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Mitochondrial function plasticity in *Acanthamoeba castellanii* during growth in batch culture

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Abstract The alterations in mitochondrial bioenergetics during growth in a batch culture of *Acanthamoeba castellanii* were studied. The capacity of cytochrome pathway-dependent respiration measured *in vitro* decreased from the intermediary phase, when cell division slowed down. The pattern of the cytochrome pathway capacity changes was paralleled from the intermediary phase by alterations in the amount of total (and reducible) membranous ubiquinone. These changes were accompanied by a decrease in mitochondrial reactive oxygen species production *in vitro* (when no energy-dissipating system was active), and almost no change in superoxide dismutase activity and protein level, thus indicating an equivalent need for this enzyme in oxidative stress defence in *A. castellanii* culture. On the other hand, a decrease in the activity and protein level of alternative oxidase and uncoupling protein was observed *in vitro*, when cells shifted from the exponential growth phase to the stationary phase. It turned out that the contribution of both energy-dissipating systems in the prevention of mitochondrial reactive oxygen species generation *in vivo* could lead to its constant level throughout the growth cycle of *A. castellanii* batch culture. Hence, the observed functional plasticity insures survival of high quality cysts of *A. castellanii* cells.

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Abbreviations AcAOX: alternative oxidase of *Acanthamoeba castellanii*· AcUCP: uncoupling protein of *Acanthamoeba castellanii* · AOX: alternative oxidase · BHAM: benzohydroxamate · BSA: bovine serum albumin · CAT: carboxyatractyloside · COX: cytochrome *c* oxidase · $\Delta\mu H^+$: proton electrochemical gradient · FCCP: carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone · FFA: free fatty acid · LA: linoleic acid · Q: ubiquinone, coenzyme Q · ROS: reactive oxygen species · SOD: superoxide dismutase · state 3: phosphorylating respiration (in the presence of ADP) · state 4: resting respiration (in the absence of added ADP) · RCR: respiratory control ratio (state 3/state 4) · TMPD: N,N,N′N′-tetramethyl-*p*-phenylenediamine · UCP: uncoupling protein

Introduction

Acanthamoeba castellanii is a small non-photosynthesizing free-living soil protozoon that has received attention as a model organism for the study of unicellular eukaryotic cell life and differentiation (Neff, 1957; Avery et al., 1994). In molecular phylogenesis, *A. castellanii* appears on a branch basal to the divergence points of plants, animal and fungi (Wainright et al., 1993). The life cycle of *A. castellanii* comprises two distinct stages, a vegetative stage as free-living amoebae (trophozoites) and a resting stage as cysts (Weisman, 1976; Hirukawa et al., 1998). Trophozoites multiply via the growth division cycle until spontaneous cyst formation occurs in the growth medium, either after reaching the stationary phase or when certain metabolic inhibitors

are added. Encystment can be also induced by transferring trophozoites to a non-nutrient medium. Mature cysts can undergo excystment when they are returned to the growth medium. The amoeba cyst formation is associated, on the one hand, with the degradation of macromolecules and on the other hand, with the synthesis of cyst-specific molecules, such as cyst wall components (Pauls and Thompson, 1978). At the resting stage the organism can withstand the lack of nutrients and desiccation with minimal metabolic activity and poor mitochondrial respiration.

The vegetative growth of *A. castellanii* in batch culture is associated with changes of certain cellular components and metabolic processes, such as cytochrome levels, adenine nucleotide pool levels, oxygen uptake rates (Edwards and Lloyd, 1977a) and cellular fatty acid composition (Aver et al., 1994). The observed alterations mainly occur during the transition between the middle exponential and stationary phases. Other studies have revealed changes in the activity of cyanide-resistant respiration of whole amoeba cells after the exponential phase of growth (Edwards and Lloyd, 1977b). So far, no studies have examined how the evolution of *A. castellanii* batch culture influences mitochondrial respiratory functions.

Mitochondria of *A. castellanii* possess a plant-type respiratory chain with additional (regarding four classical) electron carriers: external and internal NADH dehydrogenases and an alternative cyanide-resistant quinol oxidase (AcAOX) that consumes mitochondrial reducing power without energy conservation into a proton electrochemical gradient ($\Delta\mu H^+$) (Jarmuszkiewicz et al., 1997, 2005a). While in plant mitochondria the activity of AOX is stimulated by α-keto acids and regulated by the redox state of the intermolecular disulfide bond (Day et al., 1995; Siedow and Umbach, 2000), these regulations do not apply to AcAOX in amoeba mitochondria (Jarmuszkiewicz et al., 1997, 2005a). Like oxidases in some other protists and in primitive fungi, AcAOX is strongly stimulated by purine nucleoside 5′-monophosphates (mainly by GMP) (Jarmuszkiewicz et al., 1997, 2005a). The other energy-dissipating system present in *A. castellanii* mitochondria is an uncoupling protein (AcUCP) that mediates free fatty acid (FFA)-activated purine nucleotide-inhibited H^+ re-uptake driven by $\Delta\mu H^+$ (Jarmuszkiewicz et al., 1999, 2005b). It has been shown that both AcUCP and AcAOX could be cold response proteins in unicellulars since cold treatment of amoeba culture increases their activity and protein level (Jarmuszkiewicz et al., 2001, 2004a). Moreover, we have shown that in *A. castellanii*, the two energy-dissipating systems may play a role in the energetic status of the cell (decreasing yield of ATP synthsesis) (Jarmuszkiewicz et al., 1998, 1999, 2004b, 2005b) and in attenuating reactive oxygen species (ROS) production (Czarna and Jarmuszkiewicz, 2005), like in plant mitochondria (Popov, 2003; Vercesi et al., 2006).

The purpose of the present study was to examine the plasticity of mitochondrial respiratory functions during the growth of *A. castellanii* in batch culture that precedes the trophozoite/cyst phase transition in its life cycle. As mitochondria provide most of the energy for the cells, but also are the main source of ROS, our aim was to shed light on links between mitochondrial bioenergetics, including mitochondrial respiration through the energy-conserving (the cytochrome pathway) and energy-dissipating (the AcUCP-sustained respiration and AcAOX-mediated respiration) systems, the efficiency of oxidative phosphorylation, and ROS release during the growth of *A. castellanii* until the stationary phase.

Materials and methods

Cell culture and mitochondrial isolation

The soil amoeba *Acanthamoeba castellanii*, strain Neff, was cultured in a medium described by Neff (1957) with some minor modifications: 1.5% proteoso-pepton (BD), 0.15% yeast extract (BD), 30 mM $MgCl_2$, 30 mM $FeSO_4$, 27 mM $CaCl_2$, 1.5% glucose, 2.5 mg/l vitamin B_{12}, 1 mg/l vitamin B_1, 0.2 mg/l vitamin H. Cells from 72 h old cultures were inoculated (time 0) to a final density of approximately $3.5 \pm 0.2 \times 10^5$ cell/ml (Fig. 1). After about 40 h of exponential growth with a generation time (a cell doubling time) of 7–8 h, amoeba cultures reach the intermediary phase and then the stationary phase, the latter preceding transformation into
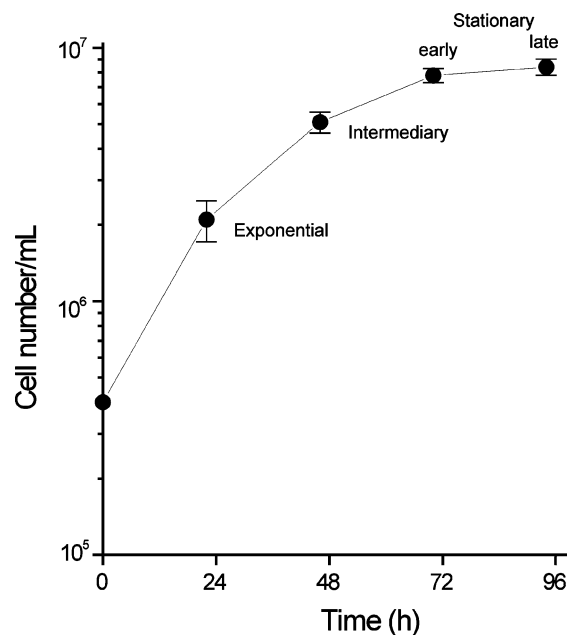

Fig. 1 Growth of *A. castellanii* cells in agitated batch culture at 28°C. Cell numbers were determined at the specified intervals. Mean values ($\pm$ SE) from three separate experiments are shown

cysts within a few hours. For the present study, trophozoites of *A. castellanii* were harvested from cultures at different phases of growth, at 24 hour intervals, i.e. 24 h ($2.1 \pm 0.3 \times 10^6$ cell/ml, the middle exponential phase), 48 h ($5.1 \pm 0.4 \times 10^6$ cell/ml, the intermediary phase), 72 h ($7.8 \pm 0.7 \times 10^6$ cell/ml, the early stationary phase) and 96 h ($8.4 \pm 0.7 \times 10^6$ cells/ml, the late stationary phase) following inoculation (Fig. 1). The cells were disrupted by a electrical homogenazer (20 strokes).

Mitochondria were isolated by differential centrifugation and purified on a self-generating Percoll gradient (30 %) as was described earlier (Jarmuszkiewicz et al., 1997). The depletion of endogenous free fatty acids (FFA) in the mitochondrial preparations was ensured by the presence of 0.4 % fatty acid-free bovine serum albumin (BSA) in isolation media except last mitochondrial washing (Jarmuszkiewicz et al., 1999). Mitochondrial protein concentration was determined by the biuret method using BSA as a standard.

Oxygen uptake

Mitochondrial oxygen consumption was measured polarographically using a Clark-type oxygen electrode (Hansatech) with 1 mM NADH or/and 7 mM succinate as respiratory substrates in 1.4 ml of a standard incubation medium (25°C) containing: 120 mM KCl, 10 mM Tris-Cl, pH 7.4, 3 mM KH_2PO_4, 2 mM $MgCl_2$, plus or minus 0.2% BSA (fatty acid free), with 1 mg of mitochondrial protein. Additional compounds added to the medium are indicated in the legends of the figures. Values of O_2 uptake are presented in nmol $O_2 \times min^{-1} \times mg^{-1}$ protein.

State 3 measurements were performed in the presence of 2 mM or 200 μM (pulse) ADP. For ADP/O ratio calculation, the total amount of oxygen consumed during pulse state 3 respiration was used. To inhibit the AcAOX and cytochrome pathway activities, 2 mM benzoxydroxamate (BHAM) and 1.5 mM cyanide were used, respectively. The AcUCP activity was inhibited with 0.2% BSA that chelates FFA. To activate AcAOX, 1 mM GMP was supplied. Different concentrations (up to 27 μM) of linoleic acid (the most efficient activator of AcUCP, Swida et al. (2007)) were used to activate AcUCP and thereby the UCP activity-sustained respiration. To exclude the activity of ATP/ADP antiporter, 1 μM carboxyatractyloside (CAT) was used in state 4 measurements.

The cytochrome *c* oxidase (COX) maximal activity was assessed with 0.25 mg of mitochondrial protein without exogenously added respiratory substrate, in the presence of antimycin A (4 μg/1 mg of mitochondrial protein), 8 mM ascorbate, 0.06% cytochrome *c* and up to 2.5 mM N,N,N′N′-tetramethyl-*p*-phenylenediamine (TMPD). The rate of oxygen consumption following the addition of TMPD reflected the maximal O_2 consumption by COX (complex IV).

Hydrogen peroxide production

Mitochondrial hydrogen peroxide (H_2O_2) production was measured fluorometrically using an Aminco Bowman-2 fluorescence spectrometer, as described previously (Czarna and Jarmuszkiewicz, 2005). Briefly, the generation of H_2O_2 was monitored by the decrease in fluorescence due to the oxidation of 0.2 μM scopoletin in the presence of 1 μg/ml horseradish peroxidase at excitation and emission wavelengths of 350 and 460 nm, respectively, in a 2 ml incubation medium (see above), with 5 mg of mitochondrial protein and constant agitation (25°C). The reaction was initiated by the addition of 10 mM succinate as a respiratory substrate.

MnSOD activity

Manganese superoxide dismutase (MnSOD) activity from isolated *A. castellanii* mitochondria was investigated spectrophotometrically using its ability to inhibit the rate of epinephrine autoxidation at a wavelength of 325 nm (Misra and Fridovich, 1972). Mitochondrial proteins were solubilized in the buffer (10 mg of protein/1 ml) containing 1% Triton X-100 and 10 mM Tris-HCl (pH 7.4) to disrupt mitochondrial membranes for 40 min in 4°C, with constant agitation. The obtained suspension was centrifuged at $15000 \times g$ for 10 min. The mitochondrial matrix protein concentration was determined in the supernatant by the Bradford method. The activity of MnSOD was measured using a UV-1602 Shimadzu spectrometer in 3 ml of 50 mM bicarbonate buffer, pH 10.2 (at 30°C) containing 0.1 mM EDTA and 0.125 mM epinephrine in the presence of up to 0.5 mg of mitochondrial matrix proteins, with constant agitation.

Determination of amount of ubiquinone

The endogenous pool of ubiquinone (Q) in the inner mitochondrial membrane was determined by an extraction technique (from 1–2 mg of mitochondrial protein), followed by HPLC detection according to Van den Bergen et al. (1994). A completely oxidized extract was obtained during incubation in the absence of respiratory substrates using an evaporation/ventilation step. A completely reduced extract was obtained when respiration in the presence of substrates (7 mM succinate and 1 mM NADH) was completely inhibited by 2 mM KCN and 2 mM BHAM. An inactive Q pool contains quinol that cannot be oxidized and quinone that cannot be reduced. To obtain an amount of active (reducible) Q, the inactive Q pool was subtracted from total Q pool. As previously found, the endogenous ubiquinone in *A. castellanii* mitochondria is Q-9 (Jarmuszkiewicz et al., 1998). For the calibration of the peaks, commercial Q-9 (Sigma) was used.

SDS-PAGE and immunoblotting

Up to 200 μg of mitochondrial protein (stored in −80°C) was solubilized in the sample buffer containing 2% (w/v) SDS, 80 mM Tris-Cl (pH 6.8), 10% glycerol, 0.04% (m/v) bromophenol blue and 2–5% mercaptoethanol (for AcAOX detection additionally containing 40 mM DTT) and boiled for 5 min. Electrophoresis (SDS-PAGE) was carried out using a 5% polyacrylamide stacking gel and a 12%–16% polyacrylamide resolving gel with 4.5 M urea followed by Western blotting. Sigma prestained low molecular mass markers or Amersham rainbow mass markers were used. Antibodies against AOX of *S. guttatum* (generously supplied by Dr. T.E. Elthon) were used at dilutions of 1:1000. Antibodies raised against UCP3 of human skeletal muscle (Alpha Diagnostic, UCP34-A) were used at dilutions of 1:500. Antibodies against subunit III of yeast cytochrome *c* oxidase (COX III, Molecular Probes, A6408) and antibodies against human manganese superoxide dismutase (MnSOD, Sigma, S5069) were used at dilutions of 1:1000. Protein bands were visualized using the Amersham ECL system and quantitated digitally. Detection of COX III, which amount was constant throughout culture growth, is considered as loading control for detections of the other proteins.

Results

The principal objective of the present study was to describe specific alterations in mitochondrial respiratory functions throughout the growth of a *A. castellanii* batch culture at 28°. Mitochondria were isolated from cells at different phases of growth, every 24 hours counting from inoculation (Fig. 1), i.e., from the exponential 24-h phase, intermediary 48-h phase, and the early and late (72-h, 96-h) stationary phases, the latter just before encystment.

Cytochrome pathway components and capacity

It turned out that growth of *A. castellanii* in batch culture was associated with marked changes in cytochrome pathway (complex III plus complex IV) activity (QH$_2$-oxygen oxidoreductase activity). The capacity of cytochrome pathway-dependent respiration was measured in the presence of BHAM (an inhibitor of AOX) and BSA (that chelates FFA)—to exclude the activity of AcAOX and AcUCP, respectively. The results obtained with external NADH (plus rotenone) as a respiratory substrate are shown in Fig. 2. External NADH has been chosen (instead of complex I or complex II substrates) in order to avoid limitation of the cytochrome pathway capacity by these two complexes (Jarmuszkiewicz et al., 2002). The rates of uncoupled (FCCP-stimulated) respiration, state 3 (ADP-stimulated), and respiratory control ra-

tio (RCR) decreased significantly as a function of culture duration, starting from the intermediary phase of growth when cell division slowed down significantly. However, there were no differences in non-phosphorylating state 4 respiration (data not shown). The pattern of the cytochrome pathway capacity changes was followed by the alterations in the amount of total (and reducible) membranous Q, although an increase (around 30%) in the amount of Q from the exponential phase to the intermediary phase was observed (Fig. 2C). On the other hand, the efficiency of oxidative phosphorylation, i.e., the ADP/O ratio (Fig. 2B), as well as the activity and amount of cytochrome *c* oxidase (complex IV) (Fig. 2D) remained constant throughout the amoeba culture growth. The constancy observed in the amount of COX protein and its activity throughout the batch culture indicates that the terminal oxidase of the respiratory chain is not rate limiting for the cytochrome pathway-sustained respiration.
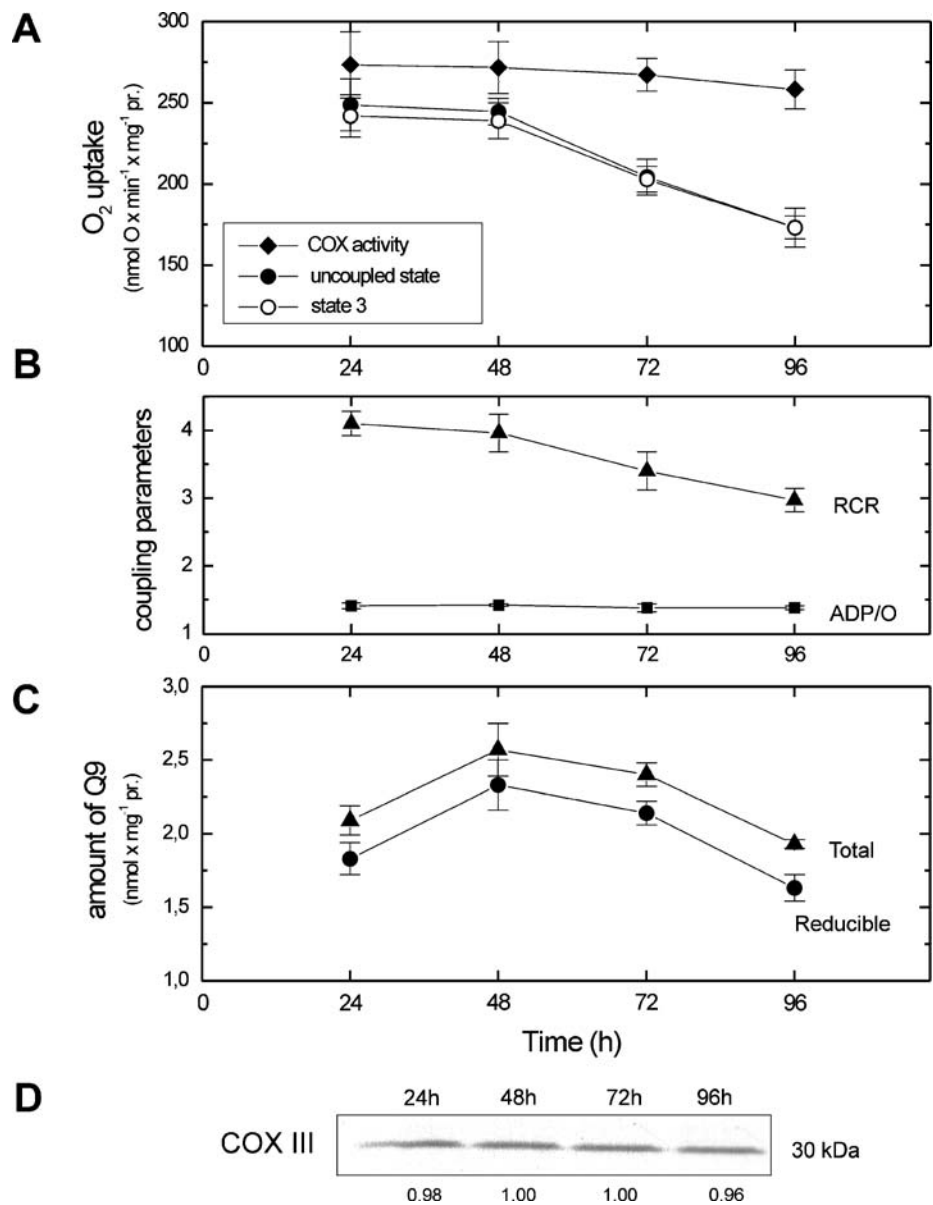
Energy-dissipating systems: AcAOX and AcUCP

We also followed the evolution pattern of the AcAOX-mediated respiration, measured as cyanide-resistant respiration in the absence (unstimulated capacity) or in the presence of the most efficient activator of the enzyme (1 mM GMP) (stimulated capacity). A significant (around 5-fold) decrease either in the AcAOX capacity (both stimulated and unstimulated) (Fig. 3A) or AcAOX protein level (Fig. 3B) was observed during the growth of *A. castellanii* in batch culture, when cells progressed from the exponential growth to the stationary phase. The effect of GMP was similar (4.5–4.8 fold stimulation) for each culture, indicating no change in allosteric stimulation.

Figure 4A shows the effect of *A. castellanii* culture duration on the linoleic acid-induced respiration that represents mainly the AcUCP capacity for sustaining respiration. The AcUCP sustained respiration activated by linoleic acid (22 μM that led to maximal stimulation) was measured in state 4 in the presence of carboxyatractyloside (to exclude ATP/ADP antiporter) and BHAM (to inhibit alternative oxidase). An approximately 50% decline (thus less pronounced compared to AcAOX) was observed in the AcUCP capacity (Fig. 4A) and protein level (Fig. 4B), when cells progressed from the exponential growth to the stationary phase. The inhibitory effect of 2 mM GDP on the linoleic acid-induced AcUCP-mediated uncoupling was partial as reported previously (Jarmuszkiewicz et al., 2004a) but quite similar for each phase of growth (around 30%), except that in the exponential phase cultures it was slightly lower (around 20%).

Since in *A. castellanii* mitochondria, the two energy-dissipating systems lead to the same final energetic effect (i.e. a decrease in ATP synthesis) and they cumulate this effect (Jarmuszkiewicz et al., 1999), we checked how AcUCP

and AcAOX working together can influence the efficiency of oxidative phosphorylation at a given phase of growth. Table 1 shows the rates of state 3 respiration and the ADP/O value obtained at submaximal activation of AcUCP (with 7 μM linoleic acid) and AcAOX (with 1 mM GMP). The rates of state 3 respiration (decreasing when cells shifted from the intermediary to stationary growth phases) did not change after activation of the two energy-dissipating systems when compared to control conditions for a given phase of growth. The lowest ADP/O ratio (around 0.7) was obtained for the exponential and intermediary phase cultures that revealed a similar state 3 rate, confirming the considerable effects of both energy-dissipating systems as expected from the capacity/activity and protein level measurements (Figs. 3 and 4). In the early and late stationary growth phases, increased

ADP/O values indicate a lowered contribution of AcUCP and AcAOX in total respiration *in vitro*

Mitochondrial ROS production

Throughout the *A. castellanii* batch culture growth, a decrease (around 40%) in *in vitro* basal mitochondrial ROS production was observed. It was estimated as the level of H_2O_2 formation in state 4 respiration (plus carboxyatractiloside and oligomycin), when no energy-dissipating systems (neither AcAOX nor AcUCP) were activated (Fig. 5, ♦).

It has been shown previously that activation of AcAOX and AcUCP lowers H_2O_2 formation in *A. castellanii* mitochondria (Czarna and Jarmuszkiewicz, 2005). In a present work, we measured the H_2O_2 formation at a given

Fig. 3 Alternative oxidase protein level and capacity. A. AcAOX activity was measured as a cyanide-resistant respiration in the presence of 1 mM NADH (as respiratory substrate), 2 mM cyanide, 0.2% BSA, 1 μM CAT, 0.5 μg/mg of protein oligomycin, minus or plus 1 mM GMP. Data deal with three different mitochondrial preparations for a given phase of growth. The mean values $\pm$ SE are shown ($n = 6$). B. Immunodetection of AcAOX protein. Mitochondrial protein equivalent to 160 μg was loaded in all lines. An example of three immunoblots (using mitochondria from different preparations) is shown. The highest intensity band was set at 1 and others calculated relative to that value
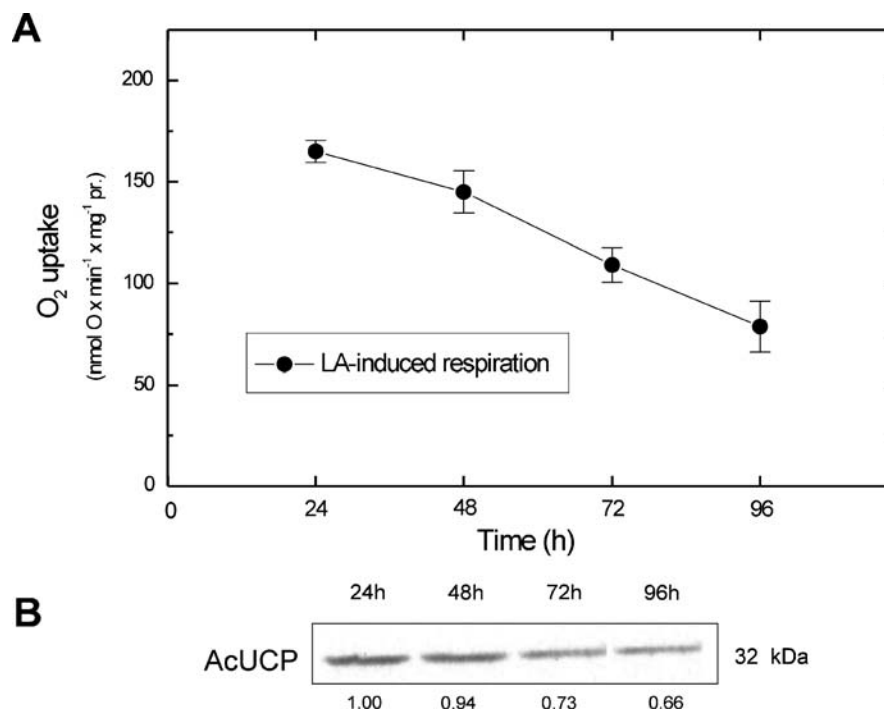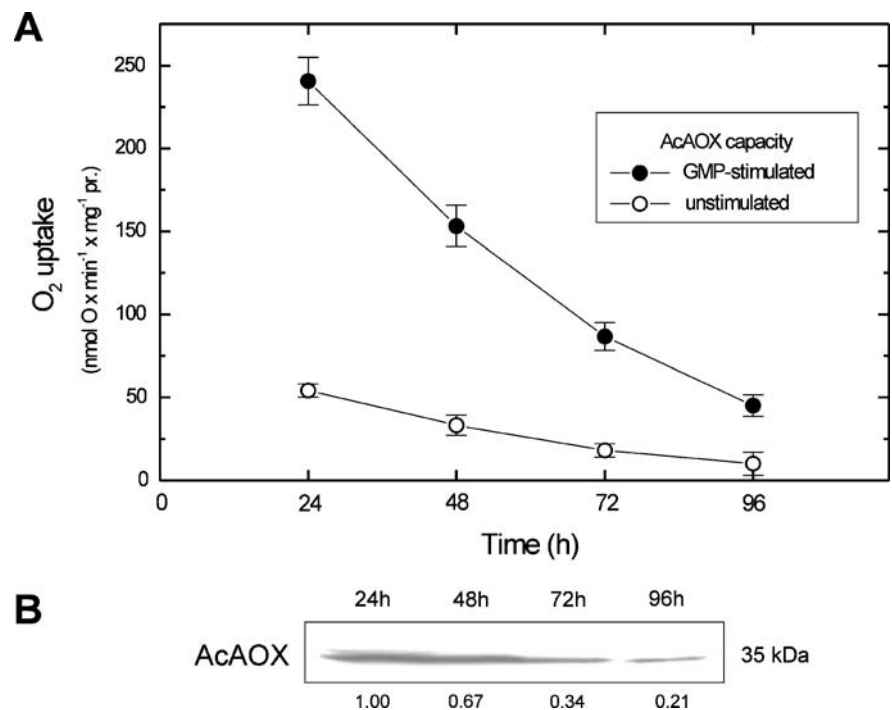
Fig. 4 Uncoupling protein level and AcUCP-activity sustained respiration. The LA-induced respiration was measured in the presence of 1 mM NADH (as respiratory substrate), 2 mM BHAM, 1 μM CAT, 0.5 μg/mg of protein oligomycin, and increasing LA concentration (up to 27 μM). The presented LA-induced respiration is a difference between respiration observed in the presence of the most effective concentration of LA (22 μM) minus respiration measured before LA addition. Data deal with three different mitochondrial preparations for a given phase of growth. The mean values $\pm$ SE are shown ($n = 6$). B. Immunodetection of AcUCP protein. Mitochondrial protein equivalent to 110 μg was loaded in all lines. An example of three immunoblots (using mitochondria from different preparations) is shown. The highest intensity band was set at 1 and others calculated relative to that value

Table 1 The effect of the uncoupling protein and alternative oxidase activations on the efficiency of oxidative phosphorylation throughout amoeba culture growth

	24h	48h	72h	96h
−LA, −GMP				
state 3	232 ± 13	229 ± 13	203 ± 10	170 ± 6
ADP/O	1.36 ± 0.03	1.38 ± 0.06	1.38 ± 0.01	1.40 ± 0.03
+LA, +GMP				
state 3	234 ± 11	230 ± 10	200 ± 10	176 ± 11
ADP/O	0.74 ± 0.03	0.71 ± 0.02	0.95 ± 0.04	1.15 ± 0.03

Note: Respiration measurements were performed with 1 mM NADH, 4 μM rotenone, and in the absence of BSA and BHAM. Pulses of 200 μM ADP were applied to obtain state 4/state 3 transformations. To activate AcAOX and AcUCP, mM GMP and 7 μM LA were added, respectively. Data deal with three different mitochondrial preparations for a given phase of growth. The mean values $\pm$ SE are shown ($n = 6$).

phase of growth, when AcAOX (Fig. 5, ●) or AcUCP (Fig. 5, ▲) were activated (by GMP or linoleic acid, respectively) and when both energy-dissipating systems were activated (Fig. 5, ◊). Activation of AcAOX by 1 mM GMP led to a similar (between 10–15%) decrease of mitochondrial H_2O_2 formation for a given culture growth phase, when compared to control conditions (no activation). In the case of AcUCP, activation of this energy-dissipating system by linoleic acid resulted in a decrease in mitochondrial H_2O_2 formation that was dependent on the phase of growth. The highest decrease (around 40%) was observed for exponential phase cultures, the lowest (around 10%)—for late stationary phase cultures.

The lowest but constant (throughout culture duration) level of H_2O_2 formation was measured with the simultaneous activation of both energy-dissipating systems (Fig. 5, ◊). Thus, in *A. castellanii* mitochondria, AcAOX and AcUCP reveal a cumulative effect on decreasing H_2O_2 formation *in vitro*, therefore they could accomplish their preventive effects *in vivo*, resulting in a stable cellular ROS level throughout the culture growth even if their protein concentration decreased. Moreover, no change in MnSOD activity and protein level was observed (Fig. 6), indicating an equivalent need for this enzyme in ROS defence in *A. castellanii* batch culture as ROS production could be maintained almost constant due to the AcAOX and AcUCP activities.

Discussion

In the present study we followed the time-course of changes in mitochondrial respiration, including the energy-conserving (the cytochrome pathway) and energy-dissipating (AcAOX-mediated respiration and AcUCP-sustained respiration) systems, and in ROS release from *A. castellanii* mitochondria, since exponential to the late stationary phase of growth. It appeared that the growth of *A. castellanii* batch culture was associated with marked changes in mitochondrial bioenergetic functions. The capacity of cytochrome pathway-dependent respiration (QH_2-O_2 oxido-reductase activity) decreased from the start of the intermediary phase (Fig. 2A). The pattern of the cytochrome pathway capacity
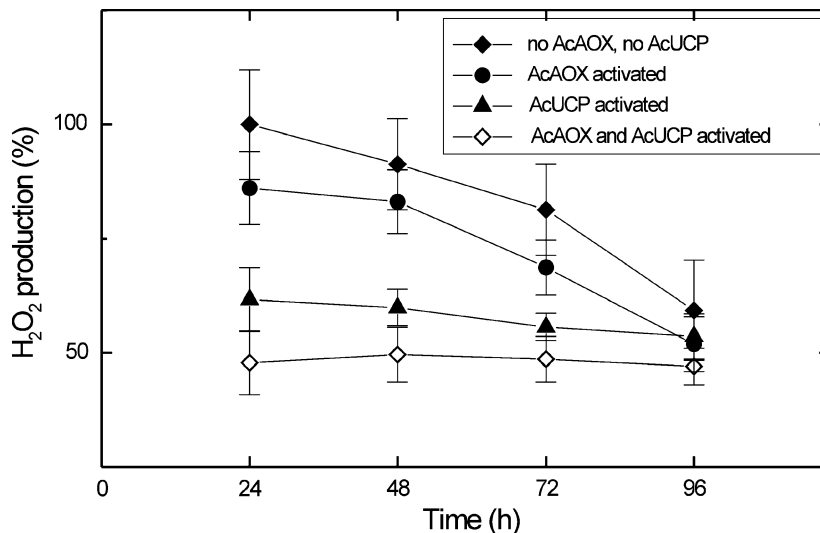

Fig. 5 The effect of AcUCP and AcAOX activations on hydrogen peroxide generation throughout amoeba culture growth. H_2O_2 measurements were performed as described under "Material and methods". Mitochondria were incubated in the presence of 10 mM succinate, 1 μM CAT, and 0.5 μg/ml oligomycin. Basal mitochondrial H_2O_2 production (control conditions, no AcUCP, no AcAOX activated) was measured in the absence of LA and GMP. To activate AcUCP and AcAOX, 20 μM LA and 1 mM GMP were applied, respectively. Hydrogen peroxide production is expressed as the percentage of fluorescence change observed in a 24 h culture in control conditions, where the highest H_2O_2 production was observed. Mean values ($\pm$S.E.) from three separate experiments are shown

Fig. 6 Manganese superoxide dismutase protein level and activity. A. MnSOD activity measurements were performed as described under "Material and methods." Data deal with three different mitochondrial preparations for a given phase of growth. The mean values ± SE are shown ($n = 6$). B. Immunodetection of MnSOD protein. Mitochondrial protein equivalent to 110 μg was loaded in all lines. An example of three immunoblots (using mitochondria from different preparations) is shown. The highest intensity band was set at 1 and others calculated relative to that value
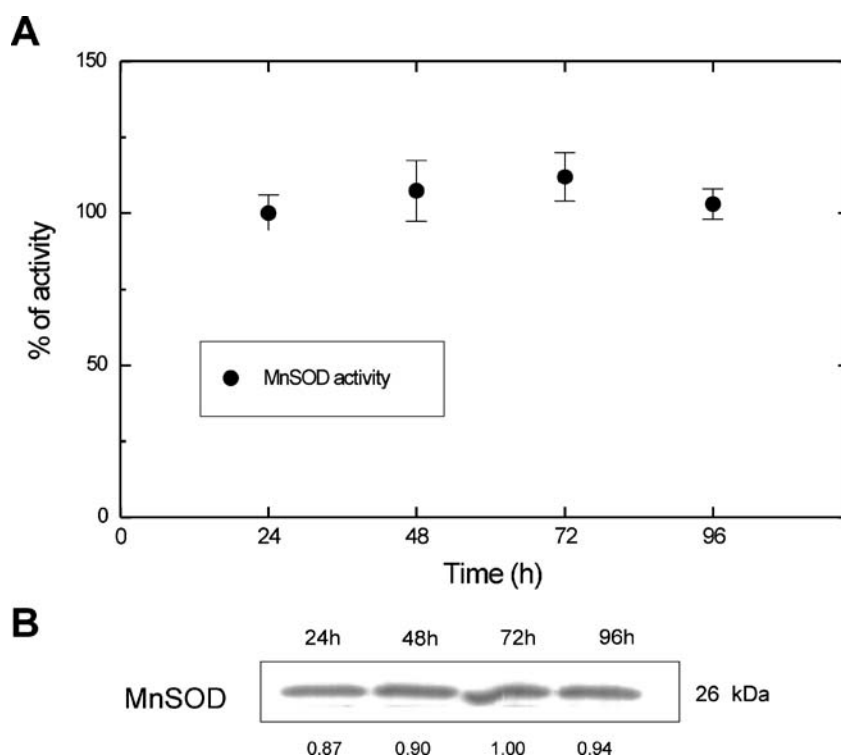
changes was concomitant with alterations in the amount of reducible (and total) membranous Q, except at the beginning of batch culture duration (Fig. 2C). Namely, a 30% increase in the amount of membranous Q accompanied by no change in the cytochrome pathway capacity occurred from the exponential phase to the intermediary phase of growth. A lower reducible Q content in intensively dividing cells (i.e. at exponential phase of growth) indicate that in this phase, Q could be more reduced at a given electron input from dehydrogenases, compared to subsequent growth phases. If this really occurs at the exponential phase of growth, a higher Q reduction level could lead to a higher AcAOX activity (that depends on Q redox state, Jarmuszkiewicz et al. (2005a)) and to a higher AcUCP activity (whose inhibition by purine nucleotides depends on Q redox state, Jarmuszkiewicz et al. (2005b). This would be reinforced by the fact that the highest capacity and protein level of AcAOX and AcUCP were observed in exponential phase cultures after 24 hours growth (Figs. 3 and 4). When the cell culture progressed to the stationary phase, activity and protein level of both energy-dissipating systems decreased significantly (around 5-fold and 50% for AcUCP and AcAOX, respectively). Moreover, Avery and his colleagues (1994) reported that during the growth of *A. castellanii* in batch culture, the total cellular fatty acid unsaturation index (an average number of double bonds per fatty acids), that reflects availability in free fatty acids—hence in AcUCP activators, also decreases, starting from the exponential phase. As our previous data showed that unsaturated free fatty acids are more effective as AcUCP

activators compared to saturated ones (Swida et al., 2007), it seems that the activity of AcUCP could decrease during batch culture evolution.

When no energy-dissipating system was active, a decrease in mitochondrial ROS production *in vitro* was observed when cells progressed from the exponential to the stationary growth phase (Fig. 5). This could be a consequence of the changes in the capacity of the cytochrome pathway-dependent respiration and in the relative amount of membranous Q (Fig. 2). Indeed, the highest basal ROS production was observed in exponential phase cultures, where the Q reduction level could be the highest and that could lead to high superoxide anion production.

In *A. castellanii* mitochondria, the two energy-dissipating systems lower ROS (Fig. 5) (Czarna and Jarmuszkiewicz, 2005) production *in vitro* and lead to the same final energetic effect i.e. a decrease in ATP synthesis (Jarmuszkiewicz et al., 1999, 2004b). Moreover, they can have a cumulative effect on both processes. It is likely that, depending on their expression level AcAOX and AcUCP, in a given phase of batch culture of amoeba, may be engaged to a different extent in the maintenance of cell energy or in the limitation of mitochondrial ROS production. At each phase of growth, activation of the two energy-dissipating systems led to a decrease in mitochondrial H_2O_2 formation *in vitro*. Surprisingly, it became apparent that, compared to AcAOX, it is AcUCP that is more efficient *in vitro* in decreasing H_2O_2 formation. Moreover, the effect of LA on H_2O_2 production paralleled the decrease of LA-stimulated respiration (+BHAM) that rein-

forces the connection between AcUCP capacity and H_2O_2 prevention.

It is well known that AOX decreases superoxide anion production at the level of complex III by decreasing the reduced state of ubiquinone and thereby the half life of the bound semiquinone on complex III, which is the source of electrons, to produce a superoxide anion from O_2. In the present study (Fig. 5), the AcAOX effect on H_2O_2 production was measured during uninhibited cytochrome pathway-sustained respiration (no cyanide), when the engagement (true contribution of AcAOX in full respiration) could be low as the Q reduced state is low (compared to conditions with cyanide, Fig. 3). Moreover, it has been shown previously that activation of AcAOX by GMP leads to only a slight effect on the Q redox state (Jarmuszkiewicz et al., 2005a). This explains why during amoeba culture duration, there is no correlation between the decrease in H_2O_2 production caused by AcAOX activation (∼10% throughout culture growth) (Fig. 5) and the significant decrease in the amount of AcAOX and its capacity (Fig. 3). On the contrary, a higher efficiency of AcUCP activation on H_2O_2 production decrease (compared to AcAOX effect) could be explained by a considerable decrease of Q redox state by linoleic acid during AcUCP activation in state 3 (Jarmuszkiewicz et al., 2005a) and in state 4 (unpublished data). This also explains a correlation between the lowering effect on decreasing H_2O_2 production caused by AcUCP activation (Fig. 5) and the decrease in the amount of AcUCP and its activity (Fig. 4) throughout amoeba culture growth.

The complex interplay between the AcAOX and cytochrome pathway capacities, Q content, FFA composition, AcUCP capacity and activation/inhibition, could lead, *in vivo*, to a rather constant level of superoxide anion production that is scavenged by a constant level of SOD throughout the cell culture duration. This stable situation can be mimicked *in vitro* when AcAOX and AcUCP are simultaneously maximally stimulated (Fig. 5).

What is described in this work is how *A. castellanii* cells can adapt their energy metabolism during the course of batch culture, leading finally to encystment. In order to succeed, the cell in such a batch culture must progressively adapt to a decrease in nutriments, increase in metabolic waste products, and, with a cell density increase, to a decrease in oxygen tension in the medium.

The first consequence of this adaptation is a progressive decrease in division rate leading to a stationary phase (Fig. 1). Nevertheless the cell quality must remain the same in order to produce cysts of high quality. Thus the metabolic adaptations are: (i) a decrease in phosphorylation respiration but with a safeguard of phosphorylation yield (ADP/O) (Fig. 2); (ii) a decrease in energy-dissipating system capacity in order to save oxygen (Figs. 3 and 4); (iii) a maintenance of MnSOD activity to protect against ROS (Fig. 6); (iv) a permanent ability to prevent ROS production by adapting the amount of AcAOX and AcUCP proteins.

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