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Basic energetic parameters of *Acanthamoeba castellanii* mitochondria and their resistance to oxidative stress

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The purpose of this study was establishing the basic energetic parameters of amoeba *Acanthamoeba castellanii* mitochondria respiring with malate and their response to oxidative stress caused by hydrogen peroxide in the presence of Fe^{2+} ions. It appeared that, contrary to a previous report (Trocha LK, Stobienia O (2007) *Acta Biochim Polon* 54: 797), H_2O_2-treated mitochondria of *A. castellanii* did not display any substantial impairment. No marked changes in cytochrome pathway activity were found, as in the presence of an inhibitor of alternative oxidase no effects were observed on the rates of uncoupled and phosphorylating respiration and on coupling parameters. Only in the absence of the alternative oxidase inhibitor, non-phosphorylating respiration progressively decreased with increasing concentration of H_2O_2, while the coupling parameters (respiratory control ratio and ADP/O ratio) slightly improved, which may indicate some inactivation of the alternative oxidase. Moreover, our results show no change in membrane potential, Ca^{2+} uptake and accumulation ability, mitochondrial outer membrane integrity and cytochrome *c* release for 0.5–25 mM H_2O_2-treated *versus* control (H_2O_2-untreated) mitochondria. These results indicate that short (5 min) incubation of *A. castellanii* mitochondria with H_2O_2 in the presence of Fe^{2+} does not damage their basic energetics.

Keywords: *Acanthamoeba castellanii*, mitochondria, hydrogen peroxide, oxidative stress

INTRODUCTION

Mitochondria are the key cellular source of superoxide ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2). There are many different exogenous inducers of reactive oxygen species (ROS) formation, such as UV irradiance and relatively high concentrations of iron (Fe^{2+}) ions in the cell. Fe^{2+} reacts with H_2O_2 in the Fenton reaction, producing the highly reactive hydroxyl radical ($^{\bullet}OH$) (Minotti & Aust, 1987; Fleury *et al*., 2002). The oxidative stress resulting from an increase in ROS generation leads to a damage of mitochondrial DNA, proteins, membranes, ageing acceleration, and in many cases to cell death (Papa & Skulachev, 1997). Hydrogen peroxide is damaging to mitochondria and other cell components because it can oxidize biomolecules directly or through the formation of hydroxyl radical. The consequences of elevated mitochondrial H_2O_2 concentrations could be lipid peroxidation, disruption of calcium homeostasis, cytochrome *c* release, inactivation of respiratory chain carriers and other mitochondrial enzymes, and uncoupling or decrease in respiration (Vladimirov *et al*., 1980; Malis & Bonventre, 1988; Zhang *et al*., 1990; Radi *et al*., 1993; Slyshenkov *et al*., 1996; Sherer *et al*., 2002; Winger *et al*., 2007).

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Abbreviations: BHAM, benzohydroxamate; BSA, bovine serum albumin; COX, cytochrome *c* oxidase; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; ROS, reactive oxygen species; RCR, respiratory control ratio; TMPD, *N*,*N*,*N*′*N*′-tetramethyl-*p*-phenylenediamine; $\Delta\mu H^+$, proton electrochemical gradient; $\Delta\Psi$, mitochondrial transmembrane electric potential; $\Delta\Psi 3$, membrane potential in state 3; $\Delta\Psi 4$, membrane potential in state 4.

Acanthamoeba castellanii is a small non-photosynthesizing free-living amoeba found in soil and in marine and freshwater environments. *A. castellanii* is also an opportunistic pathogen of clinical interest, responsible for several distinct human diseases. In molecular phylogenesis, *A. castellanii* appears on a branch basal to the divergence points of plants, animals and fungi (Wainright *et al*., 1993). Under axenic non-pathogenic conditions, *A. castellanii* has been used frequently as a model organism to study mitochondrial energy-dissipating systems such as a cyanide-resistant alternative oxidase (Jarmuszkiewicz *et al*., 1997; 1998; 2001; 2005a), an ATP-sensitive potassium channel (Kicinska *et al*., 2007), and an uncoupling protein (Jarmuszkiewicz *et al*., 1999; 2004a; 2004b; 2005b; Czarna *et al*., 2007; Swida *et al*., 2007). Mitochondria of *A. castellanii* contain a plant-type respiratory chain with additional (in addition to the four classical) electron carriers: external and internal NADH dehydrogenases and an alternative cyanide-resistant quinol oxidase that consumes mitochondrial reducing power without energy conservation in the proton electrochemical gradient ($\Delta\mu H^+$) (Jarmuszkiewicz *et al*., 1997; 2005a). We have shown that in *A. castellanii,* like in plant mitochondria (Popov, 2003; Vercesi *et al*., 2006), the two mitochondrial energy-dissipating systems, the alternative oxidase and the uncoupling protein, may play a role in the energetic status of the cell (decreasing the yield of ATP synthesis) (Jarmuszkiewicz *et al*., 1998; 1999; 2004b; 2005b) and in attenuating ROS production (Czarna & Jarmuszkiewicz, 2005). Moreover, the contribution of both energy-dissipating systems in the prevention of mitochondrial ROS generation *in vivo* could ensure their constant level throughout the growth cycle of *A. castellanii* batch culture (Czarna *et al*., 2007).

The aim of the present work was to establish the basic energetic parameters of amoeba *A. castellanii* mitochondria respiring with malate and their response to oxidative stress caused by H_2O_2 in the presence of Fe^{2+}. Our aim was to verify the results obtained recently by Trocha and Stobienia (2007) with isolated *A. castellanii* mitochondria treated for a short time (1 min) with increasing concentrations of H_2O_2 (up to 25 mM) in the presence of Fe^{2+}. The puzzling results reported by those authors included unexpectedly quite high values of coupling parameters obtained with malate under control conditions (ADP/O ratio of 2.9 and RCR of 11) and inconsistent changes observed after 1 min incubation of mitochondria with H_2O_2, i.e., up to a 3-fold increase in phosphorylating respiration accompanied by an enormous cytochrome *c* release, as well as a decrease in the coupling parameters. Therefore, we repeated all the experiments performed by Trocha and Stobienia (2007), measuring changes in respiratory rates, coupling parameters, membrane potential, Ca^{2+} uptake and cytochrome *c* release/retention in isolated *A. castellanii* mitochondria. Additionally, we checked cytochrome *c* oxidase activity and outer mitochondrial membrane integrity.

MATERIALS AND METHODS

Cell culture and mitochondria isolation. The soil amoeba *Acanthamoeba castellanii*, avirulent strain Neff, was cultured as described previously (Jarmuszkiewicz *et al*., 1997). Trophozoites of amoeba were collected 60–72 h following inoculation at the early stationary phase (at a density of about 5–6 × 10^6 cells/ml). Mitochondria were isolated as described by Trocha and Stobienia (2007). Mitochondrial protein concentration was determined by the biuret method using BSA as a standard. Mitochondria preparations were diluted to a concentration of 50 mg protein/ml. All measurements were carried out within 5–6 h after mitochondria isolation, as their bioenergetic features were found to weaken after this time.

The oxidative stress model. Oxidative stress model conditions (concentration of H_2O_2 and incubation time) were those applied by Trocha and Stobienia (2007). To trigger the Fenton reaction, samples containing 3 mg of mitochondrial protein (180 μl) were treated with H_2O_2 (0.5, 5, 15, or 25 mM) in the presence of 0.2 mM $FeCl_2$. Hydrogen peroxide (Sigma, INFARM) concentrations were determined using a molar absorbance coefficient of 43.6 M^{-1} cm^{-1} at 240 nm. Stock solution of 10 mM $FeCl_2$ was prepared daily by adding the solid iron salt to 2.5 M NaCl (plus 5 mM Tris/HCl), pH 7.0, exhaustively bubbled with argon. The solution was capped, protected from light, and used within 4 h (Minotti & Aust, 1987). A small volume (3.6 μl) of Fe^{2+} solution was added to the mitochondria incubation reaction to reach a final concentration of 0.2 mM $FeCl_2$ and 50 mM NaCl. Incubations were performed for 1 or 5 min at 0–4°C. Control (H_2O_2-untreated) mitochondria were incubated for 1 or 5 min in the absence of H_2O_2 but in the presence of 0.2 mM $FeCl_2$ and 50 mM NaCl (pH = 7.0). The reactions were stopped by a 100-fold dilution with the incubation medium (0.25 M sucrose, 10 mM Tris/HCl, pH 7.4). The samples were centrifuged at 10 000 × *g* at 0°C for 7 min to remove remaining H_2O_2. The pellets containing stressed (H_2O_2-treated) or control (H_2O_2-untreated) mitochondria were suspended gently in 180 μl of the incubation medium (to obtain a concentration of 50 mg of mitochondrial protein per ml) and used immediately for the measurements of oxygen consumption, membrane potential and calcium ion uptake. Supernatants were collected and used to estimate cytochrome *c* release. Only results obtained after 5 min incuba-

tion of mitochondria with H_2O_2 in the presence of Fe^{2+} are shown, since no significant changes were found after 1 min incubation.

Oxygen uptake and membrane potential. Oxygen uptake was measured polarographically using a Rank Bros. (Cambridge, UK) oxygen electrode or a Hansatech oxygen electrode in 3 ml or 1.5 ml (respectively) of the standard reaction medium (25°C) containing 120 mM KCl, 20 mM Tris/HCl, pH 7.4, 3 mM KH_2PO_4, 8 mM $MgCl_2$ and 0.2% BSA, with 3 or 1.5 mg of mitochondrial protein (to keep the concentration of 1 mg × ml^{-1}). Membrane potential ($\Delta\Psi$) of mitochondria was measured simultaneously with the measurements of oxygen uptake using a tetraphenylphosphonium-specific electrode according to Kamo *et al*. (1979). For calculation of the $\Delta\Psi$ value the matrix volume of amoeba mitochondria was assumed as 2.0 µl/mg protein. Values of O_2 uptake are presented in nAt O × min^{-1} × (mg protein)$^{-1}$. $\Delta\Psi$ values are presented in mV.

All measurements were made in the presence of 3 mM malate (as a respiratory substrate) and 100 nmol ADP (equivalent to 30–60 µM, prepulse). The ADP/O ratio was determined by the ADP pulse method with 450–500 nmol ADP. The total amount of oxygen consumed during state 3 respiration was used for calculation of the ratio. A prepulse of ADP was always applied before the main pulse to ensure that a true state 4 had been achieved and to activate malate dehydrogenase by the produced ATP. Measurements were made in the absence or presence of 2 mM benzohydroxamate (BHAM), an inhibitor of the alternative oxidase. At the end of each measurement, 1 µM FCCP was added to collapse $\Delta\Psi$ and to assess the rate of the uncoupled state, i.e., a maximal cytochrome pathway activity. Measurements of $\Delta\Psi$ allowed fine determination of the duration of state 3 respiration.

Cytochrome *c* oxidase activity and outer mitochondrial membrane integrity. Cytochrome *c* oxidase (COX) activity was measured with an oxygen electrode using 0.5 mg of mitochondrial protein (in 1.5 ml of the standard reaction medium), without exogenously added respiratory substrate and in the presence of 2 mM BHAM. Respiratory rates were measured during sequential additions of antimycin A (4 µg/mg mitochondrial protein), 8 mM ascorbate, 0.06% cytochrome *c* and up to 2.5 mM TMPD. The rate of oxygen consumption following the addition of TMPD reflected the maximal O_2 consumption by COX (complex IV). Outer mitochondrial membrane integrity was assayed as the latency of COX activity during the same measurements (acceleration of respiration by addition of cytochrome *c* prior to addition of TMPD).

Calcium ion uptake. The measurements of Ca^{2+} uptake and accumulation by mitochondria were carried out using a tetraphenylphosphonium-specific electrode at 25°C with 3 mg mitochondrial protein per individual measurement. Standard reaction medium (3 ml) was used with 3 mM malate as a respiratory substrate. After addition of malate (state 4 conditions), known aliquots of calcium ions (about 200 nmol/mg of mitochondrial protein) were sequentially added to the medium until the membrane potential collapsed. The total amount of accumulated calcium ions and the total time of calcium ion accumulation were measured. Total loading of mitochondria with Ca^{2+} was assumed when no rebuilding of $\Delta\Psi$ was observed.

Cytochrome *c* assay. Cytochrome *c* release was assessed by three independent spectrophotometric approaches, i.e., at 550 nm, at 550 minus 540 nm, and by spectrum measurements (420–620 nm). We used supernatants obtained after mitochondria (H_2O_2-treated or -untreated) suspension and centrifugation in a 100-fold volume of the incubation medium. Three milligrams of mitochondrial protein per reaction was used. The measurements were monitored in quartz cuvettes containing 1 ml of the supernatant solution in the absence or presence of 0.1 mM potassium ferricyanide (to oxidize the reduced cytochrome *c*) and in the absence or presence of a few grains of sodium dithionite (to reduce cytochrome *c*). Cytochrome *c* concentrations were determined using a molar absorbance coefficient of 21 mM^{-1} cm^{-1} at 550 minus 540 nm and 27.8 mM^{-1} cm^{-1} at 550 nm.

RESULTS

Respiration rates, coupling parameters and membrane potential

The capacity of cytochrome pathway-dependent respiration of isolated *A. castellanii* mitochondria was measured in the presence of BHAM, an inhibitor of the alternative oxidase, and BSA that binds free fatty acids thereby excluding uncoupling protein activity. Malate was used as a respiratory substrate. In the presence of BHAM, in control, H_2O_2-untreated mitochondria the ADP/O ratio was 2.3 ± 0.2 and respiratory control ratio (RCR) was 3.3 ± 0.3 (S.D., for five different mitochondria preparations). Table 1 shows that the incubation of mitochondria with 0.5–25 mM H_2O_2 in the presence of Fe^{2+} results in no marked changes in the cytochrome pathway activity. In particular, in the presence of BHAM, there were no differences in the rates of uncoupled (FCCP-stimulated) respiration, state 3 (ADP-stimulated) respiration, RCR and ADP/O ratio in the absence and in the presence of up to 25 mM

Table 1. Respiratory rates, coupling parameters and COX activity of *A. castellanii* mitochondria oxidizing malate.

Rates of state 3 (phosphorylating state), state 4 (nonphosphorylating state) and state U (uncoupled state) are presented. For control (no H_2O_2) and 25 mM H_2O_2-treated mitochondria measurements were additionally performed in the presence of 2 mM BHAM (values in parentheses). Values of O_2 uptake and COX activity are expressed in nAt O × min^{-1} × (mg protein)$^{-1}$. Mean values for five different mitochondria preparations ± S.D. are shown. Values marked with * are significantly different from those from control (no H_2O_2) mitochondria at the level of $P < 0.05$ (paired Student's *t*-test).

H_2O_2 (mM)	State 3	State 4	RCR	ADP/O	State U	COX activity
0	163 ± 24	63 ± 8	2.6 ± 0.2	2.2 ± 0.2	171 ± 24	409 ± 80
	(159 ± 21)	(48 ± 4)	(3.3 ± 0.3)	(2.3 ± 0.2)	(163 ± 18)	
0.5	161 ± 15	63 ± 5	2.6 ± 0.2	2.2 ± 0.1	169 ± 14	402 ± 48
5	161 ± 12	60 ± 4	2.7 ± 0.1	2.2 ± 0.1	169 ± 12	390 ± 66
15	163 ± 14	55 ± 3	3.0 ± 0.2	2.2 ± 0.1	171 ± 11	384 ± 31
25	158 ± 22	48 ± 3*	3.2 ± 0.3*	2.3 ± 0.1*	165 ± 20	373 ± 75*
	(159 ± 21)	(48 ± 4)	(3.3 ± 0.3)	(2.3 ± 0.2)	(165 ± 19)	

H_2O_2. However, in the absence of BHAM, non-phosphorylating state 4 respiration (but not state 3 and uncoupled state) progressively decreased with the increasing concentrations (0.5–25 mM) of H_2O_2 (by up to 31% with 25 mM H_2O_2, $P = 0.0086$) while the coupling parameters, RCR and ADP/O ratio slightly improved (respectively by up to 20 and 10% with 25 mM H_2O_2, $P = 0.0073$ and 0.0325). This indicates that short (5 min) incubation of mitochondria with H_2O_2 in the presence of Fe^{2+} could decrease the alternative oxidase activity. In addition, a slight (by up to 10% with 25 mM H_2O_2, $P = 0.0175$) decrease in the activity of COX (complex IV) was observed.

The values of mitochondrial ΔΨ generated during malate oxidation both in nonphosphorylating (state 4) and phosphorylating (state 3) conditions are presented in Table 2. As expected, in the absence and presence of BHAM, no significant changes in the ΔΨ4 and ΔΨ3 values for H_2O_2-treated compared to control (H_2O_2-untreated) mitochondria were observed. This confirms our before-mentioned observations that a 5-min incubation of isolated *A. castellanii* mitochondria with H_2O_2 (up to 25 mM) does not decrease the cytochrome pathway-mediated respiration.

Table 2. State 4 and state 3 membrane potential of *A. castellanii* mitochondria oxidizing malate.

For control (no H_2O_2) and 25 mM H_2O_2-treated mitochondria measurements were additionally performed in the presence of 2 mM BHAM (values in parentheses). Mean values for five different mitochondria preparations ± S.D. are shown.

H_2O_2 (mM)	ΔΨ4 (mV)	ΔΨ3 (mV)
0	186 ± 3	161 ± 3
	(186 ± 2)	(160 ± 3)
0.5	184 ± 3	160 ± 2
5	186 ± 2	158 ± 2
15	184 ± 2	158 ± 1
25	184 ± 4	158 ± 3
	(185 ± 3)	(159 ± 4)

Accumulation of Ca^{2+}

Calcium ion accumulation by *A. castellanii* mitochondria leads to an increase in oxygen consumption in the resting state (state 4) and simultaneously decreases ΔΨ. In *A. castellanii* mitochondria, ΔΨ-driven ruthenium red-sensitive Ca^{2+} uptake is mediated through a Ca^{2+} uniporter (Domka-Popek & Michejda, 1986). In the present work, active Ca^{2+} accumulation was measured when portions of 200 nmol Ca^{2+} were sequentially added to the reaction medium containing H_2O_2-treated or control (H_2O_2-untreated) mitochondria respiring under state 4 conditions (Table 3). The measurements were performed as long as

Table 3. Calcium ion uptake by mitochondria of *A. castellanii*.

Accumulation of Ca^{2+} by mitochondria respiring with malate under nonphosphorylating state 4 conditions. Mean values for five different mitochondria preparations ± S.D. are shown.

H_2O_2 (mM)	Total amount of accumulated Ca^{2+} (μmol/mg prot.)	Total time of Ca^{2+} accumulation (min/mg prot.)	Rate of Ca^{2+} accumulation (μmol/min × (mg prot.)$^{-1}$
0	2.3 ± 0.6	8.2 ± 1.0	0.27 ± 0.04
0.5	2.2 ± 0.2	8.3 ± 0.3	0.27 ± 0.02
5	2.2 ± 0.2	8.4 ± 0.6	0.26 ± 0.02
15	2.2 ± 0.3	8.4 ± 0.6	0.26 ± 0.02
25	2.2 ± 0.4	8.4 ± 0.8	0.26 ± 0.03

the mitochondrial ΔΨ was no longer restored after the uptake of a subsequent portion of Ca^{2+}, indicating total loading of mitochondria with these ions. These measurements allowed us to calculate Ca^{2+} accumulation rates expressed as the amount of Ca^{2+} (μmol) accumulated during one minute per one milligram of mitochondrial protein. Table 3 shows that the accumulation of calcium ions did not change significantly with increasing H_2O_2 concentration (up to 25 mM). Control (H_2O_2-untreated) and stressed (H_2O_2-treated) mitochondria of *A. castellanii* accumulated 2.2–2.3 μmol Ca^{2+} per mg of mitochondrial protein before ion saturation. Similarly, the rate of accumulation of calcium ions did not vary between the treatments.

Mitochondrial outer membrane integrity and cytochrome *c* retention

The influence of external cytochrome *c* on the respiratory rate during measurements of COX maximal activity was used to assess the outer mitochondrial membrane integrity of *A. castellanii* mitochondria. No significant difference in this feature was found between control (H_2O_2-untreated) and stressed (0.5–25 mM H_2O_2-treated) mitochondria. Namely, the outer mitochondrial membrane integrity averaged 98 ± 4% and 95 ± 5% for H_2O_2-untreated and 25 mM H_2O_2-treated mitochondria, respectively (for five different mitochondria preparations).

To measure cytochrome *c* release from the mitochondrial intermembrane space as a result of a possible outer membrane damage after 5 min incubation of mitochondria with H_2O_2 in the presence of Fe^{2+}, we carried out spectrophotometric measurements at 550 nm, at 550 minus 540 nm, and continuous at 420–620 nm. The absorbance values obtained at 550 minus 540 nm and at continuous spectrum measurements show no cytochrome *c* release from mitochondria of *A. castellanii* subjected to increasing H_2O_2 concentrations under 1 or 5 min incubation (not shown). The absorbance values recorded only at 550 nm were discarded as they were apparently due to some light-absorbing contaminations that was clearly indicated by the continuous spectral measurements and those at 550 minus 540 nm. In intact isolated *A. castellanii* mitochondria, total *c*-type cytochromes ($c + c_1$) content estimated from the difference spectra obtained at room temperature (substrate-reduced minus oxidized) of the α peaks at 550 minus 540 nm (using absorbance coefficient 19 mM^{-1} cm^{-1}) was 1.8 ± 0.4 nmol/mg mitochondrial protein (S.D., n = 5). The estimated content of *c*-type cytochromes is close to that found previously for *A. castellanii* mitochondria with the difference spectrum obtained at low temperature (Edwards *et al.*, 1977).

DISCUSSION

Our results show that a 5-min incubation of *A. castellanii* mitochondria with H_2O_2 at a concentration up to 25 mM (in the presence of 0.2 mM $FeCl_2$) is not associated with marked changes in cytochrome pathway activity. It is revealed by no changes in respiratory rates, ΔΨ values and coupling parameters found in the presence of the alternative oxidase inhibitor BHAM. In contrast, using similar conditions (1 min incubation with 0.5–25 mM H_2O_2, in the presence of Fe^{2+}) Trocha and Stobienia (2007) observed a significant increase in both state 3 and state 4 respiration accompanied by a considerable decrease in ΔΨ values and coupling parameters. The results described by those authors seem to be inconsistent, as the increase in phosphorylating respiration found for H_2O_2-treated mitochondria (e.g., a 3-fold increase for 25 mM H_2O_2) was accompanied by an enormous cytochrome *c* release. Moreover, the coupling parameters obtained for control conditions (H_2O_2-untreated mitochondria) with malate as the respiratory substrate are quite different in the present work (ADP/O = 2.3 and RCR = 3.3) from those of Trocha and Stobienia (2007) (ADP/O of 2.9 and RCR of 11.4). According to our results, cancellation of ΔΨ4 after Ca^{2+} accumulation indicates mitochondria saturation by these positively charged ions but not mitochondria disruption (Trocha & Stobienia, 2007). Contrary to Trocha and Stobienia (2007), in our experiments incubation of mitochondria with H_2O_2 had no effect on Ca^{2+} uptake, in agreement with unchanged ΔΨ4 values. Moreover, according to those authors, the total amount of cytochrome *c* released from *A. castellanii* mitochondria treated with 15 mM H_2O_2 would be (after calculation of the obtained absorption values) around 1.4 μmol/mg mitochondrial protein, thus three orders of magnitude higher than the amount found presently and by Edwards *et al.* (1977) with the difference spectrum obtained at room temperature and at low temperature, respectively.

Although a slight decrease in the COX activity was observed in the present study, it seems that it should not influence the overall cytochrome pathway activity, as the terminal oxidase of the respiratory chain of *A. castellanii* mitochondria is not rate-limiting for the cytochrome pathway-sustained respiration (Czarna *et al.*, 2007). However, our results point to a partial inactivation of two Fe-containing proteins, the alternative oxidase and COX (the nonheme and heme-bound proteins, respectively) in H_2O_2-treated mitochondria. This may mean that these proteins could be highly sensitive to oxidation under treatment of isolated *A. castellanii* mitochondria with H_2O_2 in the presence of $FeCl_2$.

It can be summarized that 0.5–25 mM H_2O_2-treated mitochondria of *A. castellanii* did not show substantial impairment. No marked changes in cytochrome pathway activity were found since in the presence of an alternative oxidase inhibitor no effects were observed on either the rates of uncoupled and phosphorylating respiration or on the coupling parameters. Moreover, our results indicate no H_2O_2-induced change in $\Delta\Psi$ values, Ca^{2+} uptake and accumulation ability, and the maintenance of mitochondrial integrity and cytochrome *c* content. Only in the absence of BHAM, non-phosphorylating respiration progressively decreased with increasing concentration of H_2O_2, while the coupling parameters slightly improved, which may indicate inactivation of alternative oxidase activity. Thus it can be concluded that short (5 min) incubation of isolated mitochondria with H_2O_2 (up to 25 mM) does not significantly damage the basic energetics of *A. castellanii* mitochondria. To observe an impact of oxidative stress, such as elevated H_2O_2 concentrations, on the function of *A. castellanii* mitochondria, cell culture should perhaps be treated with H_2O_2 for a couple of days. Such a treatment may allow to observe a response to stress at the level of mitochondrial protein composition and activity. Sweetlove *et al*. (2002) demonstrated for example that treatment of *Arabidopsis* cell cultures with 88 mM H_2O_2 for 7 days resulted in degradation of the tricarboxylic acid cycle and some respiratory chain proteins and led to a decrease in respiration, while mitochondrial integrity was maintained. The consequences of elevated H_2O_2 concentrations could also be lipid peroxidation, disruption of calcium homeostasis and cytochrome *c* release (Vladimirov *et al*., 1980; Malis & Bonventre, 1988; Zhang *et al*., 1990; Radi *et al*., 1993; Sherer *et al*., 2002; Sweetlove *et al*. 2002; Winger *et al*., 2007). In our opinion, to observe such a response of *A. castellanii* mitochondria, exposure of isolated mitochondria to a stronger oxidative stress (longer exposure and/or higher H_2O_2 concentration) would be required. In this study, under the stress conditions applied (5-min incubation at 4°C with up to 25 mM H_2O_2 in the presence of 0.2 mM $FeCl_2$), *A. castellanii* mitochondria displayed remarkable resistance to H_2O_2 treatment.

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