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Effect of prooxidants on yeast mitochondria

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Abstract Tightly coupled mitochondria from *Yarrowia lipolytica* and *Dipodascus* (*Endomyces*) *magnusii* yeasts were used in this study. The two yeasts are aerobes containing the fully competent respiratory chain with three energy conservation sites. Interaction of the yeast mitochondria with prooxidants (diamide, menadione, oxaloacetate, phenylarsine oxide, hydrogen peroxide, *t*-butyl peroxide, and ascorbate plus Fe^{2+}) was studied. The prooxidants, depending on their chemical nature, either caused uncoupling (e.g., activated state 4 respiration) or inhibited oxidation of respiratory substrates. All of the agents dissipated the membrane potential without megachannel formation (no large-scale swelling of mitochondria was observed). Except for combined application of ascorbate and Fe^{2+}, the prooxidant-induced decrease in the membrane potential was specifically prevented by ATP, even in the cases when classic antioxidants, e.g., N-acetylcysteine, were ineffective. No permeabilization of yeast mitochondria was observed under concerted action of prooxidants and Ca^{2+}, suggesting that an mPTP-like pore, if it ever occurs in yeast mitochondria, is not coupled with Ca^{2+} uptake.

Keywords Yeasts · *Dipodascus* (*Endomyces*) *magnusii* · *Yarrowia lipolytica* · Prooxidants · Membrane potential · Swelling · Permeability transition

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Abbreviations

Δψ	mitochondrial transmembrane potential
Alam	alamethicin
Atr	atractyloside
ANT	adenine nucleotide translocase
BKA	bongkrekic acid
BSA	bovine serum albumin
CCCP	carbonyl cyanide 3-chlorophenylhydrazone
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol-bis(aminoethyl ether) *N*,*N*,*N*′,*N*′-tetraacetic acid
ETH129	specific Ca^{2+} ionophore
mPTP	permeability transition pore
N-Ac	N-acetylcysteine
OA	oxaloacetate
Oligo	oligomycin
PhAsO	phenylarsine oxide
RC	respiratory control ratio
Tris	tris(hydroxymethyl)aminomethane

Introduction

In recent years the role of mitochondria in both apoptotic and necrotic cell death has received considerable attention. An increase of mitochondrial membrane permeability is one of the key events in apoptotic or necrotic death, although the details of the mechanism involved remain to be elucidated.

Animal mitochondria are known to be induced by a variety of agents and conditions to undergo a permeability transition (mPTP, pore), which nonselectively increases the permeability of the inner membrane to small (<1500 Da)

solutes. The pore opening threshold was profoundly affected under conditions of oxidative stress, with a shift to more negative values that may cause mPTP opening at physiological membrane potentials (Costantini et al. 1996). Some investigators suggested instead that prooxidants open a Ca^{2+}-selective channel in the inner mitochondrial membrane and that the opening of this channel, when coupled with Ca^{2+}-cycling mediated by the Ca^{2+} uniporter, leads ultimately to the observed increase in mitochondrial permeability (see, e.g., Schlegel et al. 1992). Novgorodov and Gudz (1996) proposed that the inner membrane contains a pore that, upon exposure to prooxidants, can be opened in two states, one of which conducts only H^+ and the other being the classic nonspecific mPTP inhibited by cyclosporin A (CsA). The correctness of this idea about the presence in animal mitochondria of two prooxidant-induced states of the same pore or two channels with clearly distinct properties was confirmed by Kushnareva and Sokolove (2000). Both groups agreed that opening of the low-conductance channel is insensitive to inhibitors of the classic mPTP and requires sulfhydryl group oxidation and the presence of a divalent cation (Ca^{2+} or Sr^{2+}). However, according to Kushnareva and Sokolove (2000), the low-conductance channel permits the passage of cations, including Ca^{2+}.

Results compatible with the opening of two conductance states of the mPTP were obtained by Zago et al. (2000). Acetoacetate, an oxidant of NADH, stimulated ruthenium red-insensitive Ca^{2+} efflux from rat liver mitochondria without significant release of state 4 respiration, disruption of the membrane potential, or mitochondrial swelling, which was indicative of the low conductance state of the mPTP. In contrast, diamide, a thiol oxidant, induced a fast mitochondrial Ca^{2+} efflux associated with a release of state 4 respiration, a collapse of the membrane potential, and a high-amplitude mitochondrial swelling. The addition of carbonyl cyanide p-(trifluoromethoxy)phenylhydrazone (CCCP) to samples in the acetoacetate experiments promoted a fast shift from the low to the high conductance state of the mPTP. It was proposed that the shift could be promoted by at least partial oxidation of NADPH. This would impair the antioxidant function of the NADPH-dependent glutathione reductase/peroxidase system, strongly strengthening the state of mitochondrial oxidative stress.

Bernardi's group (Chernyak and Bernardi 1996; Costantini et al. 1996) proposed an integrative mechanism encompassing the interaction of many oxidative agents affecting the mPTP, and they resolved conflicting reports on the relative role of pyridine nucleotides and glutathione oxidation in the permeability transition within the framework of mPTP (dis) regulation at two separate sites. Pyridine nucleotide oxidation was correlated with increased mPTP open probability under conditions where the glutathione pool was kept in the fully reduced state; this process was blocked by N-ethylmaleimide but not by monobromobimane, a thiol-selective reagent. Just the opposite, cross-linking of mitochondrial dithiols with arsenite or phenylarsine oxide, or treatment with *t*-butylhydroperoxide leading to complete oxidation of glutathione, increased the sensitivity of mPTP opening to Ca^{2+} under conditions where the pool of pyridine nucleotides was kept in the fully reduced state. This effect could be fully prevented by pretreatment of mitochondria with monobromobimane and restored by reduction with dithiothreitol.

Yeast and animal cells contain similar or even identical systems for induction and prevention of oxidative stress. The functioning of isolated complex I from the respiratory chain of *Y. lipolytica* was associated with the production of superoxide radical (Galkin and Brandt 2005). Highly active antioxidant systems were found in cells of *S. cerevisiae*: thioredoxin peroxidase, two isoforms of catalase, thioredoxin reductase, thioredoxin, Cu^{2+}/Zn^{2+}- and Mn^{2+}-dependent superoxide dismutases (localized in the cytosol and mitochondria, respectively), cytochrome *c* peroxidase (in the intermembrane space), glutathione and the enzymes of its synthesis, and metallothionein (Coleman et al. 1999; Pedrajas et al. 1999; 2000; 2002; Gasch et al. 2000; Kowaltowski et al. 2000; Miranda-Vizuete et al. 2000; Garrido and Grant 2002; Monteiro et al. 2004; Jiménez et al. 2007; Lushchak 2006, 2007, 2010, 2011; Lushchak and Lushchak 2009; Lushchak et al. 2009). The genetic tractability of *Y. lipolytica* (Kerscher et al. 2002) allows us to use bioinformatic approaches to reveal components analogous to those involved in the antioxidant defense in *S. cerevisiae* (unpublished data).

Oxidative stress in yeasts is accompanied by the following metabolic changes. (i) Increase in the stationary content of ROS (Davidson et al. 1996; Agarwal et al. 2005). (ii) Changes in the glutathione status (Lee et al. 2001; Pócsi et al. 2004), the decline in the total glutathione content often being accompanied by the increased content of its oxidized form and the respective increase in the [GSSG]/[GSH ratio] (Lushchak and Lushchak 2008, 2009). (iii) Changes in the activity of oxidation-sensitive enzymes. Among all examined yeast enzymes, aconitase seems to be the most sensitive to activated oxygen and nitrogen species (Lushchak and Lushchak 2008). Because of such sensitivity this enzyme is used most often as a marker of oxidative stress (Lushchak and Lushchak 2008, 2009); (iv) Protein carbonylation. Analysis of free-radical protein oxidation in yeasts conducted in several studies (see Lushchak 2010) revealed characteristic protein modifications. (v) Changes in plasma membrane permeability to oxidants. According to Branco et al. (2004) and Sousa-Lopes et al. (2004), hydrogen peroxide does not readily permeate through the membrane of *S. cerevisiae,* but this permeation can be

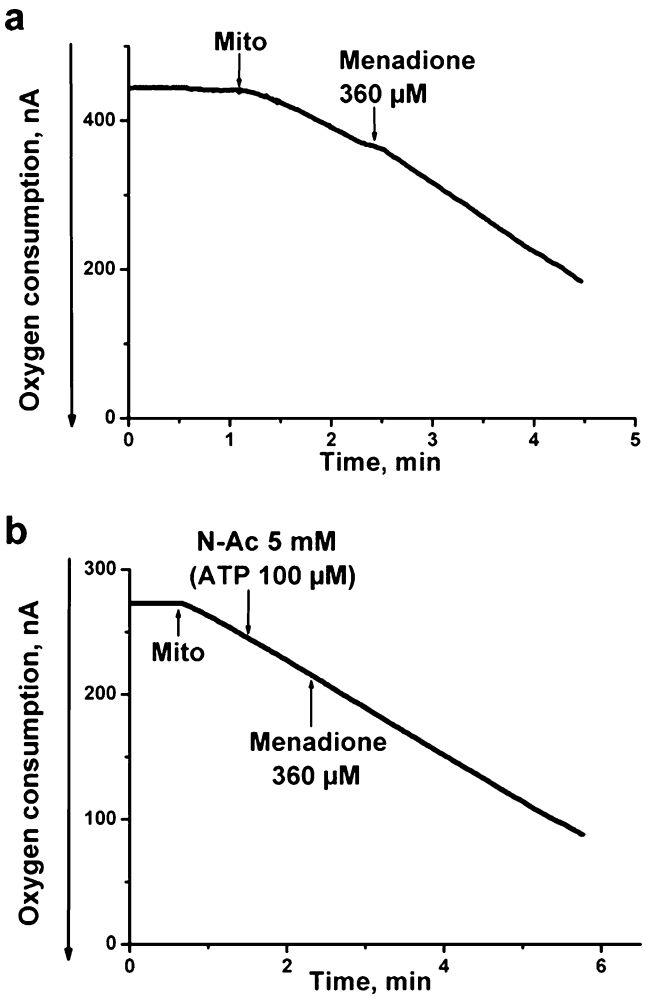


Fig. 1 Effects of menadione, N-acetylcysteine (N-Ac), and ATP on state 4 respiration by *D. magnusii* mitochondria. **a** Menadione stimulated state 4 respiration; **b** 5 mM N-acetylcysteine or 100 μM ATP completely prevented the uncoupling effect of menadione (therefore ATP is placed in the figure in parentheses). The incubation medium contained 0.6 M mannitol, 20 mM Tris–pyruvate, 5 mM Tris–malate, 2 mM Tris–phosphate buffer, 1 mM EDTA (pH 7.2–7.4), and mitochondrial protein (0.5 mg/ml)

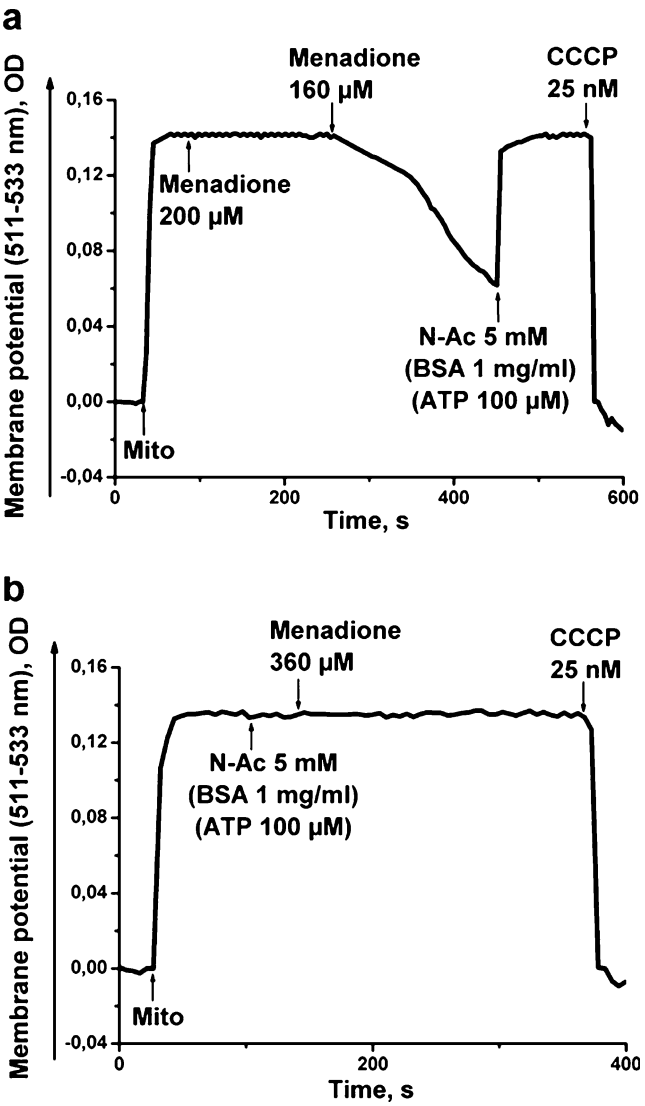


Fig. 2 Effect of menadione, ATP, and N-acetylcysteine (N-Ac) on the membrane potential generated by mitochondria of *D. magnusii* respiring on succinate. The incubation medium contained 0.6 M mannitol, 0.2 mM Tris–phosphate buffer (pH 7.2–7.4), 20 mM Tris–succinate, and mitochondrial protein (0.5 mg/ml). CCCP (25 nM) was added as indicated. Additions and amounts are given in the figure. **a** The membrane potential was dissipated by 360 μM menadione and was entirely restored by the addition of 5 mM N-acetylcysteine, BSA (1 mg/ml) or 100 μM ATP. **b** The addition of 5 mM N-acetylcysteine, BSA (1 mg/ml) or 100 μM ATP completely prevented the menadione-induced decrease in the membrane potential

regulated in response to other exogenous oxidants. The authors supposed that regulation of the ergosterol biosynthesis rate might be a mechanism that adjusts the membrane permeability of the yeast cells to hydrogen peroxide. (vi) Damage to nucleic acids. Detection of DNA damage in yeast is impeded by the presence of a powerful DNA repair system that prevents mutations. However, DNA damage can be detected in strains defective in repair systems. Doetsch et al. (see e.g., Rowe et al. 2008; Griffiths et al. 2009) described in detail the molecular bases of yeast defense against deleterious impact of oxidants, hydrogen peroxide in particular. The defense mechanisms include base excision repair, nucleotide excision repair, recombination, and translesion synthesis. (vii) Activation of transcription factors. Transcription factor Yap1p of *S. cerevisiae* plays a key role in the cell response to oxidative stress. It activates transcription by virtue of binding to specific DNA sequences that are localized in the promoter regions of regulated genes. A number of such genes are known to be involved in the oxidative stress response, namely *TRX2* (thioredoxin), *GSH1* (γ-glutamylcysteine synthase), *GSH2* (glutathione synthase), *TRR1* (thioredoxin reductase),

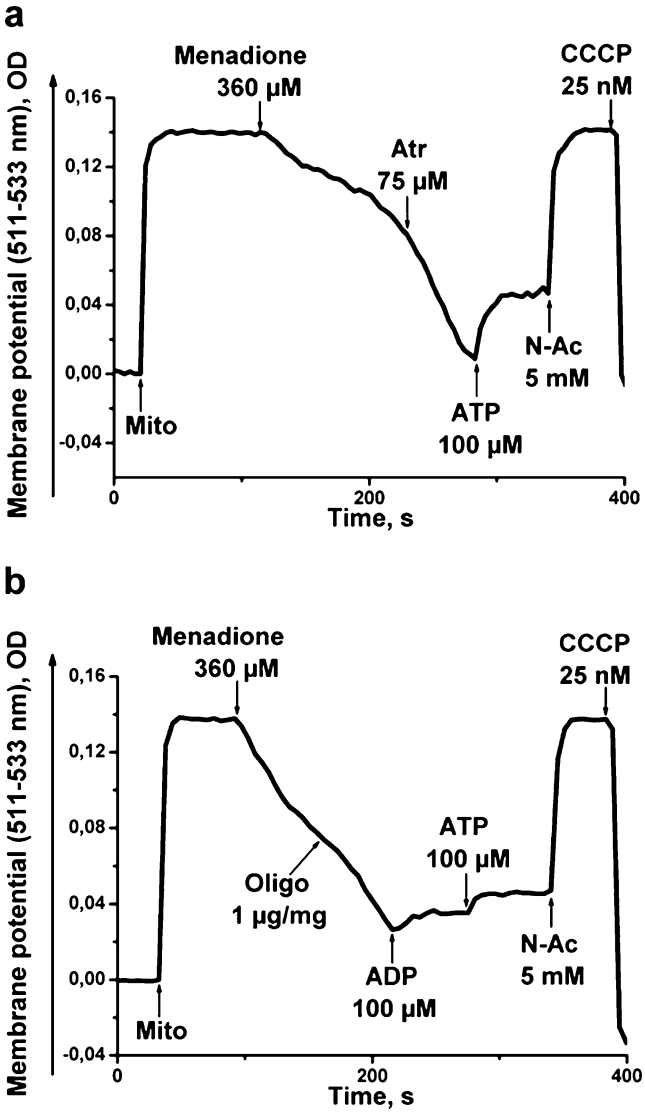


Fig. 3 Effects of menadione, ATP, Atractyloside (Atr), and oligomycin (Oligo) on the membrane potential generated by mitochondria of *D. magnusii* respiring on succinate. Experimental conditions were as in Fig. 2. Additions and amounts are given in the figure. Atractyloside **(a)** and oligomycin **(b)** almost completely abolished the ATP-induced membrane repolarization after its depolarization produced by menadione

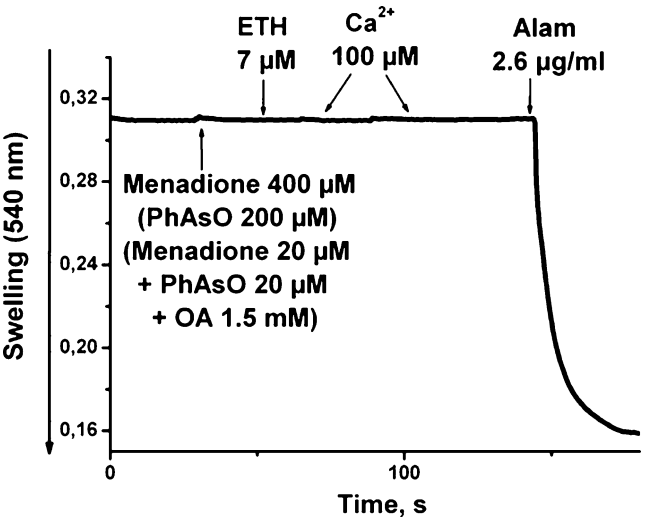


Fig. 4 Menadione, PhAsO, and the combination of menadione, PhAsO, and oxaloacetate (OA) did not cause swelling of *D. magnusii* mitochondria suspended in a slightly hypotonic medium supplemented with KCl even in the presence of the Ca^{2+} ionophore ETH129 (ETH) and moderate Ca^{2+} concentrations. The incubation medium contained 0.4 M mannitol, 40 mM KCl, 20 mM Tris-succinate, 0.2 mM Tris-phosphate buffer (pH 7.2–7.4), and mitochondrial protein (0.5 mg/ml). Alamethicin (2.6 μg/ml) was added as indicated (Alam). Additions and amounts are given in the figure

GPX2 (glutathione peroxidase), *TSA1* (thioredoxin peroxidase), and *AHP1* (alkyl hydroperoxide reductase). In the Yap family of yeast cells at least eight proteins have been identified and named Yap1p-Yap8p. These proteins have slightly different but overlapping DNA-binding specificities; they participate in regulation of cell responses to oxidative stress and the action of cytotoxic agents, including pharmaceutical preparations and heavy metal ions, but the functions of these proteins may differ appreciably (Lushchak 2010).

The impact of prooxidants on yeast mitochondria remains poorly investigated. It is only known that mitochondria of *Dipodascus* (*Endomyces*) *magnusii* yeast were resistant to the action of prooxidants applied at concentrations sufficient to induce the permeability transition pore in animal mitochondria (Deryabina et al. 2004). However, it is possible that prooxidants added to yeast mitochondria at rather low concentrations induced no oxidative stress because of the high natural activity of antioxidant systems in yeast mitochondria compared to those in animal mitochondria.

The goal of the present study was to examine interactions of prooxidants – menadione, oxaloacetate, and phenylarsine oxide (applied separately and in combinations), as well as diamide, hydrogen peroxide, *t*-butyl peroxide, and Fe^{2+} plus ascorbate – in mitochondria of yeasts with aerobic type of energy conservation (*D. magnusii* and *Y. lipolytica*). An additional goal was to determine whether nonspecific permeability can be induced in yeast mitochondria upon the combined action of prooxidants and Ca^{2+}. The results show that, depending on the chemical nature of prooxidants, they either produced uncoupling action or inhibited respiration. The membrane potential was collapsed by all prooxidants without mega-channel formation, and in most cases it was specifically restored by ATP.

No permeabilization of yeast mitochondria was observed under concerted action of prooxidants and Ca^{2+}, suggesting that an mPTP-like pore, if it ever occurs in yeast mitochondria, is not coupled with Ca^{2+} uptake.

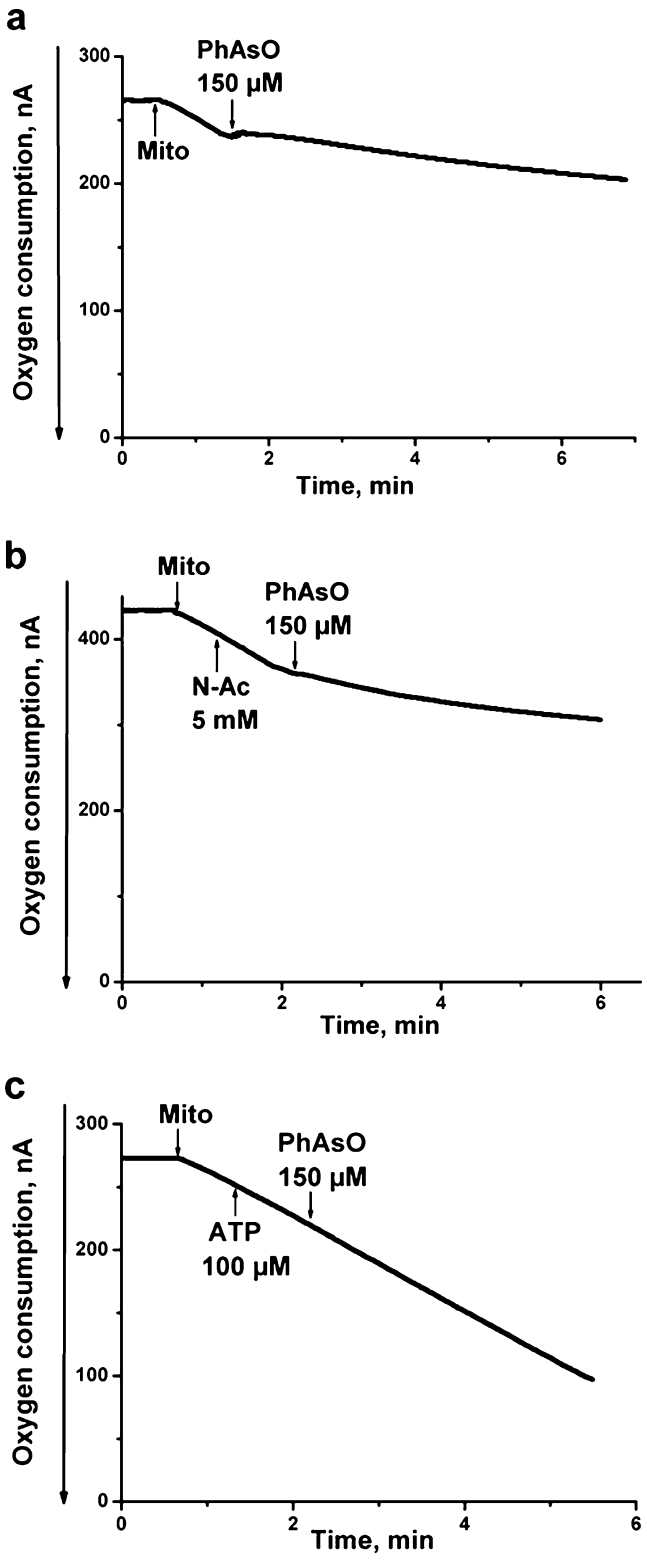


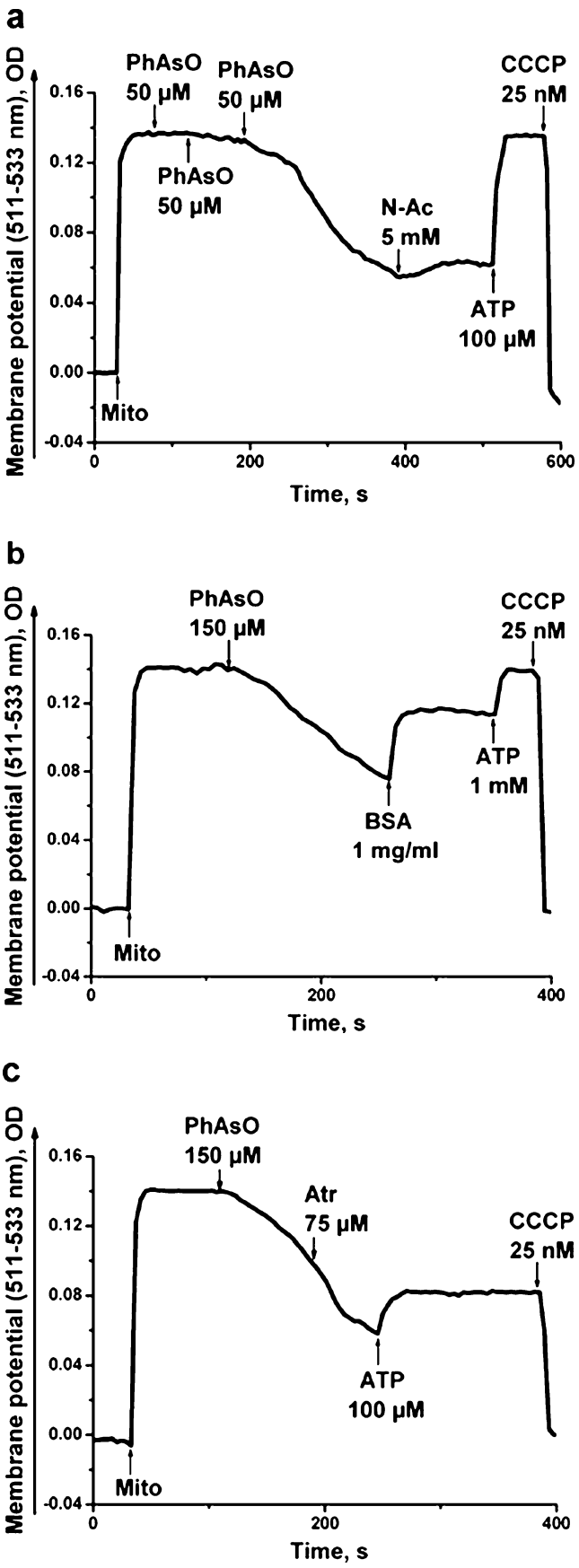
Fig. 5 Effects of phenylarsine oxide (PhAsO), N-acetylcysteine (N-Ac), and ATP on state 4 respiration of *D. magnusii* mitochondria respiring on pyruvate+malate. Experimental conditions were as in Fig. 1. Additions and amounts are given in the figure. **a** 150 μM PhAsO inhibited mitochondrial respiration in state 4; **b** 5 mM N-acetylcysteine only partially abolished the PhAsO-induced inhibition of respiration; **c** 100 μM ATP entirely prevented the inhibitory effect of PhAsO on respiration

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. Cells were harvested in the late exponential growth phase (OD_{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) it has been demonstrated that during cultivation of yeast cells (*Candida utilis*) on succinate as the sole source of carbon and energy, the 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 the 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. 1998) 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 a 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



◀ **Fig. 6** Effects of phenylarsine oxide (PhAsO), N-acetylcysteine (N-Ac), ATP, BSA, and atractyloside (Atr) and on the membrane potential generated by *D. magnusii* mitochondria respiring on succinate. Experimental conditions were as in Fig. 2. Additions and amounts are given in the figure. **a** 150 μM PhAsO produced a decrease in the membrane potential that was insensitive to N-Ac but abolished by ATP. **b** BSA only partially restored the membrane potential diminished by PhAsO. **c** Atractyloside prevented the recoupling effect of ATP

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.

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, 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 are expressed as ng-atoms O/(min per mg mitochondrial protein).

The membrane potential was measured with safranine O as a $\Delta\Psi$-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.

Intramitochondrial levels of reduced glutathione (GSH) were determined using the Glutathione Assay Kit as described by suppliers.

All data traces shown are representative of at least four to six replicates.

Mannitol, sorbitol, pyruvic acid, malic acid, succinic acid, ADP, ATP, EDTA, EGTA, fatty-acid-free BSA, atractyloside, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), Tris, dithiothreitol, alamethicin, phenylarsine oxide, menadione, oxaloacetate, oligomycin, N-acetylcysteine, and Glutathione Assay Kit 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

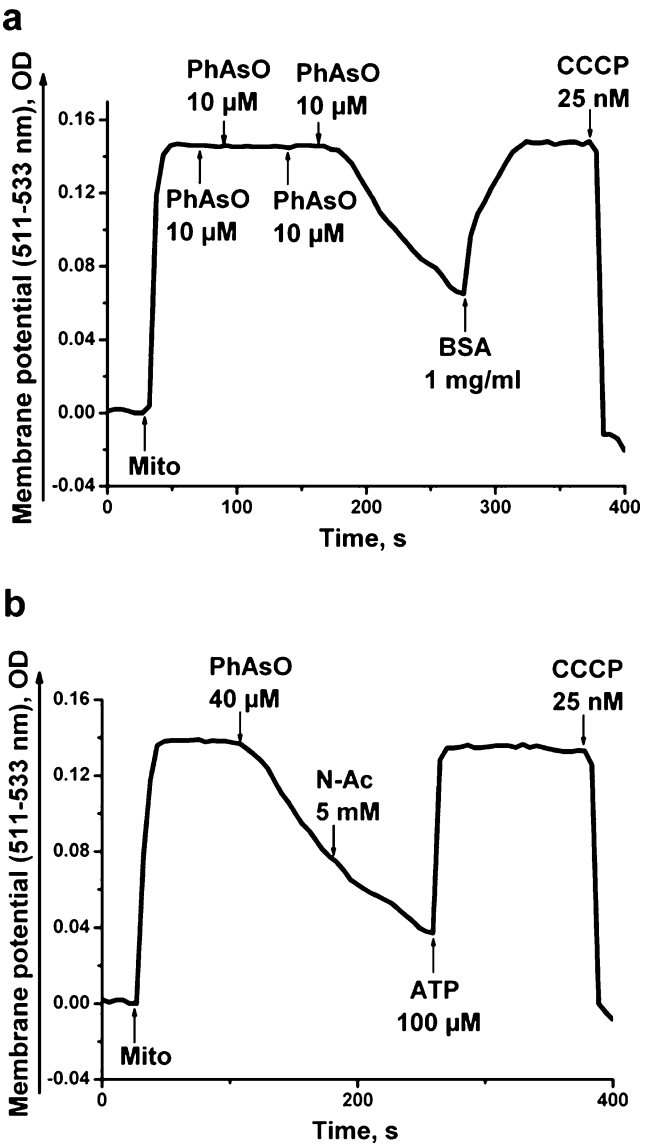


Fig. 7 Effects of phenylarsine oxide (PhAsO), N-acetylcysteine (N-Ac), BSA, and ATP on the membrane potential generated by *D. magnusii* mitochondria isolated in BSA-free medium. Experimental conditions were as in Fig. 2. Additions and amounts are given in the figure. 40 μM PhAsO decreased the membrane potential, which was fully abolished by BSA **(a)** or ATP **(b)**

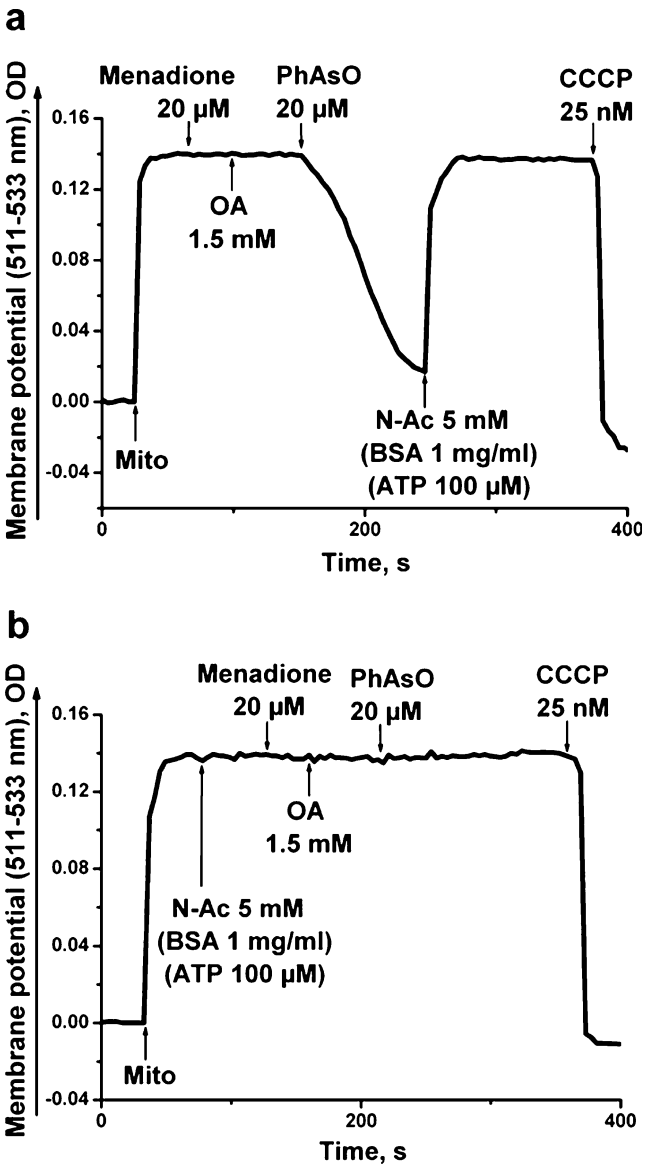
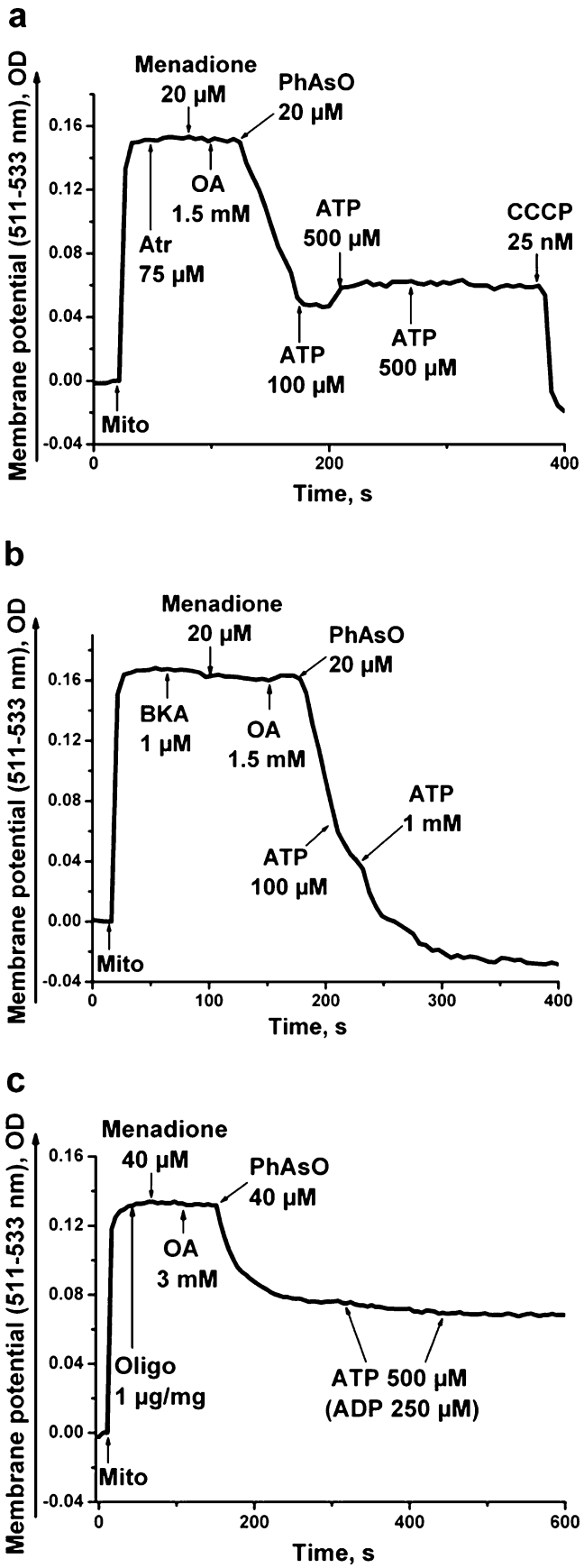


Fig. 8 Effects of combined action of menadione, oxaloacetate (OA), and phenylarsine oxide (PhAsO), as well as N-acetylcysteine (N-Ac), BSA, and ATP on the membrane potential generated by *D. magnusii* mitochondria respiring on succinate. Experimental conditions were as in Fig. 2. Additions and amounts are given in the figure. Concerted action of relatively low concentrations of menadione, oxaloacetate, and phenylarsine oxide caused a collapse of the membrane potential entirely abolished **(a)** and prevented **(b)** by N-acetylcysteine (N-Ac), BSA, and ATP

Fluka (Germany). α-Glycerophosphate, KCl and $CaCl_2$ were from Merck (Germany). Other reagents of the highest quality available were from domestic suppliers. The 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, and from some additional tests on structural integrity of the outer and inner mitochondrial membranes. Respiring mitochondria kept their Δψ constant for prolonged periods without any change in the intensity of the light scattering signal even in hypotonic mannitol-based incubation medium (Kovaleva



◀ **Fig. 9** Effects of combined action of menadione, oxaloacetate (OA) and phenylarsine oxide (PhAsO), as well as ATP, ADP, atractyloside (Atr), bongkrekic acid (BKA), and oligomycin (Oligo) on the membrane potential generated by *D. magnusii* mitochondria respiring on succinate. Experimental conditions were as in Fig. 2. Additions and amounts are given in the figure. The ATP-dependent repolarization of mitochondria depolarized under combined action of menadione, OA, and PhAsO was almost fully prevented by atractyloside **(a)**, bongkrekic acid **(b),** or oligomycin **(c)**

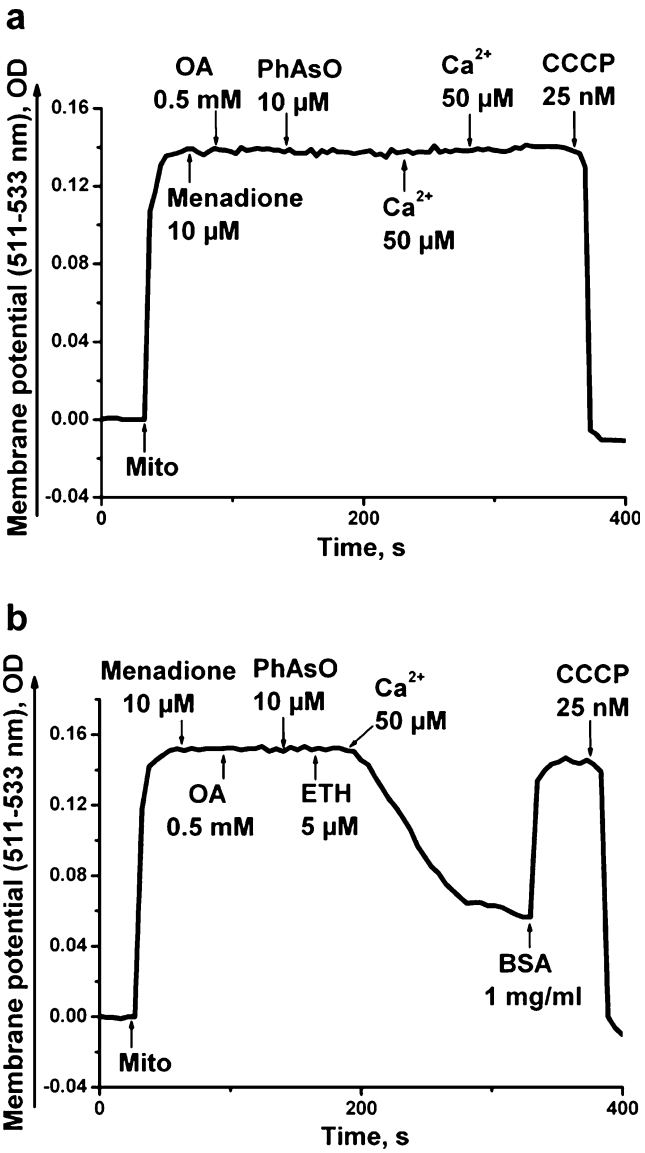
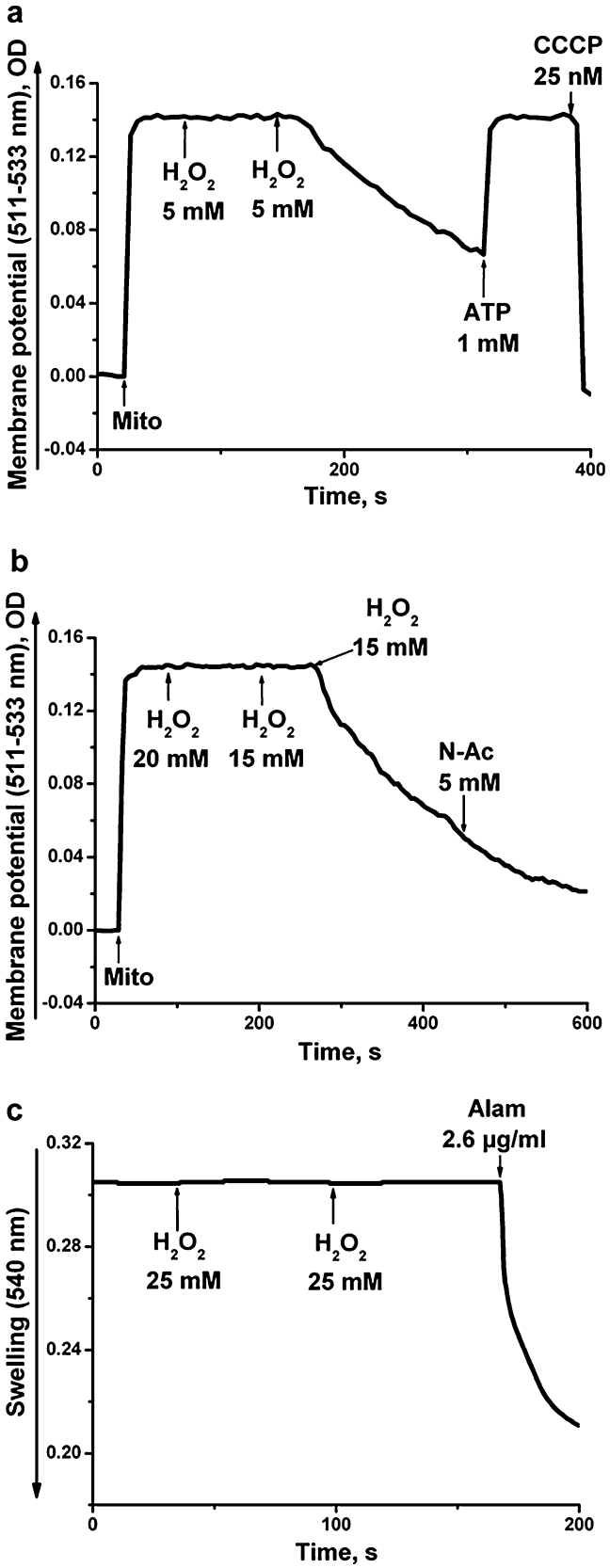


Fig. 10 Effects of menadione, oxaloacetate (OA), phenylarsine oxide (PhAsO), Ca^{2+}, ETH129 (ETH), EGTA, and BSA on generation of membrane potential in *D. magnusii* mitochondria isolated in BSA-free medium. Experimental conditions were as in Fig. 2. Additions and amounts are given in the figure. **a** The addition of 100 μM Ca^{2+} in the presence of concentrations of menadione, OA, or PhAsO that did not uncouple did not cause dissipation of the membrane potential; **b** The addition of 5 μM ETH129 and 50 μM Ca^{2+} in the presence of concentrations of menadione, OA, or PhAsO that did not uncouple induced a decrease in the membrane potential that was almost fully reversed upon the addition of BSA



◀ **Fig. 11** Effects of hydrogen peroxide (H_2O_2), ATP, and N-acetylcysteine (N-Ac) on the membrane potential and swelling of *D. magnusii* mitochondria. Additions and amounts are given in the figure. **a** Experimental conditions were as in Fig. 2. Hydrogen peroxide (10 mM) depolarized the membrane of *D. magnusii* mitochondria isolated in BSA-free medium; this depolarization and was fully prevented by the addition of ATP. **b** Experimental conditions were as in Fig. 4. Only relatively high concentrations of hydrogen peroxide produced a decrease in the membrane potential generated by *D. magnusii* mitochondria isolated in BSA-containing medium. **c** Experimental conditions were as in Fig. 4. Hydrogen peroxide did not induce swelling of *D. magnusii* mitochondria in a slightly hypotonic medium supplemented with 40 mM KCl

et al. 2009, 2010). Besides, the respiring mitochondria contained a high level of reduced glutathione (up to 40 nmol/mg protein in state 3 respiration), which implies that yeast cells used for mitochondria isolation maintained "normal" intracellular redox balance.

The opening of a nonspecific pore was judged from the concurrent occurrence of two events: decrease in $\Delta\Psi$ and high-amplitude swelling of mitochondria caused by unrestricted entry of sugars and osmotically active solutes with molecular mass of up to 1500 Da into the matrix, followed by the consequent uptake of water.

In the first step of our study, we investigated the influence of prooxidants on energetic parameters of the mitochondria.

Menadione added to mitochondria of *D. magnusii* induced moderate uncoupling (activated state 4 respiration) only at a concentration as high as 360 µM (Fig. 1a). The uncoupling action of menadione was fully prevented by the water-soluble antioxidant N-acetylcysteine and by ATP (Fig. 1b).

Menadione applied at the same concentration induced dissipation of the membrane potential (Fig. 2a). The membrane depolarization was inhibited (Fig. 2a) and precluded (Fig. 2b) by N-acetylcysteine, BSA, and ATP. The recoupling effect of ATP was specific because it was substantially reduced by atractyloside, an inhibitor of the adenine nucleotide translocase (Fig. 3a). The addition of ADP in the presence of oligomycin, an inhibitor of ATP-synthase, produced no recoupling effect (Fig. 3b).

Menadione did not cause swelling of yeast mitochondria (Fig. 4a). The lack of swelling was unrelated to structural constraints because high-amplitude swelling of yeast mitochondria was induced by the peptide alamethicin, which is known to form ion channels with a diameter up to 1 nm upon its incorporation into the membrane.

Phenylarsine oxide (PhAsO) at 150 µM concentration inhibited respiration (Fig. 5a) and depolarized the mitochondrial membrane (Fig. 6a). These changes were only slightly sensitive to N-acetylcysteine (Figs. 5b and 6a) but fully reversed by ATP (Figs. 5c and 6a). Atractyloside, an inhibitor of adenine nucleotide translocase, prevented the

repolarizing action of ATP (Fig. 6c). BSA enabled only partial recovery of the membrane potential (Fig. 6b).

We noted that the PhAsO concentration of 150 μM, depolarizing the membrane potential in mitochondria of *D. magnusii,* was almost an order of magnitude higher than the concentration collapsing the membrane potential in animal mitochondria (around 30 μM). We assumed that this resistance of yeast mitochondria might be due, among other reasons, to the antioxidant action of BSA. Yeast mitochondria are usually isolated in the presence of fatty acid-free BSA because they are enriched in endogenous fatty acids, which act as uncouplers. However, BSA is also a powerful antioxidant (Roche et al. 2008). To check this assumption we isolated yeast mitochondria in the absence of BSA. The membrane potential of these mitochondria was rather high (Fig. 7a), but it was diminished by significantly lower PhAsO concentrations, which were comparable to those dissipating the membrane potential in animal mitochondria (Fig. 7a). In this case the membrane potential decrease was completely prevented by BSA (Fig. 7a) and ATP (Fig. 7b), but it was still insensitive to the water-soluble antioxidant N-acetylcysteine (Fig. 7b). Similarly to menadione, PhAsO did not cause swelling of the yeast mitochondria (Fig. 4a), which implies the lack of induction of nonspecific permeability of the mitochondria.

Oxaloacetate (OA) inhibited the state 4 respiration of *D. magnusii* mitochondria only at very high concentration (15 mM), but the mitochondrial membrane potential remained unchanged at even higher concentrations, and no high-amplitude swelling was observed (Data not shown).

Next, in an attempt to reduce the prooxidant concentrations required, we examined their combined action on yeast mitochondria.

Menadione, phenylarsine oxide, and oxaloacetate added together at low (empirically adjusted) concentrations significantly depolarized the membrane potential (Fig. 8a); this depolarization was entirely inhibited and prevented by N-acetylcysteine, BSA, and ATP (Figs. 8a and b). The protective effect of ATP was specific because it was completely or almost completely eliminated by atractyloside (Fig. 9a), bongkrekic acid (Fig. 9b), or oligomycin (Fig. 9c). The addition of ADP as well as ITP and GTP to oligomycin-treated mitochondria produced only partial "recoupling" of mitochondria, whereas CTP had no effect on membrane depolarization caused by the combined action of the prooxidants (Data not shown). The addition of Mg^{2+} partially removed the recoupling effect of ATP (Data not shown), indicating that ATP was active in its free form.

Moderate Ca^{2+} concentrations applied in the presence of prooxidant concentrations had no effect on the membrane potential (Fig. 10a). Decline of the membrane potential was observed only in the presence of the Ca^{2+} ionophore ETH129 (Fig. 10b), which is known to form a Ca^{2+}-permeable membrane channel. Based on our recent data, we recognized that this Ca^{2+} permeation is mediated by the Ca^{2+}/H^{+} exchange dependent on endogenous fatty acids (Kovaleva et al. 2009, 2010).

Mitochondria of *D. magnusii* treated with a combination of menadione, phenylarsine oxide, and oxaloacetate did not

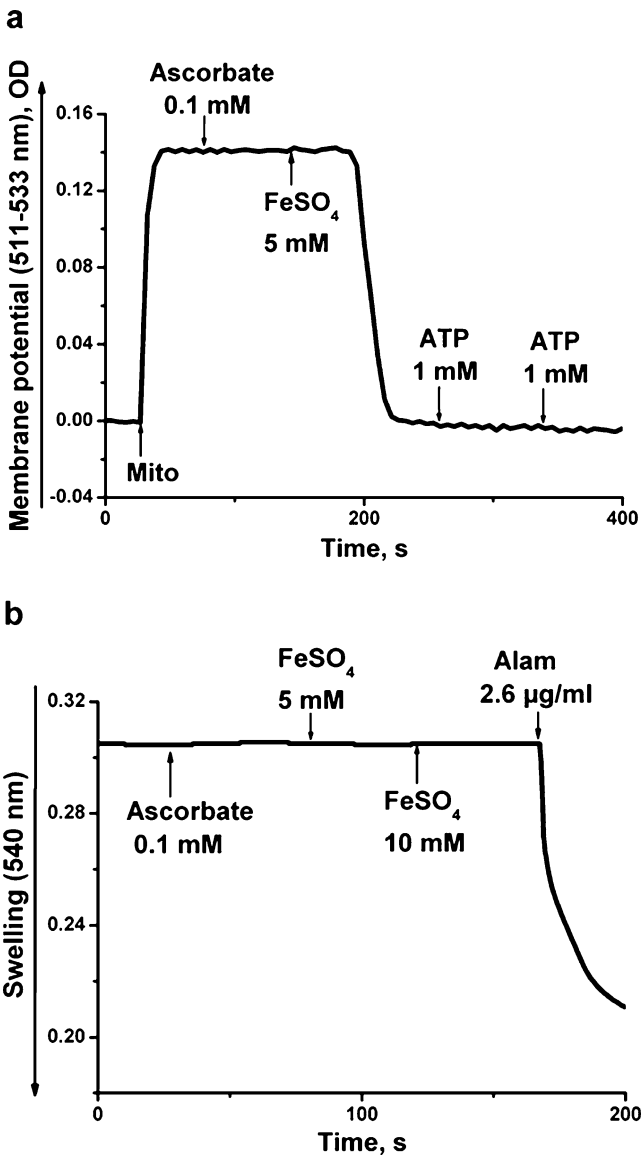


Fig. 12 Effects of ascorbate, $FeSO_4$, and ATP on the membrane potential and swelling of *D. magnusii* mitochondria. Additions and amounts are given in the figure. **a** Experimental conditions were as in Fig. 2. Ascorbate (0.1 mM) applied in combination with 5 mM $FeSO_4$ caused the collapse of the membrane potential that was insensitive to ATP. **b** Experimental conditions were as in Fig. 4. The addition of the ascorbate+$FeSO_4$ system did not induce swelling of *D. magnusii* mitochondria in a slightly hypotonic medium supplemented with 40 mM KCl

show high-amplitude swelling even in a slightly hypotonic medium containing 40 mM KCl (Fig. 4a). Hence, this treatment produced only the low-conductance (H^+-permeable) channel without formation of a megachannel.

The addition of 10 mM hydrogen peroxide to *D. magnusii* mitochondria isolated in the absence of BSA led to lowering of the membrane potential (Fig. 11a). When the mitochondria were isolated in the presence of BSA, membrane depolarization was observed at much higher concentrations of this agent (Fig. 11b). Repolarization of the membrane was achieved by the addition of ATP (Fig. 11a) but not by N-acetylcysteine (Fig. 11b). Similar data were obtained upon treatment of mitochondria with *t*-butyl peroxide (Data not shown). Neither hydrogen peroxide nor *t*-butyl peroxide, even at high concentrations, induced high-amplitude swelling of the mitochondria (Fig. 11c). Hence, these experimental conditions did not lead to formation of a high-conductance channel.

Finally, the exposure of yeast mitochondria to the ascorbate plus $FeSO_4$ system with the purpose of producing hydroxyl anion-radical, a short-lived strong oxidant, caused a considerable decrease in the membrane potential (Fig. 12a) that was insensitive to ATP (Fig. 12a) and antioxidants but was not accompanied by the large scale swelling of mitochondria (Fig. 12b).

Thus, we examined thoroughly, for the first time, the effect of prooxidants on energy parameters of yeast mitochondria and analyzed the possibility of inducing a membrane pore in yeast mitochondria under conditions of oxidative stress. We showed that the prooxidants menadione, oxaloacetate, and phenylarsine oxide (taken separately or applied in various combinations), as well as hydrogen peroxide, t-butyl peroxide, and the ascorbate+$FeSO_4$ system caused dissipation of the membrane potential without formation of a megachannel; this was evident from the lack of mitochondrial swelling after the addition of the prooxidants at concentrations inducing the decrease in membrane potential. Unlike animal mitochondria, yeast mitochondria showed no swelling after the addition of prooxidants in combination with moderate Ca^{2+} concentrations and ETH129, providing additional evidence for the lack of pore opening.

The decrease in membrane potential induced by prooxidants was prevented by the addition of antioxidants as well as by ATP (an original observation) and, to a much lesser degree, by other nucleotides. The recoupling action of ATP was eliminated and prevented by atractyloside and bongkrekic acid (inhibitors of the adenine nucleotide translocase), oligomycin (energy transfer inhibitor), and partially by Mg^{2+}. Interestingly, the recoupling action of ATP was observed even in those cases (e.g., at high concentrations of phenylarsine oxide or hydrogen peroxide) when the classic antioxidant N-acetylcysteine was ineffective. The only exception was observed under the action of hydroxyl radical as the most powerful oxidant. There are grounds to believe that ATP acts as a universal "recoupling" agent.

Two possible explanations of this phenomenon can be considered. The first is that ATP addition puts into operation a second source of energization, i.e. its generation of $\Delta\Psi$ at the expense of ATP hydrolysis. Thus, mitochondria of *D. magnusii* and *Y. lipolytica* yeasts can generate membrane potential either during substrate oxidation in the respiratory chain or at the expense of ATP hydrolysis. The validity of this proposal is supported by the fact that oligomycin at low concentrations (1 μg/mg protein) almost fully prevented the recoupling action of ATP (Figs 3b and 9c).

The results could be also explained from the idea that the adenine nucleotide translocase is the main target for prooxidant action. This enzyme is the main protein of the inner mitochondrial membrane and contains four cysteine residues in the lipid bilayer (Kihira et al. 2007). One can suppose that the protein acquires a specific conformation in the presence of ATP that makes the SH-groups inaccessible for the attack of prooxidants.

It is still difficult to make a choice between these two explanations. It is well possible that the above two alternatives are not mutually exclusive.

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