regulated cell death. Moreover, it has been recognized that yeasts, relatively simple unicellular organisms, vigorously growing on simple and inexpensive media, having well-characterized genetically tractable genomes, are invaluable systems for functional analysis not only of known pro- and antiapoptotic factors, but also for revealing novel ones (see, for example, Smardova et al. 2005; Eisenberg et al. 2007; Cymerman et al. 2008). Several crucial regulators of apoptosis are conserved between metazoans and yeast. Some of them are normally located in the intermembrane space. For animal mitochondria, two major mechanisms for release of mitochondrial apoptotic factors into the cytosol have been suggested. One involves recruitment, activation, conformational rearrangement, and insertion in the mitochondrial outer membrane of proapoptotic Bax, a member of the Bcl-2 family proteins. Insertion of Bax in the mitochondrial outer membrane is modulated by the mitochondrial complex of the translocase of the outer membrane (called TOM) (Colin et al. 2009) and requires cardiolipin (Lucken-Ardjomande et al. 2008). The other mechanism relies on increased mitochondrial conductance due to opening of some pores in the inner mitochondrial membranes (for details see Kovaleva et al. 2009, 2010), including a classical nonspecific cyclosporin A (CsA)-sensitive pore known as the mitochondrial permeability transition pore (mPTP). Although the regulation of pore gating varies depending on the tissue studied, accumulation of Ca^{2+} in the matrix is an obligatory and most important trigger for its opening (Zoratti and Szabò 1995; Bernardi 1999; Bernardi et al. 2006). Several factors, including the type of substrate used, the prevailing membrane potential, redox state of pyridine nucleotides, reactive oxygen species, phosphate concentrations, pH, and addition of saturated fatty acids can, however, act as coregulators by affecting the sensitivity of the pore to Ca^{2+} (Zoratti and Szabò 1995; Bernardi 1999; Bernardi et al. 2006).

Pore opening allows free passage of protons across the inner membrane leading to dissipation of the proton-motive force, i.e. the mitochondrial transmembrane potential ($\Delta\psi$) and the ΔpH gradient, causing mitochondrial dysfunction, disruption of ionic homeostasis, massive swelling of mitochondria, and release of several mitochondrial proapoptotic proteins including cytochrome *c*, AIF, Smac/Diablo, endonuclease G, and Omi/HtrA2 (Di and Bernardi 1998; Bernardi 1999; Bernardi et al. 1999; Crompton 1999; Suleiman et al. 2001).

In yeasts, some of the abovementioned apoptotic factors are also located in the mitochondrial intermembrane space, like in animal mitochondria. However, the longstanding principal question of how apoptotic factors are released from yeast mitochondria and how these apoptotic factors initiate apoptosis in yeast cells lacking proteins of the Bcl-2 family (the yeast genome does not contain genes encoding these proteins) remains open. We still do not know the role of cristae structure remodeling in release of apoptotic factors from yeast mitochondria. Information on an mPTP-like pore in yeast mitochondria has been until recently scarce, fragmentary, and contradictory (see Kovaleva et al. 2009, 2010).

Previously (Kovaleva et al. 2009, 2010) we have shown that mitochondria from *Y. lipolytica*, lacking a natural mitochondrial Ca^{2+} uptake pathway, and *D. magnusii*, harboring a high-capacity, regulated mitochondrial Ca^{2+} transport system (Bazhenova et al. 1998a; Bazhenova et al. 1998b; Deryabina and Zvyagilskaya 2000; Deryabina et al. 2001) subjected to almost all conditions known to induce permeability transition in animal mitochondria, i.e., elevated [Ca^{2+}] in different combinations with varying concentrations of P_i, palmitic acid, SH-reagents, carboxyatractyloside (an inhibitor of ADP/ATP translocator), as well as depletion of intramitochondrial adenine nucleotide pools, deenergization of mitochondria, and shifting to acidic pH values in the

presence of high [P_i] did not undergo the permeability transition. It was concluded that in yeast mitochondria an mPTP-like pore, if it ever occurs, is differently regulated compared to the mPTP of animal mitochondria and is not coupled with Ca^{2+} uptake. However, at that time we did not examine another condition also known for inducing an mPTP, namely, anaerobiosis (hypoxia).

In wheat (*Triticum aestivum* L.) root mitochondria, the onset of anoxia caused rapid dissipation of the $\Delta\Psi$, initial shrinkage of the mitochondrial matrix, and the release of previously accumulated Ca^{2+}. Anoxia alone failed to induce cytochrome *c* release from the mitochondria. Treatment of mitochondria with high micromolar and millimolar Ca^{2+} concentrations under anoxic conditions induced high amplitude swelling and the release of cytochrome c, which is indicative of mPTP opening. Changes in mitochondrial volume were confirmed by transmission electron microscopy. The mitochondrial swelling was not sensitive to cyclosporin A (CsA), an inhibitor of mammalian PTP (Virolainen et al. 2002).

In animal mitochondria, studies of mPTP activation under low oxygen tension have produced conflicting results, possibly due to the diversity of PTP activators used in these studies (Chávez et al. 1997). In elegant experiments where the $\Delta\Psi$, oxygen consumption, swelling, and calcium transport were recorded simultaneously, it was shown that injection of 100 μM Ca^{2+} into anaerobic rat liver mitochondria supplemented with 500 μM ATP or the ATP-regenerating system to support succinate-driven $\Delta\Psi$ produced the CsA-sensitive permeability transition (Kuzminova et al. 1998). Similar results were obtained by the same authors with anaerobic mitochondria energized by ferricyanide (Krasnikov et al. 1997). Under these conditions the permeability transition was induced even by lower Ca^{2+} concentrations, possibly due to ferricyanide-mediated oxidation of components of the respiratory chain, which is known to facilitate PTP opening (Halestrap 2009). It was concluded that oxygen is not a necessary factor for mitochondria to undergo permeability transitions.

The goal of this paper was to examine the response of yeast (*Y. lipolytica* and *D. magnusii*) mitochondria to concerted action of anaerobiosis and Ca^{2+}. No permeabilization of the mitochondria was observed under these conditions, suggesting that an mPTP-like pore, if it ever occurs in yeast mitochondria, is not coupled with Ca^{2+} uptake.

Materials and methods

Dipodascus magnusii yeast, strain VKM Y261, was cultivated at 28 °C in agitated (220 rpm) 750-ml Erlenmeyer flasks in 100 ml of a semi-synthetic medium (Zvyagilskaya et al. 1981) containing 1% glycerol as the sole source of carbon and energy. The cells were harvested in the late exponential growth phase (A_{590}=2.0–2.2, 10–13 g wet weight/l).

The *Yarrowia lipolytica* strain was obtained by RZ as a pure isolate from epiphytic microflora of salt-excreting leaves of arid plants (Negev Desert, Israel) and identified as an anamorph of *Y. lipolytica* (Wick.) van der Walt and Arx. (Zvyagilskaya et al. 2001). The *Y. lipolytica* cells were routinely grown at 28 °C in agitated (220 rpm) succinate-containing semi-synthetic medium (Andreishcheva et al. 1997) to the late exponential growth phase (OD_{590}=3.4–3.6). Previously (Zvyagilskaya et al. 1988) we demonstrated that during cultivation of yeast cells (*Candida utilis*) on succinate as the sole source of carbon and energy, mitochondria possess invariantly functioning complex I of the respiratory chain because the reverse electron transport in the respiratory chain is the predominant, if not exclusive, way to produce reduced equivalents for anabolic reactions. Additionally, under these conditions activity of the terminal alternative oxidase is reduced to a minimum.

Mitochondria from *D. magnusii* cells were prepared according to the protocol described in (Bazhenova et al. 1998a) with minor modifications. Briefly, cells were harvested by centrifugation, washed twice with ice-cold distilled water, resuspended (0.1 g wet cells/ml) in pre-spheroplast buffer (50 mM Tris–HCl buffer, pH 8.6, 10 mM dithiothreitol), incubated at room temperature for 10–15 min, pelleted at 3000 g for 5 min, washed twice to remove excess dithiothreitol, and incubated at 28 °C under gentle stirring for 15–20 min in spheroplast buffer (1 M sorbitol, 50 mM EDTA, 20 mM citrate-phosphate buffer, pH 6.0) with complex of lytic enzymes from *Helix pomatia* and Zymolyase 20 T from *Arthrobacter luteus* added to a final concentration of 20 and 3.5 mg/g cells, respectively. Spheroplast formation was monitored by measuring the osmotic fragility of a 200-μl sample of cells after dilution 1:10 in distilled water. The spheroplasts were rapidly cooled, pelleted by centrifugation at 3000 g for 10 min, washed gently twice in post-spheroplast buffer (1.2 M sorbitol, 10 mM EDTA, pH adjusted to 6.0), resuspended (0.1 g wet cells/ml) in grinding buffer (10 mM Tris–HCl, pH 7.2, containing 0.4 M mannitol, 0.4 mM EDTA, 0.5 mM EGTA, 4 mg/ml BSA, and protease inhibitor cocktail), and disrupted in an all-glass Dounce homogenizer (Kontes, Vineland, NJ, USA) with a low-clearance pestle. The suspension was diluted with isolation buffer (10 mM Tris–HCl, pH 7.2, 0.6 M mannitol, 0.5 mM EDTA, 0.5 mM EGTA, and 4 mg/ml BSA) and centrifuged at 2000 g for 12 min. The supernatant was centrifuged once more at 7000 g for 20 min. The resulting pellet was washed in 10 mM Tris–HCl, pH 7.2, containing 0.6 M mannitol and 4 mg/ml BSA, resuspended in a smaller volume of the same buffer, and stored on ice until use. Mitochondrial preparations thus obtained were fully active for at least 4 h.

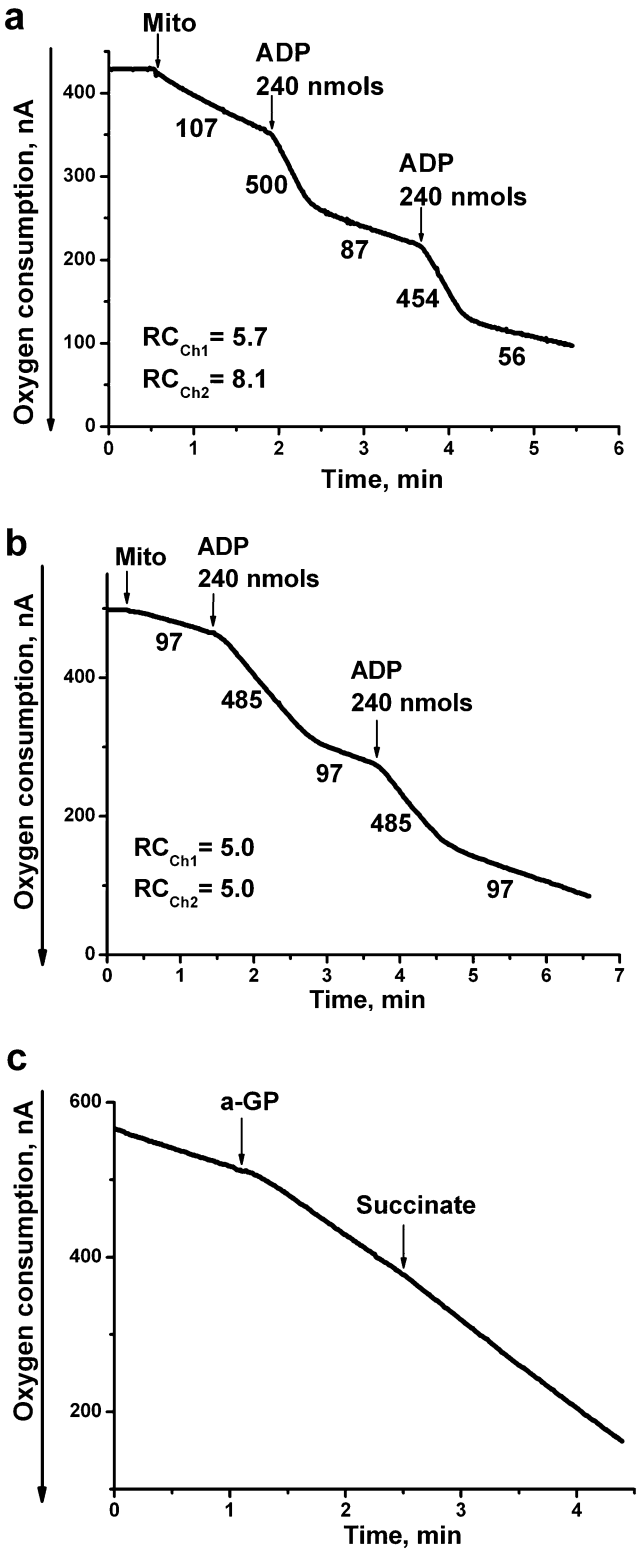

Fig. 1 Amperometric recordings of oxygen consumption by mitochondria isolated from *D. magnusii* **(a)** and *Y. lipolytica* **(b–c)**. The basal incubation medium contained 0.6 M mannitol, 0.2 mM Tris-phosphate, pH 7.2, 20 mM Tris-pyruvate, 5 mM Tris-malate, and mitochondria corresponding to 0.5 mg mitochondrial protein added at "Mito". In **(c)** the basal medium was supplemented with 10 mM Tris-α-glycerophosphate and 10 mM Tris-succinate. Additions and amounts are given in the figures. Numbers adjacent to traces are respiration rates in ng-atoms of O/min/mg mitochondrial protein. Respiratory control ratios (determined as a ratio of state 3 respiration to state 4 respiration) upon successive additions of ADP were **a** 5.7 and 8.1, **b** 5.0 and 5.0

Mitochondria from *Y. lipolytica* cells were prepared as described previously (Kovaleva et al. 2009).

Oxygen consumption by mitochondria was monitored amperometrically at room temperature using a Clark-type oxygen electrode. Unless otherwise specified in figure legends, the incubation medium contained 0.6 M mannitol, 20 mM Tris-pyruvate, 5 mM Tris-malate, 10 mM Tris-α-glycerol phosphate, 10 mM Tris-succinate, 0.2 mM Tris-phosphate, pH 7.2–7.4, and mitochondria corresponding to 0.5 mg protein/ml. Respiratory control and ADP/O ratios were calculated according to Chance et al. (1955).

Respiratory rates were expressed as ng-atoms O/(min per mg mitochondrial protein).

ΔΨ was measured with safranine O as a Ψ-related probe with a Beckman dual beam spectrophotometer using 511 and 533 nm as the measuring and reference wavelength, respectively (Åkerman and Wikström 1976). The basal incubation medium was supplemented with 20 μM safranine O.

Mitochondrial swelling was monitored spectrophotometrically with a Hitachi-557 spectrophotometer (Japan) by recording changes in apparent absorbance at 540 nm.

Mitochondrial protein was determined using the Bradford method (Bradford 1976) with BSA as standard.

All data traces shown are representative of at least 4 to 6 replicates.

Mannitol, sorbitol, pyruvic acid, malic acid, succinic acid, 2-oxoglutaric acid, α-glycerophosphate, ADP, ATP, EDTA, EGTA, fatty-acid-free BSA, atractyloside, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), Tris, dithiothreitol, alamethicin, pyruvate kinase, and CsA were purchased from Sigma-Aldrich (USA); Coomassie G-250 and Zymolyase 20 T (from *Arthrobacter luteus*) were from MP Biomedicals (USA); yeast extract was from Difco (USA); safranine O and ETH129 were from Fluka (Germany). Other reagents of the highest quality available were from domestic suppliers. Complex of lytic enzymes from *Helix pomatia* was a gift from Dr. I. Morgunov.

Results and discussion

All yeast mitochondrial preparations used exhibited a high degree of intactness as inferred from high respiratory rates in state 3 respiration (in the presence of ADP), metabolic

state regulation upon successive additions of ADP, high respiratory control ratios (RC) ranging from 5 to 8 upon oxidation of NAD-dependent substrates, ADP/O ratios close to the theoretically expected maxima (Fig. 1a and b), and from some additional tests on structural integrity of the outer and inner mitochondrial membranes (see Burbaev et al. 1983). Respiring mitochondria kept their Δψ constant for prolonged periods without any disturbance in the intensity of the light scattering signal even in hypotonic mannitol-based incubation medium (Kovaleva et al. 2009, 2010).

High-amplitude swelling and collapse of the ΔΨ were used as parameters for demonstrating induction of the mitochondrial permeability transition due to opening of a pore (mPTP) (Bernardi et al. 2006).

Oxygen depletion was accomplished by allowing mitochondrial respiration supported by a mixture of substrates (Tris-pyruvate, Tris-malate, and Tris-α-glycerophosphate) to consume the residual oxygen. Under these conditions mitochondrial respiration proceeded at its highest rate and was not further accelerated upon the subsequent addition of succinate (Fig. 1c). This accelerated respiration in the absence of stirring should result in rapid oxygen depletion from the incubation medium, thus causing anaerobiosis (hypoxia). In some experiments medium saturated with N_2 or argon was used. As already noted above, the ΔΨ built up in mitochondria of *Y. lipolytica* and *D. magnusii* upon oxidation of NAD-linked substrates (pyruvate+malate) remained constant for at least 10 min. The ΔΨ generated during oxidation of the mixture of the aforementioned substrates (pyruvate, malate, and α-glycerophosphate) by mitochondria of *Y. lipolytica* (as well as by mitochondria of *D. magnusii*) declined already within 200–300 s. This decline was likely caused by the onset of anaerobiosis in the measuring cell (Fig. 2a) because the addition of micromolar concentrations of hydrogen peroxide (as an oxygen source) was followed by complete restoration of the initial ΔΨ (Fig. 2a). When hydrogen peroxide was added to mitochondria at an early stage of incubation, it prevented the occurrence of anaerobiosis (Fig. 2b). Although yeast catalase is mainly located in the cytosol and peroxisomes, discernible catalase activity was also observed in mitochondria, and its level might be sufficient for prevention of anaerobiosis after adding hydrogen peroxide at micromolar concentrations.

Interestingly, the ΔΨ, diminished upon anaerobiosis, was restored not only by hydrogen peroxide but also (almost completely) upon the addition of ATP (Fig. 3a). To exclude the possible influence of comparatively high ATP concentrations on the subsequent induction of a Ca^{2+}-dependent pore, we used an ATP-regenerating system containing low concentration of ATP, phosphoenolpyruvate, and pyruvate kinase. This system added to mitochondria of *Y. lipolytica* was found fully competent to ensure complete restoration of the ΔΨ collapsed during anaerobiosis (Fig. 3b). Similar results were obtained with mitochondria of *D. magnusii*. The addition of the ATP-regenerating system at an early stage of incubation of mitochondria entirely prevented the membrane depolarization induced by anaerobiosis (Fig. 3c). The effect of ATP was specific because the addition of atractyloside and bongkrekic acid (inhibitors of the adenine

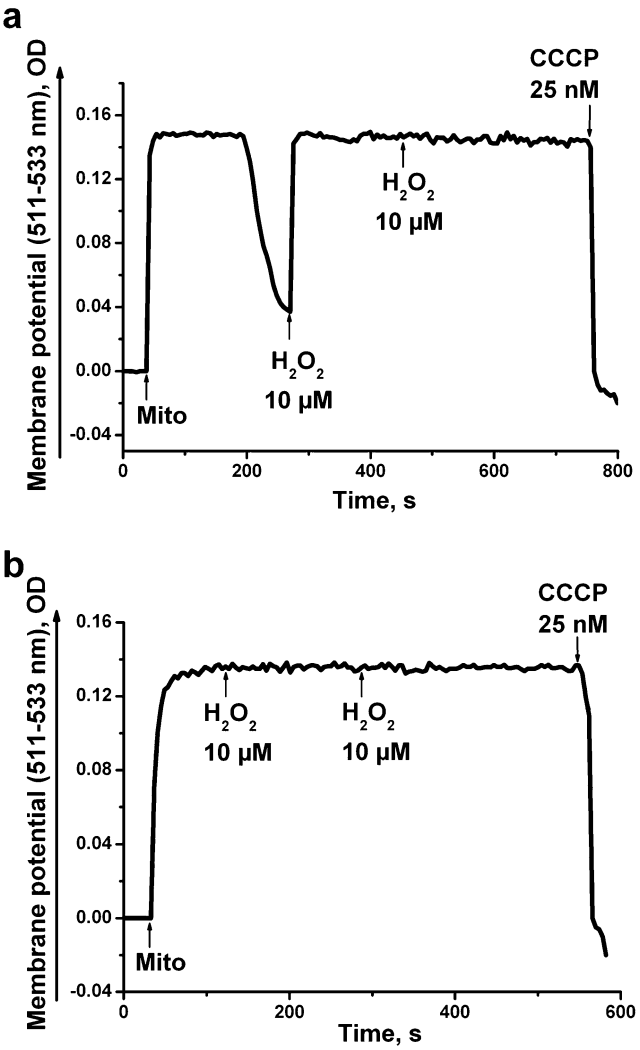

Fig. 2 Recording of Δψ generated by *Y. lipolytica* mitochondria respiring on a mixture of substrates. The incubation medium contained 0.6 M mannitol, 0.2 mM Tris-phosphate, pH 7.2, 20 mM Tris-pyruvate, 5 mM Tris-malate, 10 mM Tris-α-glycerophosphate and mitochondria corresponding to 0.5 mg mitochondrial protein added at "Mito". Where indicated, 25 nM CCCP was added. Additions and amounts are given in the figures. **a** Spontaneous decline of the ΔΨ related to the onset of anaerobiosis (during oxidation of the aforementioned mixed substrates) and the complete restoration of membrane potential after the addition of hydrogen peroxide. **b** Hydrogen peroxide prevented the occurrence of anaerobiosis and the decrease of the ΔΨ

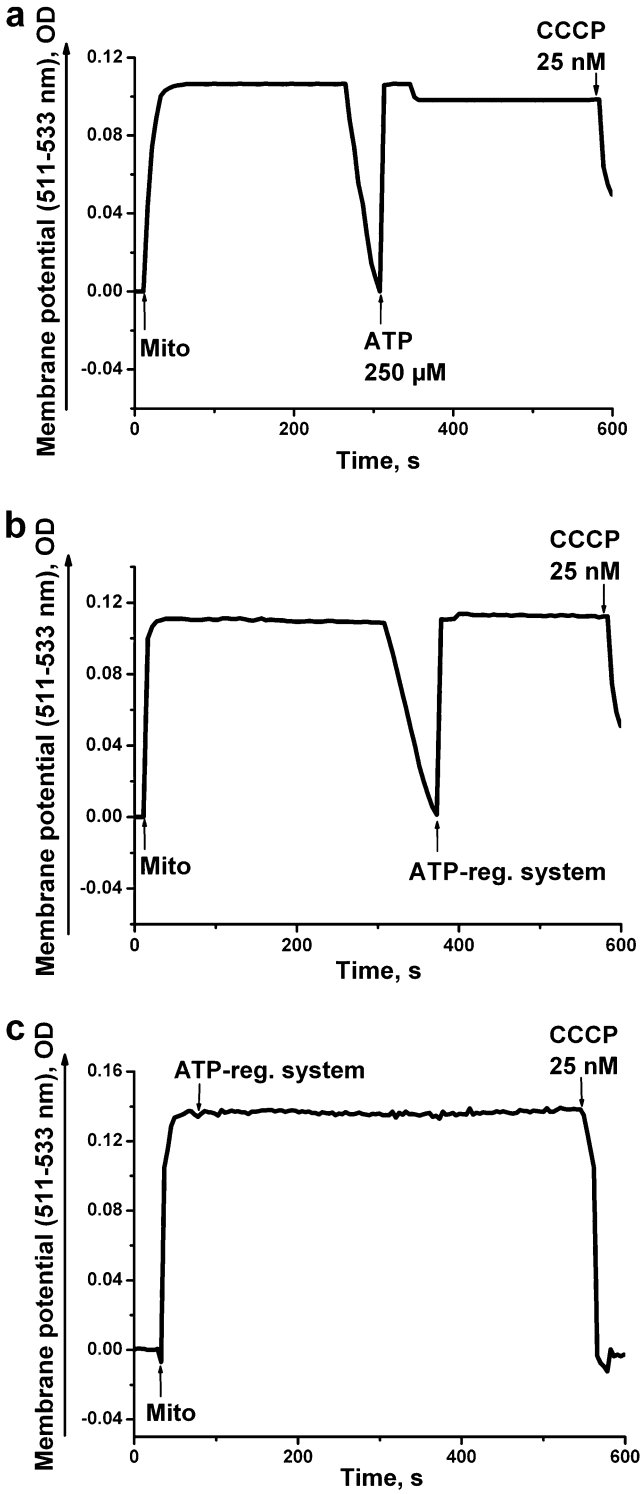

Fig. 3 The ΔΨ, diminished upon the increasing anaerobiosis, recovered almost completely after ATP addition **(a)** and was fully restored by the addition of an ATP-regeneration system containing 1.5 mM phosphoenolpyruvate and 2 μl of pyruvate kinase **(b)**. Addition of the ATP-regenerating system at an early stage of incubation entirely prevented the anaerobiosis-induced depolarization of *Y. lipolytica* mitochondrial membrane **(c)**. The basal incubation medium was as in Fig. 2a. Additions and amounts are given in the figures

nucleotide translocase) (Fig. 4a and b) and oligomycin (an inhibitor of ATP synthesis) (Fig. 4c) entirely eliminated the protective action of ATP. Furthermore, the addition of ADP (in the presence of oligomycin), unlike ATP addition, did not avert the anaerobiosis-induced membrane depolarization (Data not shown).

Thus, we were able to show for the first time that yeast mitochondria can be energized under anaerobiosis by ATP hydrolysis.

When mitochondria of *Y. lipolytica* or *D. magnusii* were incubated at moderate Ca^{2+} concentrations under anaerobic condition in the presence of the ATP-regenerating system, no decline of the ΔΨ was observed during the incubation (Fig. 5a). Substantial membrane depolarization was observed only upon the subsequent addition of the Ca^{2+} ionophore ETH129, which is known to produce a Ca^{2+} channel after its incorporation into the mitochondrial membrane. We showed earlier (Kovaleva et al. 2009, 2010) that this depolarization was due to Ca^{2+}/H^{+} exchange, whose activation depended on endogenous fatty acids. The ΔΨ was restored under the action of the calcium chelator EGTA (Fig. 5a). Unlike animal and plant mitochondria, yeast mitochondria exposed to anaerobiosis in the presence of the substrate mixture did not swell upon the combined application of Ca^{2+} and ETH129, which indicates that the megachannel was not assembled under these conditions (Fig. 5b). Mitochondrial swelling is usually interpreted as reflecting an expansion of the matrix that can culminate in the physical rupture of the outer membrane. The ΔΨ diminished upon the onset of anaerobiosis in mitochondria of *D. magnusii* (similar data were obtained with mitochondria of *Y. lipolytica*) was fully restored by the addition of 8 mM ferricyanide as an alternative electron acceptor (Fig. 6a). The concentration of $Fe(CN)_6^{3-}$ required for restoration and maintenance of the membrane ΔΨ was adjusted empirically. It should be mentioned that rat liver mitochondria subjected to hypoxia (the media was saturated with nitrogen gas and contained succinate and ferricyanide as an oxidation substrate and electron acceptor, respectively) responded to Ca^{2+} addition by opening of the CsA-dependent pore (Krasnikov et al. 1997). Furthermore, the Ca^{2+} concentrations sufficient for opening the pore under anaerobic conditions were much lower than under aerobic conditions. The elevated Ca^{2+}-sensitivity of the system was apparently due to oxidation of respiratory chain components in the presence of ferricyanide. Unlike animal mitochondria, mitochondria of *D. magnusii* incubated at moderate Ca^{2+} concentrations (under anaerobiosis in the presence of $Fe(CN)_6^{3-}$) did not show any decay of the ΔΨ (Fig. 6a). The ΔΨ was collapsed only upon

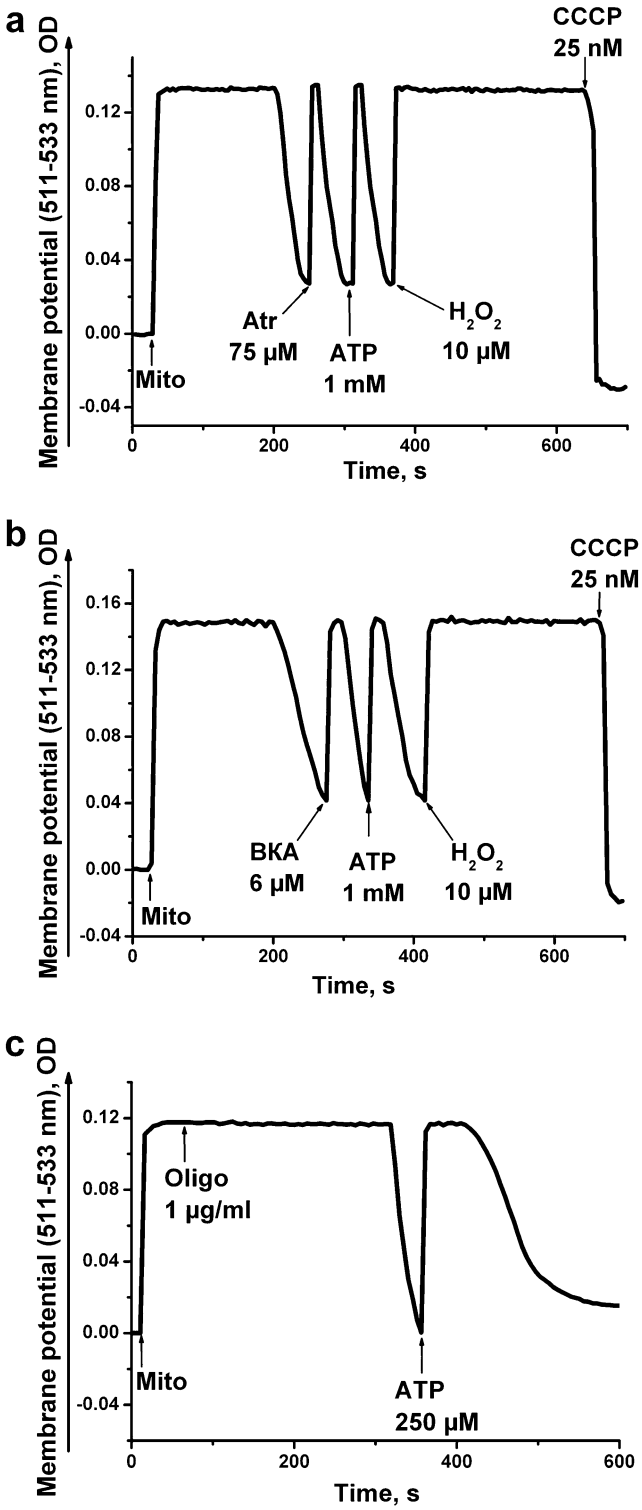

Fig. 4 The recoupling effect of ATP was specific as it was totally abolished by atractyloside **(a)** and bongkrekic acid **(b)** (inhibitors of the adenine nucleotide translocase), as well as by oligomycin **(c)**, an inhibitor of ATP synthesis. The basal incubation medium was as in Fig. 2a. Additions and amounts are given in the figures

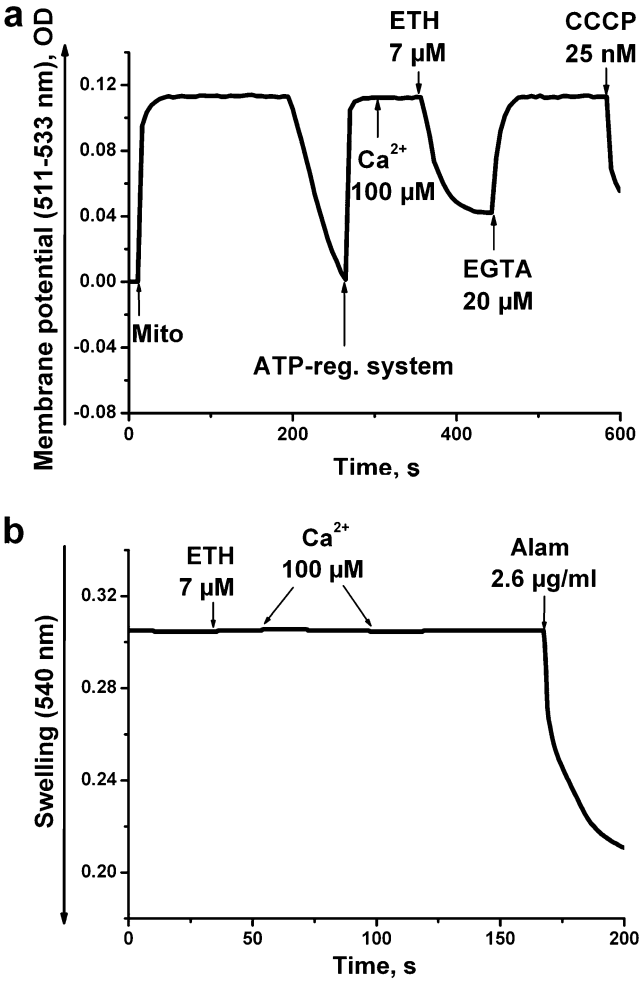

Fig. 5 Effect of a mixture of substrates, the ATP-generating system, ETH129 (ETH), Ca^{2+}, and EGTA on the Δψ generated by *Y. lipolytica* mitochondria. **a** The ATP-regenerating system totally prevented anaerobiosis-induced ΔΨ collapse. Under these conditions moderate Ca^{2+} concentrations did not promote a decrease in Δψ, which was profound upon successive addition of the Ca^{2+} ionophore ETH129. Membrane depolarization was almost totally abolished by EGTA. The basal incubation medium was as in Fig. 2a. Additions and amounts are given in the figures. **b** Anaerobiosis alone or in a combination with moderate Ca^{2+} concentrations and ETH129 did not induce a high-amplitude swelling of *Y. lipolytica* mitochondria even in a slightly hypotonic medium. The incubation medium contained 0.4 M mannitol, 40 mM KCl, 0.2 mM Tris-phosphate, pH 7.2, 20 mM Tris-pyruvate, 5 mM Tris-malate, 10 mM Tris-α-glycerophosphate, and mitochondria corresponding to 0.5 mg mitochondrial protein. Additions and amounts are given in the figures

simultaneous addition of moderate Ca^{2+} concentrations and the Ca^{2+} ionophore ETH129 (Fig. 6a). The ΔΨ was only partially restored by the addition of hydrogen peroxide (Fig. 6a).

Anaerobiosis alone or in combination with moderate Ca^{2+} concentrations supplemented with ETH129 did not promote the high-amplitude swelling of yeast mitochondria (Fig. 6b). The absence of response of yeast mitochondria was not simply due to structural limitations since high-amplitude swelling occurred in the presence of alamethicin, a hydro-

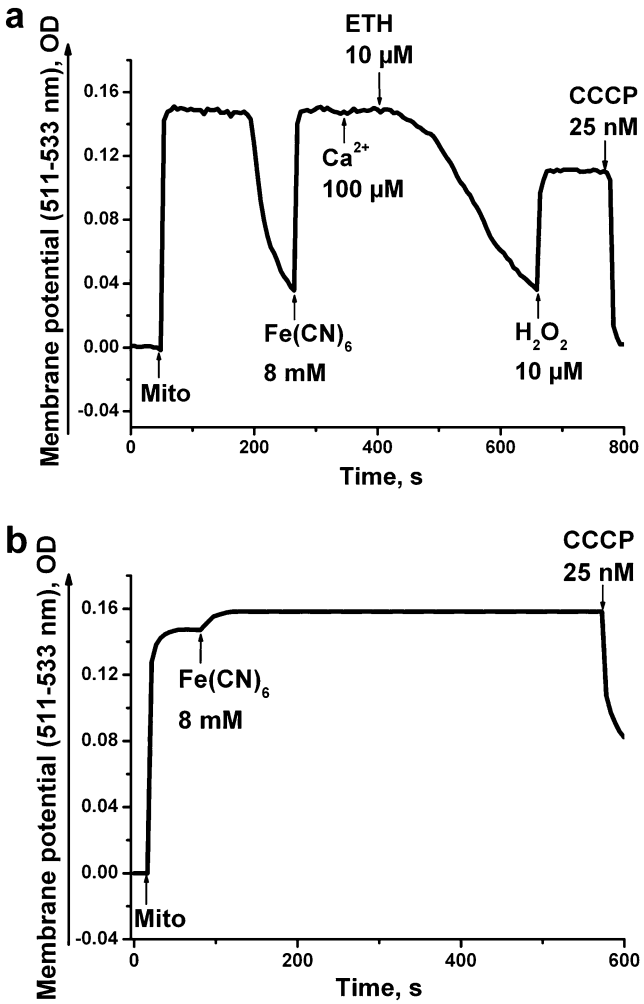

Fig. 6 Effect of a mixture of substrates, $Fe(CN)_6^{3-}$, and Ca^{2+} on $\Delta\psi$ generation and swelling of *D. magnusii* mitochondria. **a** The membrane potential, diminished upon the increasing anaerobiosis, was fully restored by the addition of $Fe(CN)_6^{3-}$ and was not collapsed by the subsequent introduction of moderate Ca^{2+} concentrations. The basal incubation medium was as in Fig. 2a. Additions and amounts are given in the figures. **b** The addition of $Fe(CN)_6^{3-}$ entirely prevented the anaerobiosis-induced depolarization of the *Y. lipolytica* mitochondrial membrane. The basal incubation medium was as in Fig. 2a. Additions and amounts are given in the figures

phobic helical peptide, which being incorporated into mitochondrial membranes forms voltage-sensitive ion channels with a diameter of 1 nm that are permeable to low molecular weight compounds. This observation serves as an important positive control by documenting the capacity of yeast mitochondria to swell and our ability to detect it.

Thus, we were unable to observe Ca^{2+}-induced high permeability of the inner membrane of *D. magnusii* or *Y. lipolytica* yeast mitochondria under anaerobic conditions. These results provide the first demonstration of ATP-dependent energization of yeast mitochondria under conditions of anaerobiosis.

We believe that all yeast mitochondria, regardless of the type of their energy metabolism, lack Ca^{2+}-dependent permeabilization. For the majority of yeast species (including *Y. lipolytica* and *S. cerevisiae*) the lack of the Ca^{2+}-dependent permeabilization (pore) in mitochondria can be easily explained as they do not possess a natural uniporter to take Ca^{2+} up and therefore are unable to attain sufficient Ca^{2+} concentration in the mitochondrial matrix needed for pore induction. However, as we have shown previously (Deryabina et al. 2004; Kovaleva et al. 2009) and here, mitochondria from the yeast *D. magnusii*, harboring a high-capacity finely regulated Ca^{2+} uniporter (Zvyagilskaya et al. 1983; Votyakova et al. 1990; Votyakova et al. 1992; Bazhenova et al. 1989; Bazhenova et al. 1998a, 1998b; Deryabina and Zvyagilskaya 2000; Deryabina et al. 2001), did not exhibit Ca^{2+}-dependent permeabilization. This infers that the ability of mitochondria to transport and accumulate Ca^{2+} is not a precondition for Ca^{2+}-induced permeabilization.

Interestingly, yeast mitochondria are not unique in this respect and are similar to invertebrate mitochondria. Calcium did not induce swelling in mitochondria from ghost shrimp, suggesting that a regulated mPTP did not exist (Holman and Hand 2009). The absence of an mPTP was confirmed by the Ca^{2+} uptake/release data, showing that mitochondria of *L. louisianensis* actively accumulated Ca^{2+} with no release of matrix Ca^{2+} (diagnostic of mPTP opening). The lack of a Ca^{2+}-induced permeability transition and the high Ca^{2+} uptake capacity was also observed in *Artemia franciscana* (Menze et al. 2005) and may be a general feature of invertebrates.

The lack of Ca^{2+}-dependent permeabilization of yeast mitochondria prompted us to look for other feasible means of permeabilizing yeast mitochondria in a Ca^{2+}-independent manner, specifically through opening of the yeast mitochondrial unspecific channel (YMUC) detected in *S. cerevisiae* (Prieto et al. 1992; Guerin et al. 1994; Prieto et al. 1995; Prieto et al. 1996; Manon and Guerin 1997; Roucou et al. 1997; Manon and Guerin 1998; Manon et al. 1998; Castrejon et al. 2002; Perez-Vazquez et al. 2003; Gutierrez-Aguilar et al. 2007). This channel, in contrast to the mitoK_{ATP}-channel of animal mitochondria, opened in response to ATP addition and closed upon ATP depletion (Manon et al. 1998). The YMUC exhibited a similar cutoff size to that of the mammalian mPTP and was active in situ (Manon et al. 1998). We could not reveal the presence of the YMUC in mitochondria from *D. magnusii* and *Y. lipolytica* yeasts. Just the opposite, we succeeded, to our knowledge for the first time, in detecting in these mitochondria an ATP-dependent K^+-channel of "animal type", closed, as in animal mitochondria, by ATP (in detail, experimental data will be published elsewhere). Exactly the opposite effect of ATP on mitochondria in *S. cerevisiae*, on

one hand, and *Y. lipolytica* and *D. magnusii*, on the other hand, presumably reflects the principle difference in mechanisms underlying energy supply in these yeast species. *Saccharomyces cerevisiae* is a facultative anaerobe, whereas *Y. lipolytica* and *D. magnusii* are aerobes (*Y. lipolytica* is an obligate aerobe), with mitochondria being vitally important for supporting their life. In mitochondria of facultative anaerobes, a decrease in the intracellular ATP level would assist in closing of the ATP-dependent channel, thus ensuring transition to the more effective mitochondrial type of energy transduction. Conversely, under "normal" conditions the ATP-dependent K^+-channel of mitochondria from *Y. lipolytica* and *D. magnusii* (and quite possibly from other yeasts of aerobic type), inhibited by micromolar ATP, should be closed. However, it seems very likely that a considerable decrease in the intracellular level of ATP (as a result of unfavorable stress conditions) can serve as a signal for ATP-dependent K^+-channel opening and, ultimately, for triggering a reaction cascade causing yeast cell apoptosis.

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