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We dedicate this work to the memory of Professor Jacek Augustyniak

Effect of growth at low temperature on the alternative pathway respiration in *Acanthamoeba castellanii* mitochondria✪

Wiesława Jarmuszkiewicz✉, Olaf Frączyk and Lilla Hryniewiecka

Department of Bioenergetics, Institute of Molecular Biology and Biotechnology, Adam Mickiewicz University, Poznań, Poland

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Mitochondria of amoeba *Acanthamoeba castellanii* in addition to the conventional cytochrome pathway possess, like plant mitochondria, a cyanide-resistant alternative quinol oxidase. In mitochondria isolated from amoeba batch culture grown temporarily at low temperature (6°C), higher respiration was accompanied by lower coupling parameters as compared to control culture (grown at 28°C). In the presence of benzohydroxamate, respiratory rates and coupling parameters were similar in both types of mitochondria indicating that growth in cold conditions did not disturb the cytochrome pathway. Increased contribution of alternative oxidase in total mitochondrial respiration in low-temperature-grown amoeba cells was confirmed by calculation of its contribution using ADP/O measurements. Furthermore, in mitochondria from low-temperature- grown cells the content of the alternative oxidase was increased and correlated with the increase in the unstimulated and GMP-stimulated cyanide-resistant respiratory activity. A possible physiological role of higher activity of alternative oxidase as response to growth at a low temperature in unicellular organisms, such as amoeba, is discussed.

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✉To whom correspondence should be addressed: Wiesława Jarmuszkiewicz, Department of Bioenergetics, Institute of Molecular Biology and Biotechnology, Adam Mickiewicz University, A. Fredry 10, 61-701 Poznań, Poland; phone: (48 61) 829 4531; fax: (48 61) 829 4503; e-mail: wiesiaj@main.amu.edu.pl

Abbreviations: AOX, alternative oxidase; BHAM, benzohydroxamate; LTG cultures, low-temperature-grown cultures; state 3, phosphorylating state in the presence of ADP; state 4, respiration in the absence of ADP; RC, respiratory control ratio; $\Delta\Psi$, mitochondrial transmembrane electrical potential.

The mitochondrial respiratory chain of the amoeba *Acanthamoeba castellanii*, like that of plant mitochondria, possesses both cyanide- and antimycin-resistant alternative oxidase (AOX) and the conventional cytochrome *c* oxidase [1, 2]. Furthermore, *A. castellanii*, a non-photosynthetic amoeboid protozoon, and higher plants share other common features at the level of respiratory chain of the inner mitochondrial membrane, like the presence of the non-phosphorylating rotenone-insensitive internal (matricial face) and external (cytosolic face) NADH dehydrogenases [1, 3].

As in higher plant mitochondria, the alternative pathway of amoeba mitochondria branches from the main respiratory chain at the level of ubiquinone, and electron flux through alternative oxidase is not coupled to ADP phosphorylation. While in plant mitochondria the activity of AOX is stimulated by α-keto acids and regulated by the redox state of intermolecular disulfide bond (the reduced state is more active) [4–7], these regulations do not concern AOX in amoeba mitochondria [8]. The amoeba cyanide-resistant AOX is strongly stimulated by purine nucleoside 5′-monophosphates AMP, GMP (the lowest K_m of stimulation) and IMP [1, 9]. A similar effect of purine mononucleotides on the cyanide-resistant alternative pathway was observed in other microorganisms: *Euglena gracilis* [10], *Monilella tomentosa* [11], *Neurospora crassa* [12], *Paramecium tetraurelia* [13], and *Hansenula anomala* [14]. Despite important differences between plant and amoeba AOX at the level of regulation, monoclonal antibodies developed against *Suaromatum guttatum* cross-react with the AOX protein of *A. castellanii* mitochondria [8], as they do with proteins of wide range of thermogenic and non-thermogenic plant species, some fungi, and trypanosomes [4–7, 15]. This indicates that the AOX protein is well conserved throughout various species.

The only obvious physiological function of the cyanide-resistant alternative pathway has been recognized in specialized plant thermogenic tissues (spadices of *Araceae*) to consist in heat generation related to reproductive processes [16]. In non-thermogenic plant tissues and unicellular organisms, the physiological role of this energy-dissipating pathway remains unclear. AOX could play a central role in the maintaining of cell energy metabolism balance related to regulation of ATP production, control of the NADH/NAD^+ ratio, and limitation of the production of mitochondrial reactive oxygen species [6, 7, 17–22].

Mitochondrial respiration rates are affected by numerous factors, including growth temperature. Most, if not all, studies on the effect of growth temperature on the mitochondrial respiration, especially on the activity of the alternative respiratory pathway, concerned higher plants. The increased rates of respiration observed in plants grown at low temperature were attributed to a greater participation of the alternative pathway [17]. Growth of plants at low temperatures often results in higher cyanide-resistant respiratory activity [23] which could be correlated with increased synthesis of AOX protein [24, 25] or *aox* gene mRNA [26]. Increased activity of the alternative pathway as a response to low temperature may prevent the formation of toxic active oxygen species that can originate from ubiquinone overreduction accompanying inhibition of the cytochrome pathway [17, 19]. However, this adaptation to temperature is not a general feature of plants [27, 28].

The aim of this study was to determine the effect of low temperature of growth on the amoeba mitochondrial respiration and AOX activity and protein content.

MATERIALS AND METHODS

Cell culture and isolation of mitochondria. Soil amoeba *A. castellanii*, strain Neff, was cultured for 72 h as described by Jarmuszkiewicz *et al.* [8]. Control amoeba batch culture was routinely grown at 28°C. From batch cultures grown at 28°C for 12 h amoeba

cells were transferred to 6°C for 24 h (low-temperature-grown cultures, LTG) and then placed again at 28°C for 12 h (Fig. 1). To isolate mitochondria, trophozoites of amoeba were from 48 h cultures when both control and LTG cells reached a similar density, i.e. $6–7 \times 10^6$ cells/ml. Mitochondria were isolated as described before [8].

Oxygen uptake and membrane potential. Oxygen uptake was measured polarographically with a Rank Brothers (Cambridge, U.K.) oxygen electrode in 3 ml of the medium (25°C) consisting of 120 mM KCl, 10 mM Tris/Cl, pH 7.4, 3 mM KH_2PO_4, 8 mM $MgCl_2$, and 0.2% bovine serum albumin (BSA), and 1–2 mg of mitochondrial protein. The measurement temperature was 25°C both for control and LTG mitochondrial preparations. The membrane potential ($\Delta\Psi$) of mitochondria was measured simultaneously with oxygen uptake using a tetraphenylphosphonium-specific electrode according to Kamo *et al.* [29]. For calculation of $\Delta\Psi$ value, the matrix volume of amoeba mitochondria was assumed as 2.0 μl/μg protein. Succinate (10 mM) in the presence of rotenone (15 μM) was used as respiratory substrate. Cyanide (1.5 mM) and benzohydroxamate (BHAM, 1.5 mM) were used as inhibitors of the cytochrome pathway and of the alternative oxidase, respectively. To fully activate alternative oxidase, 0.8 mM GMP was added to the incubation medium. Values of O_2 uptake and $\Delta\Psi$ are expressed in nmol O $\times$ min^{-1} per mg protein and mV, respectively.

Protein was estimated by the biuret method [30] with BSA fraction V as a standard.

SDS/PAGE and immunoblotting. Up to 80 μg of freshly isolated mitochondrial protein was solubilized in the sample buffer (1% (w/v) SDS, 60 mM Tris/Cl (pH 6.8), 10% glycerol, 0.004% (w/v) bromophenol blue, and 8% mercaptoethanol) and boiled for 4 min. Mitochondrial samples were subjected to SDS/PAGE (12.5% non-urea gel), followed by Western blotting [8]. Bio-Rad prestained low molecular mass markers were used. Antibodies against alternative oxidase proteins of *S. guttatum* (generously supplied by Dr. T.E. Elthon) were used at dilutions of 1:1000. Alternative oxidase bands were visualized using the Amersham ECL system.

RESULTS

Effect of low temperature on growth of *A. castellanii* in batch culture

As shown previously, in amoeba *A. castellanii*, mitochondrial respiratory activities including the cyanide-resistant respiration change with the age of amoeba batch culture revealing a maximum at the exponential phase of growth [8]. Changes in membrane fatty acid composition during growth of amoeba in batch culture were also observed [31]. Therefore, to isolate mitochondria, amoeba trophozoites were harvested 48 h after inoculation when both, control and cold-exposed, cultures reached the early stationary phase of growth ($6–7 \times 10^6$ cells/ml) (Fig. 1).

Respiratory characteristics of mitochondria isolated from amoeba cells grown at various temperatures

Respiratory rates (measured at 25°C), both in state 3 and state 4, in the absence of BHAM (an inhibitor of AOX), were significantly higher in mitochondria isolated from low-temperature-grown cells than in mitochondria from control high-temperature-grown cells (Fig. 2). The increase in respiration of mitochondria from cells grown at low temperature was accompanied by a decrease in respiratory control ratio (RC) and ADP/O values, indicating lowering of mitochondrial coupling. In the absence of BHAM, in mitochondria isolated from low-temperature-grown cells ADP/O was 0.85 ± 0.15 (S.D., n = 8, for four different mitochondrial preparations) and RC was 1.3 ± 0.2 (S.D., n = 8), while in control mitochondria ADP/O was 1.18 ± 0.10 (S.D., n = 6, for three

different mitochondrial preparations) and RC was 1.6 ± 0.2 (S.D., n = 6). In the presence of BHAM, respiratory rates and coupling parameters were similar (ADP/O = 1.40 ± 0.04, RC = 2.3 ± 0.3, S.D., n = 8 for mitochondria from low-temperature-grown cells, and ADP/O = 1.40 ± 0.03, RC = 2.4 ± 0.2, S.D., n = 6 for control mitochondria indicating that BHAM-resistant respiratory activity of the cytochrome pathway was not sensitive to the growth temperature of amoeba culture. Thus, the effect of growth temperature on amoeba mitochondrial respiration could not be attributed to an increase in the cytochrome pathway respiration. Furthermore, in the presence of BHAM, there was no difference in state 3 and state 4 membrane potential values measured in mitochondria from both control and low-temperature-grown amoeba cultures (Fig. 2).

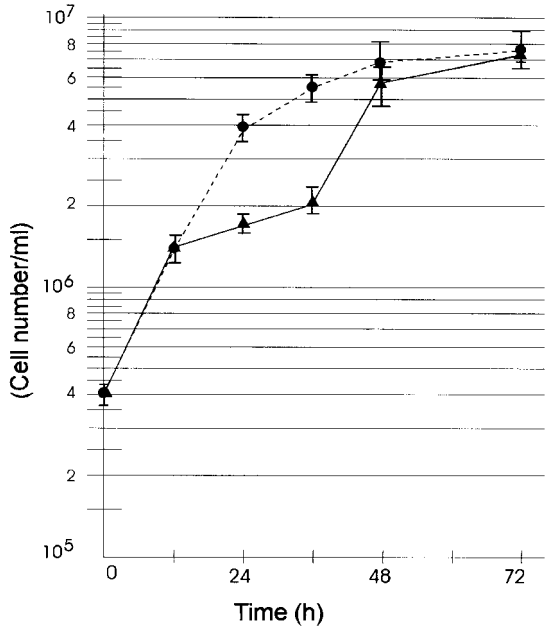

Figure 1. Growth of *A. castellanii* cells in batch culture at 28°C and during 24 h at 6°C.

Cells from 72 h old cultures were inoculated (time 0) to a final density of approximately 4×10^5 cells/ml. Control batch culture were grown at 28°C (•, dashed line). To obtain low-temperature-grown batch culture (•, solid line), 12 h after inoculation amoeba cells were transferred from 28°C to 6°C for 24 h and then placed again at 28°C. To isolate mitochondria amoeba trophozoites were harvested 48 h after inoculation. Cell numbers were determined at the specified intervals. Mean values (± S.D.) from three separate experiments are shown.

On the other hand, a higher sensitivity of respiratory rates to the AOX inhibitor (BHAM) accompanied by a more significant improvement of coupling parameters were observed in mitochondria from low-temperature-grown cells. This observation could suggest a higher contribution of AOX to total respiration in these mitochondria. The AOX and cytochrome pathway contributions in state 3 respiration can be calculated using the ADP/O measurements as described previously [32]. In the example shown in Fig. 2, taking the ADP/O ratio without BHAM (0.91), the contribution of AOX represents 35%, and the contribution of the cytochrome pathway 65% of state 3 respiration in mitochondria from low-temperature-grown cells. In mitochondria from control cells grown permanently at 28°C, in which the ADP/O value in the absence of BHAM was 1.23 (Fig. 2), the contribution of AOX and cytochrome pathways was 14% and 86%, respectively.

Thus, it can be concluded that growth of amoeba cells at low temperature led to the increased respiration through the AOX pathway. When malate or external NADH was used as a respiratory substrate instead of succinate, comparable results were obtained (not shown).

As reported previously by Avery *et al.* [33], chilling of amoeba cultures (late-exponential and stationary-phase cultures) to 15°C resulted in a change in the membrane fatty acid composition (i.e. increased polyunsaturated fatty acid synthesis). However, no change in the fatty acid composition was observed following chilling of early- and mid-exponential-phase cultures. In the present study, when AOX activity was blocked by BHAM, similar respiratory rates and ADP/O values and no difference in the value and stability of mitochondrial membrane potential (especially for state 4 respiration) were observed for control cultures and mid-exponential-phase cultures chilled to 6°C. This indicates that a possible

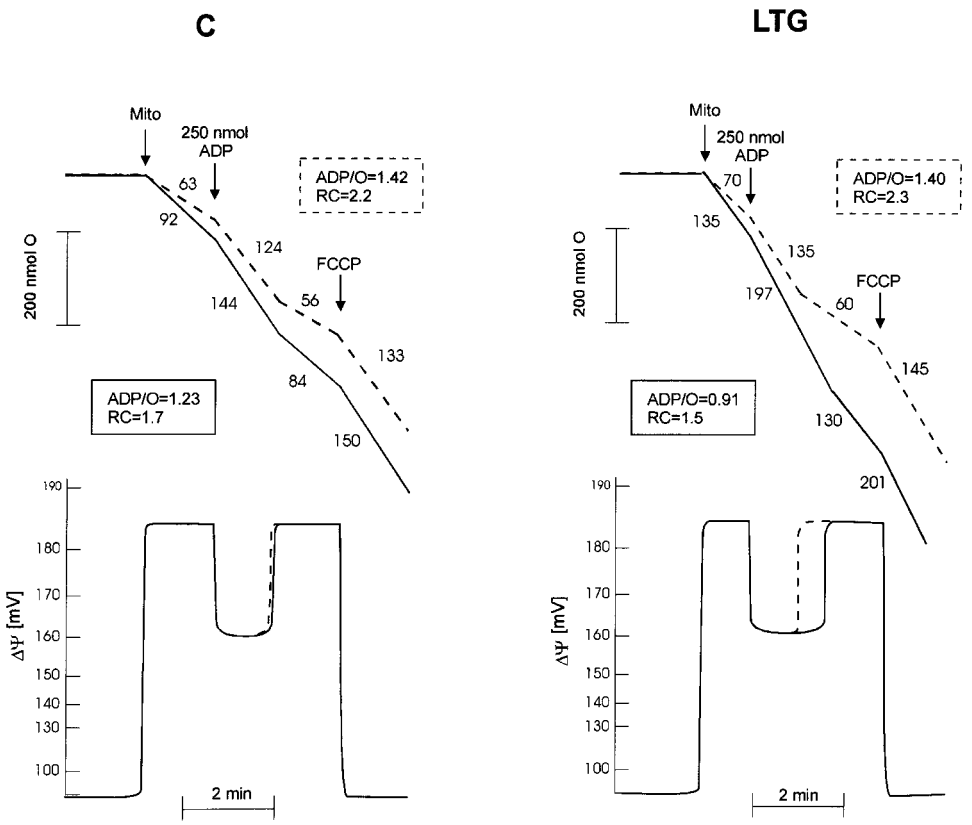

Figure 2. Effect of growth temperature on respiration and coupling parameters in amoeba mitochondria.

Examples of oxygen uptake and $\Delta\Psi$ measurements in mitochondria isolated from control (C) cultures grown at 28°C and low-temperature-grown (LTG) cultures. Mitochondria (mito) were incubated in the presence of 10 mM succinate, 0.1 mM ATP, 15 μM rotenone, and in the absence (solid lines) or presence (dashed lines) of 1.5 mM BHAM. Additions (where indicated): 250 nmoles ADP, 1 μM carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP). Numbers at the traces refer to O_2 consumption rates in nmol O × min^{-1} per mg protein. Membrane potential changes are presented in mV. Values of the respiratory control ratio (RC) and ADP/O are shown.

change in fatty acid composition of the inner mitochondrial membrane caused by cold exposure did not lead to increased permeability of this membrane to protons.

Effect of growth temperature on the activity and protein level of amoeba alternative oxidase

The cyanide-resistant alternative pathway-mediated respiration was higher in mitochondria isolated from low-temperature-grown cells compared to those isolated from control cells. This observation concerns both unstimulated and GMP-stimulated AOX activity (Table 1). The degree of stimulation of cyanide-resistant mitochondrial respiration by GMP was similar for both types of cultures (around 7-fold stimulation), indicating that the increase in AOX activity observed in mitochondria from cells grown at low temperature could be related to the increase in the level of the protein. When antimycin A was used in-

Table 1. Unstimulated and GMP-stimulated cyanide-resistant respiration in amoeba mitochondria from control and low-temperature-grown.

Succinate (10 mM) oxidation was inhibited by 1.5 mM KCN in the absence or presence of 0.8 mM GMP. Values of O_2 uptake are presented in nmol O × min^{-1} per mg protein. Data are mean values ±S.D. of three determinations.

Cyanide-resistant respiration	Control	LTG
- GMP	17 ± 3	30 ± 7
+ GMP	112 ± 18	210 ± 24

stead of cyanide as an inhibitor of the cytochrome pathway respiration, similar results were obtained (not shown).

Immunodetection showed that the amount of 35 kDa AOX protein increased markedly in the mitochondria of amoeba cells grown at 6°C as compared with cells grown permanently at 28°C (Fig. 3). Thus, in cold-grown

Figure 3. Immunoblots of the alternative oxidase protein in isolated amoeba mitochondria from control (C) cultures grown at 28°C and low-temperature-grown (LTG) cultures.

Mitochondrial protein equivalent to 80 μg was loaded in both lanes. An example of three immunodetections (using mitochondria from different preparations) is shown. When the total density of 35-kDa bands was measured, a 1.7 ± 0.2 (S.D., n = 3) fold increase in the amount of AOX was observed in mitochondria from LTG cultures.

amoeba cells the level of AOX protein in mitochondria was up-regulated (on average, an 1.7-fold increase as determined by total density measurements) that could be correlated with the increase in the cyanide-resistant respiratory activity and with a greater electron partitioning to AOX (i.e. increased contribution in total state 3 respiration).

DISCUSSION

The results presented above clearly indicate that growth of amoeba *A. castellanii* cells at low temperature resulted in increased rates of respiration measured at 25°C that can be attributed to a greater activity of the cyanide-resistant pathway, not accompanied by changes in the cytochrome pathway activity. Furthermore, the amoeba cells responded to exposition to a low temperature by an increase in AOX protein synthesis which resulted in a higher AOX contribution to total respiration. The effects of chilling on mitochondrial respiration, AOX activity and on up-regulation of AOX protein observed in amoeba mitochondria are similar to those found in some plants exposed to low temperature [17, 23–25, 28].

Free-living soil amoeba *A. castellanii* are relatively tolerant to the low growth temperature [34]. However, their optimal growth temperature is around 28°C. The question arises what can be the physiological role of up-regulation of AOX protein in response to cold adaptation during the growth of amoeba cells. The cyanide-resistant alternative pathway, the free redox energy-dissipating pathway present in higher plants, some fungi and protozoa, leads to decreased ATP synthesis accompanied by heat production and thermogenesis in some specialized (thremogenic) plant tissues. Since in some plants exposed to chilling temperatures increased heat production was observed, it has been suggested that the alternative pathway could play a local thermoregulatory role [35, 36]. However, the concept of the alternative pathway as "a local thermal balance regulator" that would respond to environmental thermal pressure cannot be extended to unicellular organisms because of their microscopic size that does not allow a local increase in temperature [37]. Moreover, Breidenbach *et al.* [38] postulated that any temperature increase in plant tissue in which electron flux would be entirely shifted to the AOX pathway would be too small to serve a thermoregulatory role. Therefore, the increased rate of heat production observed after exposure of chilling-sensitive plants to low-temperature [35, 36] must be due to other metabolic factors [28, 38]. Heat production, leading to increase in temperature that results from the activity of AOX, is observed only when total respiration increases tremendously like in specialized plant thermogenic tissues. In non-thermogenic plant tissues and in unicellular organisms heat production by

energy-dissipating systems may be only a minor side event. In low-temperature-grown amoeba cells, AOX could be considered as response protein to cold exposure (translational regulation) allowing increased oxygen consumption leading to some improvement of biosynthesis and growth at low external temperature. The cost of that improvement would be paid by the free redox energy-dissipation. Amoeba AOX could have a subtle role (more pronounced during chilling) in energy metabolism control working as safety valve when the redox potential or/and phosphate potential become overloaded [37, 39]. Operation of AOX, increased during low temperature exposure, could diminish the rise in reducing power, thus providing NAD^+ to Krebs cycle and glycolysis and, consequently, carbon skeleton for biosynthesis. Furthermore, similarly as it was previously postulated for plants [28], the increased AOX activity (accompanied by increased protein level) would maintain the rate of the mitochondrial electron transport to oxygen at low temperature, compensating for the higher sensitivity of the cytochrome pathway to cold and thereby preventing production of toxic reactive oxygen species.

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