Biochimica et Biophysica Acta 1787 (2009) 264–271

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbabio

Regulation of *Acanthamoeba castellanii* alternative oxidase activity by mutual exclusion of purine nucleotides; ATP's inhibitory effect

Andrzej M. Woyda-Ploszczyca [a], Francis E. Sluse [b], Wieslawa Jarmuszkiewicz [a,*]

[a] *Laboratory of Bioenergetics, Faculty of Biology, Adam Mickiewicz University, Umultowska 89, 61-614 Poznan, Poland*
[b] *Laboratory of Bioenergetics and Molecular Physiology, Liege University, Sart Tilman, B-4000 Liege, Belgium*

ARTICLE INFO

Article history:
Received 4 November 2008
Received in revised form 7 January 2009
Accepted 23 January 2009
Available online 31 January 2009

Keywords:
Alternative oxidase
Mitochondria
Purine nucleotides
Acanthamoeba castellanii
Kinetics
Mutual exclusion

ABSTRACT

The effects of different adenine and guanine nucleotides on the cyanide-resistant respiration (i.e. alternative oxidase (AcAOX) activity) of mitochondria from the amoeba *A. castellanii* mitochondria were studied. We found that guanine nucleotides activate AcAOX to a greater degree than adenine nucleotides, and that nucleoside monophosphates were more efficient activators than nucleoside di- or triphosphates. The extent of the nucleotides' influence on AcAOX was dependent on the medium's pH and was more pronounced at pH 6.8, which is optimal for AcAOX activity. In contrast to other purine nucleosides, we demonstrate, for the first time, that ATP has an inhibitory effect on AcAOX activity. Since we also observed the inhibition by ATP in the mitochondria of another protozoon, such as *Dictyostelium discoideum*, and the yeast, *Candida maltosa*, it may be a regulatory feature common to all purine nucleotide-modulated non-plant AOXs. The physiological importance of this discovery is discussed. Kinetic data show that the binding of GMP (a positive allosteric effector) and the binding of ATP (a negative allosteric effector) to AcAOX are mutually exclusive. ATP's inhibition of the enzyme can be overcome by sufficiently high concentrations of GMP, and conversely, GMP's stimulation can be overcome by sufficiently high concentrations of ATP. However, an approximately three times lower concentration of GMP compared to ATP gives a half maximal effect on AcAOX activity. This is indicative of a higher binding affinity for the positive effector at the same or, at least overlapping, nucleotide-binding sites on AcAOX. These results suggest that AcAOX activity in *A. castellanii* mitochondria might be controlled by the relative intracellular concentrations of purine nucleotides.

1. Introduction

Acanthamoeba castellanii is a small non-photosynthesizing free-living amoeba found in soil and in marine and freshwater environments. In molecular phylogenesis, *A. castellanii* appears on a branch basal to the divergence points of plants, animals and fungi [1]. The mitochondrial respiratory chain of *A. castellanii* contains a plant-type respiratory chain with additional (in addition to four classical) electron carriers: external and internal NADH dehydrogenases and an alternative cyanide- and antimycin-resistant quinol oxidase (AcAOX) that consumes mitochondrial reducing power without energy conservation in the proton electrochemical gradient [2,3]. We have shown that AcAOX may play a role in the energetic status of the cell (by decreasing the yield of ATP synthesis) [4] and in attenuating reactive oxygen species production [5]. Moreover, the contribution of AcAOX to the preventing the generation of mitochondrial reactive oxygen species *in vivo* could lead to their constant level throughout the growth cycle of an *A. castellanii* batch culture [6]. The activity and protein content of AcAOX is clearly increased by amoeba cells growing at low temperature, indicating that the oxidase may be a cold-response protein in unicellular organisms [7].

As in higher plant mitochondria, the alternative pathway of amoeba mitochondria branches from the main respiratory chain at the level of ubiquinone, and the electron flux through AOX is not coupled to ADP phosphorylation. While the activity of AOX in plant mitochondria is stimulated by α-keto acids and regulated by the redox state of an intermolecular disulphide bond (with the reduced state being more active) [8–10], these regulatory mechanisms do not apply to AOX in amoeba mitochondria [2]. It has been shown that the amoeba cyanide-resistant AOX is stimulated by purine nucleoside 5′-monophosphates (with GMP being the most efficient) [11,12]. Purine nucleotides (PNs) have also been observed to have a similar effect on the cyanide-resistant alternative pathway in other protists and some primitive fungi [13–21]. Our previous results indicate that, in isolated *A. castellanii* mitochondria, the binding of GMP, the redox state of ubiquinone, as well as the matrix pH determine the activity of the GMP-stimulated AcAOX [4,22,23]. Moreover, AcAOX's apparent

Abbreviations: AcAOX, alternative oxidase of *A. castellanii*; AOX, alternative oxidase; BHAM, benzohydroxamate; BSA, bovine serum albumin; DQH_2, duroquinol; $N_{0.5}$, concentration of negative allosteric effector that gives a half maximal effect; PN, purine nucleotide; $P_{0.5}$, concentration of positive allosteric effector that gives a half maximal effect; $\Delta\Psi$, mitochondrial membrane potential

* Corresponding author. Tel.: +48 61 8295881; fax: +48 61 8293656.
E-mail address: wiesiaj@amu.edu.pl (W. Jarmuszkiewicz).

0005-2728/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbabio.2009.01.017

affinity for oxygen, which is much lower than that of cytochrome *c* oxidase, depends on the ubiquinone reduction level and changes with the availability of the activator (GMP) and the respiratory substrate [3].

The aim of this work was to study the ability of various purine nucleoside phosphates to regulate AcAOX activity in *A. castellanii* mitochondria. In contrast to guanosine nucleotides (GMP, GDP, GTP) and adenosine mono- and di-nucleotides (AMP, ADP), which stimulate cyanide-resistant respiration, we found that ATP inhibits AcAOX activity. The results indicate that PNs regulate AcAOX in *A. castellanii* mitochondria by means of a mutual exclusion from a common binding site.

2. Materials and methods

2.1. Cell culture and mitochondrial isolation

The avirulent Neff strain of the soil amoeba *A. castellanii* was cultured as described previously [2]. Trophozoites of amoeba were collected 48 h after inoculation, during the exponential phase (at a density of about $3.5–4.5\times10^6$ cells/ml). Mitochondria were isolated and purified on a self-generating Percoll gradient (31%) [2].

Free-living amoeboid cells of the *Dictyostelium discoideum* AX-2 strain were grown axenically at 20 °C as described previously [20]. The generation time in continuously agitated cultures was 9–10 h. Cells were harvested in the middle of the exponential phase, i.e., 36 h after inoculation (at a density of about $2–3\times10^6$ cells/ml). Mitochondria were isolated and purified on a self-generating Percoll gradient (28%) [20].

The yeast strain *Candida maltosa* EH 15 was grown at 28 °C under vigorous aeration in complete liquid medium (3% glycerol, 2% bacto-peptone and 1% bacto-yeast extract). Cells were collected in the early exponential phase (with an absorbance of 1.0–1.4 at 546 nm). The generation time in continuously agitated cultures was 1.5 h. Mitochondria were isolated according to a procedure described for *Candida parapsilosis* mitochondria isolation [24].

2.2. Assay procedures with isolated A. castellanii mitochondria

Oxygen uptake was measured polarographically using a Rank Bros. (Cambridge UK) oxygen electrode or a Hansatech oxygen electrode in 2.8 ml or 1.4 ml (respectively) of the standard reaction medium (25 °C) containing 120 mM KCl, 10 mM Tris–HCl, 5 mM MES, 3 mM KH_2PO_4, 0.8 mM $MgCl_2$, and 0.5 mM EGTA, with 1 or 0.5 mg of mitochondrial protein (to keep the concentration of 0.36 mg×ml^{-1}). The pH values of the reaction medium were adjusted according to experimental needs. Values of O_2 uptake are presented in nmol O×min^{-1}×mg^{-1} protein. In *A. castellanii* mitochondria, no correction of respiratory rates is needed, as there is no residual rate in the presence of inhibitors of both AOX and the cytochrome *c* oxidase. The membrane potential ($\Delta\Psi$) of mitochondria was measured simultaneously with oxygen uptake (in 2.8 ml of the standard medium) using a tetraphenylphosphonium-specific electrode according to Kamo et al. [25]. For calculation of $\Delta\Psi$, the matrix volume of the amoeba mitochondria was assumed to be 2.0 µl×mg^{-1} protein. $\Delta\Psi$ values are presented in mV.

Measurements of AcAOX activity were made in the presence of 1.5 mM NADH or 1 mM duroquinol (DQH_2) (as the respiratory substrates), 0.2% BSA (to prevent free fatty acid-induced activity of the uncoupling protein), 4 µM rotenone as well as 1.8 µM carboxyatractyloside and 0.5 µg×ml^{-1} oligomycin (to inhibit ATP/ADP antiporter and ATP synthase, respectively). State 3 (phosphorylating) respiration measurements were performed with 1.5 mM NADH, in the absence of carboxyatractyloside and oligomycin. The ADP/O ratio was determined by an ADP pulse method with 450 nmol of ADP (0.16 mM). The total amount of oxygen consumed during state 3 respiration was used to calculate the ratio. Measurements of $\Delta\Psi$ allowed fine control of the duration of the state 3 respiration. The cytochrome pathway was inhibited with cyanide (1.5 mM), and the NADH- or DQH_2-dependent AcAOX activity was inhibited with 1.5 mM BHAM.

The effect of PNs on cyanide-resistant AcAOX-mediated respiration sustained by complex I was measured with 7 mM malate as the respiratory substrate (plus 0.2 mM ATP to activate complex I), in the presence of 0.2% BSA, 1.8 µM carboxyatractyloside and 0.5 µg×ml^{-1} oligomycin.

Detergent-solubilised mitochondria were prepared according to the method of Elthon and McIntosh [26]. Mitochondrial membranes were solubilised with 1% cholate, 0.1% Triton X-100, 0.5% *N*,*N*-bis-(3-D-glucoamidopropyl)-deoxycholamide (BIGCHAP), 0.5% deoxycholate or 0.1% sodium dodecyl sulphate (SDS). *A. castellanii* mitochondria (0.5 mg) were added to 1 ml of the above detergent solutions which contained 1 mM EDTA, 30 mM Tes (pH 6.8), and 1 mM GMP (to stabilise AcAOX). The AcAOX activity was measured as BHAM-sensitive DQH_2-supported O_2 uptake (at 25 °C). Of the detergents evaluated, cholate, BIGCHAP, and Triton X-100 yielded active solubilised AcAOX. However, solubilisation of the AcAOX resulted in a more than 90% loss of the potential DQH_2-dependent cyanide-resistant activity measured with intact mitochondria. The presence of GMP did not aid in the stabilisation of the solubilised AcAOX, relative to an experiment performed without the nucleotide. After solubilisation, the supernatant resulting from centrifugation (100 000 *g* for 45 min) was used for AOX assays, and no AcAOX activity was detected (data not shown).

2.3. Measurements of AOX activity in isolated D. discoideum and C. maltosa mitochondria

Cyanide-resistant AOX-mediated respiration was measured in *D. discoideum* and *C. maltosa* mitochondria in 1.4 ml of reaction medium with 1 mg of mitochondrial protein, and in the presence of 1 mM DQH_2 or 1.5 mM NADH (as the respiratory substrates), 4 µM rotenone, 1.8 µM carboxyatractyloside and 0.5 µg×ml^{-1} oligomycin. In the case of *C. maltosa*, 10 mM cyanide (instead of 1.5 mM) was used in order to inhibit a cytochrome *c* oxidase of the classical respiratory chain and a putative terminal cytochrome *c* oxidase of the parallel respiratory chain described in *Candida parapsilosis* mitochondria [24]. The reaction medium (25 °C) for *D. discoideum* mitochondria contained 120 mM KCl, 20 mM Tris–HCl (pH 7.0), 3 mM KH_2PO_4, 0.8 mM $MgCl_2$, and 0.1% BSA. Reaction medium (28 °C) for *C. maltosa* mitochondria contained 125 mM sucrose, 65 mM KCl, 10 mM Tris–HCl (pH 7.0), 3 mM KH_2PO_4, 0.8 mM $MgCl_2$, and 0.1% BSA.

2.4. Chemicals

All of the PNs used (GTP, GDP, GMP, ATP, ADP, and AMP) were purchased from Sigma. DQH_2 was dissolved in dimethyl sulphoxide (DMSO) or in methanol for the experiments with isolated or solubilised mitochondria, respectively.

3. Results

3.1. The effect of purine nucleotides on the alternative oxidase activity of A. castellanii; nucleotide specificity

The influence of adenine and guanine nucleotides on cyanide-resistant respiration was measured in isolated *A. castellanii* mitochondria with external NADH as the respiratory substrate. Representative curves illustrating the measurements are presented in Fig. 1. External NADH was chosen (instead of a complex I or complex II substrate) in order to avoid limiting the capacity of the AcAOX-mediated respiration by these two complexes or their activation by ADP [23]. DQH_2, a reduced quinol, was not used as an electron donor for AcAOX for these

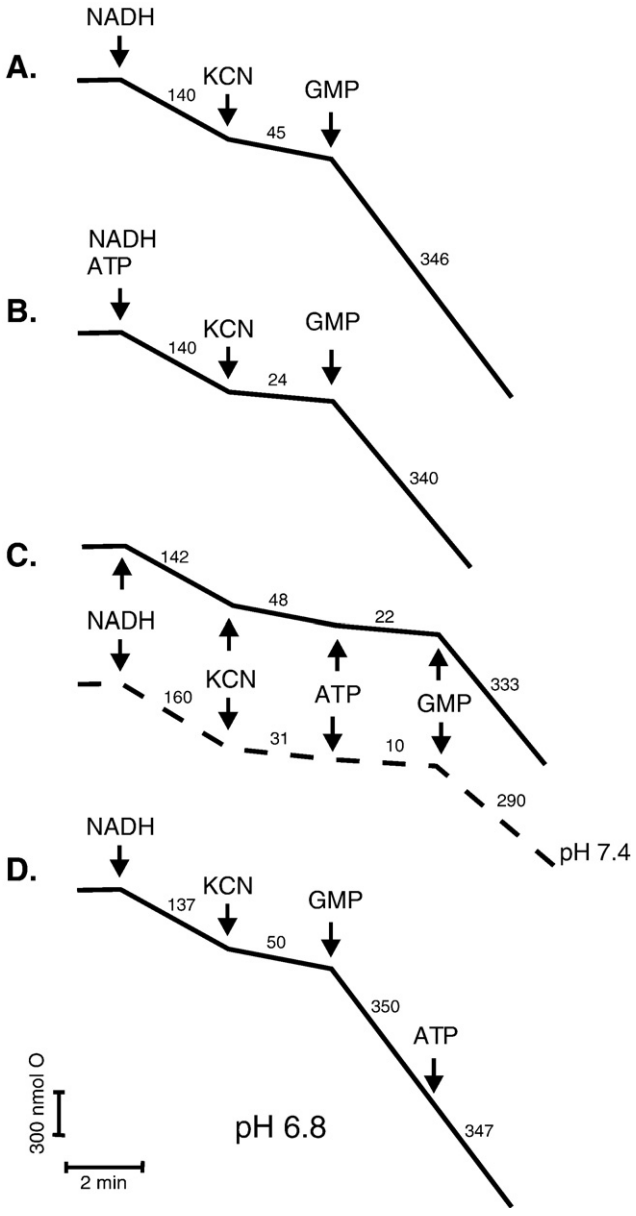

Fig. 1. The influence of PNs on cyanide-resistant respiration. Respiration measurements were performed with 1.5 mM NADH as the respiratory substrate. Measurements at pH 6.8 are shown, except one at pH 7.4 (dashed line). Additions (where indicated): 1.5 mM cyanide (KCN), 1.5 mM GMP, 0.5 mM ATP. Numbers on the traces refer to O_2 consumption rates in nmol $O \times min^{-1} \times mg^{-1}$ protein.

experiments since it led to a lower enzyme capacity (cyanide-resistant respiration in the presence of GMP) when compared to NADH. Measurements were performed at two different pH values (6.8 and 7.4) corresponding to the optimal pHs for AOX activity and cytochrome pathway activity in *A. castellanii* mitochondria, respectively [23]. The presence of oligomycin and carboxyatractyloside in the reaction medium did not alter the influence of the PNs on cyanide-resistant respiration. We ensured that the concentration of carboxyatractyloside used (1.8 μM) blocked the nucleotide transport (at the applied PN concentrations). We also checked to see if the concentrations of the inhibitor (up to 25 μM) influence the PN effect on AcAOX activity, and we found that they do not (data not shown).

Table 1 summarises the effect of different purine nucleotide phosphates on AcAOX activity in *A. castellanii* mitochondria. In general, guanine nucleotides activate AcAOX activity to a greater degree than adenine nucleotides, and nucleoside monophosphates cause more activation than nucleoside di- or triphosphates. These effects appear to

Table 1
Effect of various PNs on the cyanide-resistant respiration in *A. castellanii* mitochondria

	Effect of PN on AcAOX activity			
	pH 6.8		pH 7.4	
PN (1.5 mM)	–	+0.5 mM ATP	–	+0.5 mM ATP
GMP	×7.7±0.6	×14.6±1.1	×7.8±0.6	×28.2±2.1
GDP	×5.7±0.3	×11.0±1.0	×6.1±0.4	×21.8±1.8
GTP	×5.0±0.4	×9.5±1.0	×4.8±0.5	×18.8±1.5
AMP	×1.1±0.1	×1.9±0.7	×1.0±0	×3.5±0.2
ADP	×1.0±0	×1.9±0.6	×1.0±0	×3.6±0.3
ATP	×0.50±0.02	–	×0.29±0.1	–
Control rates	47±6	23±4	31±4	10±2

Factors of stimulation (or inhibition in the case of ATP) and control rates of cyanide-resistant respiration (in nmol $O \times min^{-1} \times mg^{-1}$ protein) are shown. Mean values±S.D. for three different mitochondria preparations are given. Assay conditions were as in Fig. 1A, C.

be independent of the assay's pH. The PNs exhibit a stimulatory effect in the following descending order: GMP>GDP>GTP>AMP>ADP. The stimulatory effects of AMP and ADP are revealed only when a lower concentration of ATP is added beforehand, indicating the presence of both endogenous AMP and ADP in the isolated mitochondria. Surprisingly, we observed that ATP had a clear inhibitory effect on the external NADH-dependent cyanide-resistant respiration (Table 1, Fig. 1). Since this effect was also observed when other respiratory substrates were used (data not shown), we deduced that ATP modulates AcAOX activity in *A. castellanii* mitochondria. As shown in Table 1, stimulation factors of cyanide-resistant respiration observed with 1.5 mM PNs (except ATP) were higher (or revealed in the case of ADP and AMP) at both tested assay pH values when AcAOX activity was first inhibited by 0.5 mM ATP. This indicates that the inhibition by ATP can be overcome. In the presence of ATP (0.5 mM), a higher degree of stimulation for a given PN was observed at a pH of 7.4 than at pH 6.8, which resulted from a lower ATP-insensitive cyanide-resistant respiration rate (AcAOX activity) (Fig. 1C).

In *A. castellanii* mitochondria, cyanide-resistant AcAOX-mediated respiration receiving electrons from coupling site I (e.g., with malate as the respiratory substrate) generated $\Delta\Psi$ up to 140 mM. As shown in Fig. 2, malate-sustained respiration was first stimulated by the addition of a low concentration of ATP (0.2 mM). In a reaction medium supplemented with carboxyatractyloside and oligomycin, $\Delta\Psi$ can be further increased by stimulation of AcAOX with either GMP or

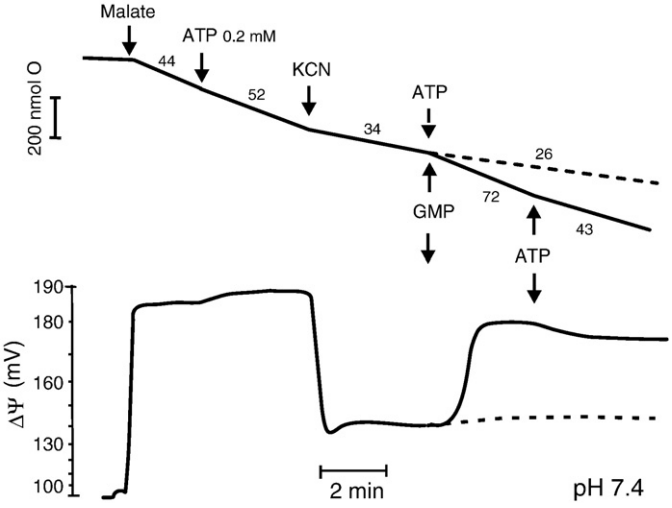

Fig. 2. The effect of PNs on cyanide-resistant AcAOX-mediated respiration sustained by complex I activity. Respiration measurements were performed with malate as the respiratory substrate. Measurements performed at pH value of reaction medium of 7.4 are shown as example. Additions (in a application order): 7 mM malate, 0.2 mM ATP, 1.5 mM cyanide (KCN), 1 mM GMP, 4.5 mM ATP. Numbers on traces refer to O_2 consumption rates in nmol $O \times min^{-1} \times mg^{-1}$ protein.

Table 2
Effect of PNs on respiration and coupling parameters in *A. castellanii* mitochondria

	pH 6.8				pH 7.4			
	State 4	State 3	ADP/O	RCR	State 4	State 3	ADP/O	RCR
GMP	218±14	410±34	0.84±0.03	1.88±0.1	238±15	520±24	1.02±0.05	2.18±0.2
GTP	161±11	404±20	1.09±0.01	2.51±0.3	188±14	517±34	1.17±0.06	2.75±0.3
ATP	135±10	411±31	1.40±0.08	3.09±0.4	156±11	522±26	1.41±0.09	3.35±0.2
GMP ATP	196±12	407±26	1.03±0.03	2.08±0.2	197±9	520±29	1.24±0.04	2.64±0.3
GMP BHAM	130±9	408±26	1.41±0.02	3.14±0.2	156±10	518±29	1.42±0.05	3.32±0.2
No additions	137±10	408±18	1.40±0.02	2.98±0.2	159±9	519±20	1.40±0.04	3.26±0.2

State 3 (phosphorylating) respiration measurements were performed with 1.5 mM NADH as the respiratory substrate in reaction medium with pH values of either 6.8 or 7.4. The concentrations used were 0.6 mM for PN and 1.5 mM for BHAM. RCR, respiratory control ratios. Assay conditions as in Fig. 4. The data are presented as the mean±S.D. for three independent experiments.

another PN (except ATP) due to the acceleration of electron flux through complex I. We observed that the extent of the increase in $\Delta\Psi$ (up to around 180 mV with GMP) was dependent on PNs and confirmed the order of efficiency for AcAOX stimulation by PNs (data not shown) established for external NADH-sustained cyanide-resistant respiration (GMP>GDP>GTP>AMP>ADP) (Table 1). As expected, the addition of ATP to mitochondria respiring with malate in the presence of cyanide (plus or minus GMP) resulted in a decrease in AcAOX-mediated respiration that led to a decrease in $\Delta\Psi$ (for GMP-stimulated respiration) depending on the PN concentrations applied (Fig. 2). These results indicate a competition-like mechanism for the regulation of AcAOX activity by different PNs, namely, activation by GMP>GDP>GTP>AMP>ADP (in descending order of stimulatory efficiency) and inhibition by ATP. Moreover, in *A. castellanii* mitochondria, pyrimidine nucleotides have no effect on AcAOX activity (data not shown).

3.2. Effect of purine nucleotides on A. castellanii alternative oxidase activity during phosphorylating respiration

In *A. castellanii* mitochondria, AcAOX as an energy-dissipating system leads to a decrease in oxidative phosphorylation efficiency [4]. In the present work, the ADP/O ratio, the best parameter for estimating oxidative phosphorylation efficiency was measured in the presence of purine nucleotides (0.6 mM) influencing AcAOX activity, i.e., GMP or GTP (as activators) and ATP (as a potential inhibitor) at two assay pH values (6.8 and 7.4) (Tab. 2). Measurements in the presence of GDP or AMP were omitted due to their influence on oxidative phosphorylation (availability of ADP as ATP synthase substrate) through interaction with ATP/ADP antiporter or myokinase, respectively. When GMP or GTP was added to mitochondria, the ADP/O ratio decreased depending on the efficiency of the nucleotides to stimulate AcAOX-mediated respiration (Table 2). Therefore, the decrease in oxidative phosphorylation efficiency caused by the nucleotides was more pronounced at pH 6.8 that is an optimum for AcAOX activity. The effect of both nucleotides on oxidative phosphorylation was revealed by prolonged state 3 respiration, decreased respiratory control ratio (state 3 versus state 4) and ADP/O ratio. Fig. 3 shows, as an example, the effect of GMP on oxygen consumption and $\Delta\Psi$ during phosphorylating respiration. In the presence of ATP, the ADP/O ratio measured with external NADH (1.40±0.08 S.D., n=3) was the same as that measured under control conditions, i.e. in the presence of BHAM, an inhibitor of alternative oxidase. When both GMP and ATP were added to mitochondria at 0.6 mM concentration, uncoupling of oxidative phosphorylation by GMP was less pronounced compared to conditions when GMP was applied alone. This suggests that, as noted above, an effect of GMP and ATP on AcAOX activity could depend on their concentrations. Moreover, results obtained with phosphorylating *A. castellanii* mitochondria confirm those from non-phosphorylating conditions and indicate that ATP is a physiological inhibitor of protist AOX.

3.3. Regulation of AcAOX activity by the mutual exclusion of ATP (a negative allosteric effector) and GMP (a positive allosteric effector)

The results presented above indicate that the regulation of AcAOX activity by PNs could be based on their mutually exclusive binding to the enzyme. In order to elucidate the opposing effects of the different PNs on AcAOX activity, namely, activation by the guanine and adenine nucleotides (except ATP) and inhibition by ATP, we determined the apparent concentrations of the positive allosteric effector GMP (an apparent $P_{0.5}$) and the negative allosteric effector ATP (an apparent $N_{0.5}$) that gave a half maximal effect on cyanide-resistant respiration by varying the concentrations of these nucleotides (Figs. 4 and 5). Fig. 4 shows GMP's concentration-dependent stimulatory effect on cyanide-resistant AcAOX-mediated respiration in reaction media at pH 6.8 or 7.4, in the absence or presence (at pH 6.8) of two different concentrations of ATP. As calculated from the linear regression (Fig. 4B), the concentration of GMP in the absence of ATP that causes a half maximal effect on respiration ($P_{0.5}$ for GMP) at pH 6.8 was half that needed at pH 7.4 (0.11 and 0.21 mM, respectively), but the maximal rate of the GMP-stimulated respiration was similar at both

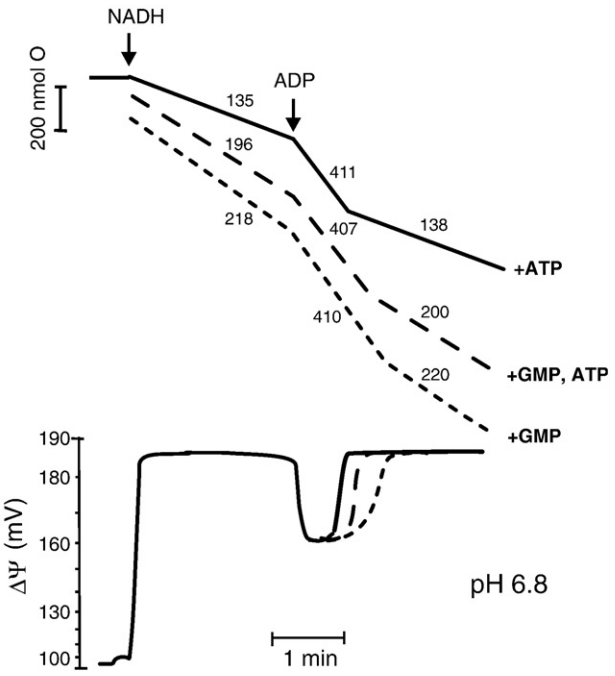

Fig. 3. The effect of PNs on coupling parameters. State 3 (phosphorylating) respiration measurements were performed with 1.5 mM NADH as the respiratory substrate with 450 nmol of ADP as a pulse. Measurements performed at pH value of reaction medium of 6.8 are shown as example. The concentrations used were 0.6 mM for all PNs. Example measurements of oxygen uptake and membrane potential in the presence of ATP (solid line), GMP and ATP (dashed line) or GMP (dotted line) are shown.

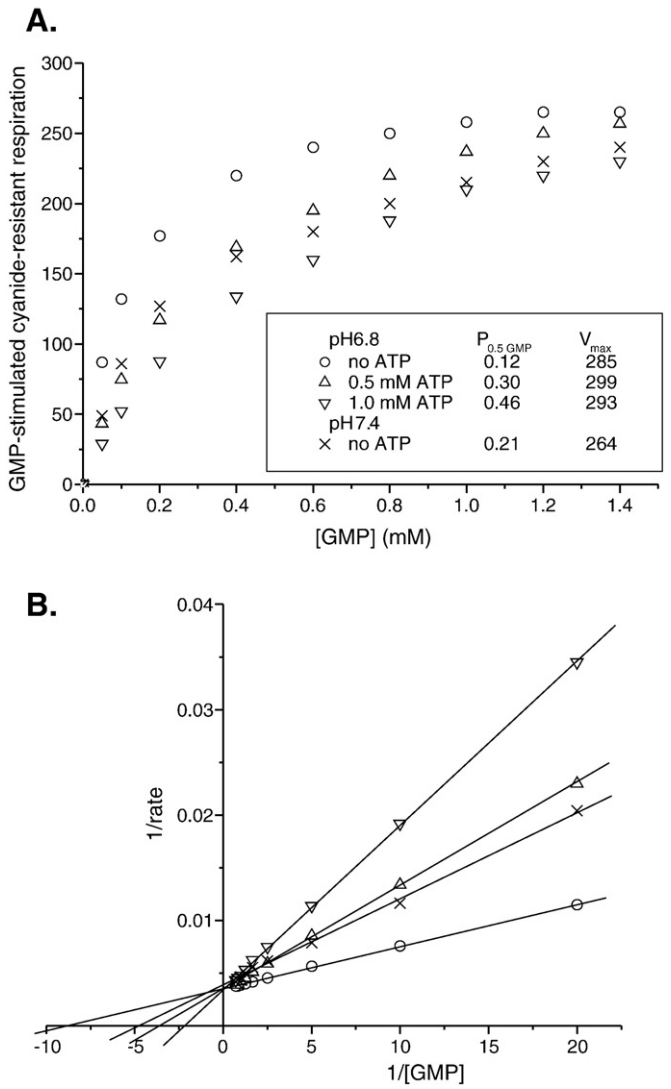

Fig. 4. (A) The stimulatory effect of GMP concentration on cyanide-resistant AcAOX-mediated respiration in reaction media with pH 6.8 or 7.4, and in the absence or presence (pH 6.8) of two different concentrations of ATP (0.5 and 1 mM). The effect of GMP is presented as the difference between the cyanide-resistant respiration in the presence of GMP and the cyanide-resistant respiration in the absence of the nucleotide. Assay conditions were as in Fig. 1A and B. Increasing concentrations of GMP were obtained by successive additions of GMP when a steady-state respiration rate had been achieved. Several oxygen traces were needed to cover the entire range of GMP concentrations investigated. (B) The double reciprocal plot of GMP-stimulated respiration versus GMP concentration. Values of the concentration required for 50% of the maximum stimulation by a positive allosteric effector ($P_{0.5\ GMP}$) and of the rates of maximal GMP-stimulated respiration (V_{max}), calculated from the linear regression, are shown. Linear regressions ($y=A+BX$) revealed: for pH 6.8 with no ATP, $A=3.51\cdot10^{-3}\pm2.79\cdot10^{-5}$, $B=4.02\cdot10^{-4}\pm3.6\cdot10^{-6}$, $r=0.999$, $n=9$; for pH 6.8 with 0.5 mM ATP, $A=3.34\cdot10^{-3}\pm7.24\cdot10^{-5}$, $B=9.89\cdot10^{-4}\pm9.37\cdot10^{-6}$, $r=0.999$, $n=9$; for pH 6.8 with 1 mM ATP, $A=3.41\cdot10^{-3}\pm8.34\cdot10^{-5}$, $B=1.56\cdot10^{-3}\pm1.08\cdot10^{-5}$, $r=0.999$, $n=9$; for pH 7.4 with no ATP, $A=3.84\cdot10^{-3}\pm1.15\cdot10^{-4}$, $B=8.19\cdot10^{-4}\pm1.49\cdot10^{-5}$, $r=0.998$, $n=9$.

pH values (285 and 264 nmol $O\times min^{-1}\times mg^{-1}$). These results clearly indicate that the affinity of GMP for the nucleotide-binding site of AcAOX depends on pH. At pH 6.8, in the presence of two different concentrations of ATP (0.5 mM and 1 mM), the slope of the double-reciprocal plot was increased and the apparent $P_{0.5}$ values for GMP were higher (0.30 and 0.46 mM, respectively), while the enzyme had a maximal rate similar to the experiment in the absence of ATP (Fig. 4B). This regulatory effect, which was also observed at pH 7.4 (data not shown), clearly indicates the mutual exclusion of GMP and ATP from the same AcAOX PN-binding site as positive and negative allosteric effectors, respectively.

Fig. 5 shows the concentration-dependent inhibitory effect of ATP on cyanide-resistant AcAOX-mediated respiration in a reaction medium at pH 6.8 and 7.4, and in the absence or presence (pH 6.8) of two different concentrations of GMP. As calculated from the linear regression (Fig. 5B), the concentration of ATP needed to cause half the maximal effect on respiration ($N_{0.5}$ for ATP) in the absence of GMP was lower at pH 6.8 than at pH 7.4 (0.33 and 0.47 mM, respectively), while the maximal rate of the ATP-inhibited respiration was similar at both pH values (115 and 111 nmol $O\times min^{-1}\times mg^{-1}$, respectively). These

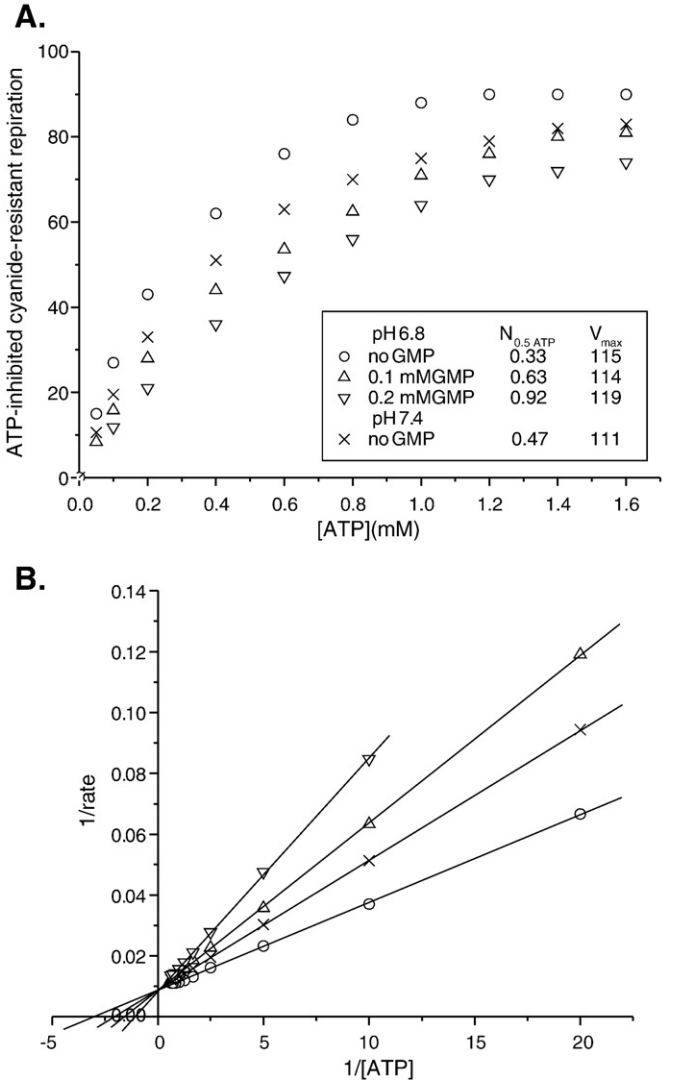

Fig. 5. (A) The inhibitory effect of ATP concentration on cyanide-resistant AcAOX-mediated respiration in reaction media of pH 6.8 or 7.4, and in the absence or presence (pH 6.8) of two different concentrations of GMP. The effect of ATP is presented as the difference between the cyanide-resistant respiration before ATP addition and the cyanide-resistant respiration in the presence of the nucleotide. Assay conditions were as in Fig. 1C, D. The rates of cyanide-resistant respiration were 128 and 93 nmol $O\times min^{-1}\times mg^{-1}$ protein, at pH 6.8 and 7.4, respectively. Increasing concentrations of ATP were obtained by successive additions of ATP when a steady-state respiration rate had been achieved. Several oxygen traces were needed to cover the entire range of ATP concentrations investigated. (B) The double reciprocal plot of ATP-inhibited respiration versus ATP concentration. Values of the concentration required for 50% of the maximum inhibition by the negative allosteric effector ($N_{0.5\ ATP}$) and of the rates of maximal ATP-inhibited respiration (V_{max}), calculated from the linear regression, are shown. Linear regressions ($y=A+BX$) revealed: for pH 6.8 with no GMP, $A=8.71\cdot10^{-3}\pm1.53\cdot10^{-4}$, $B=2.88\cdot10^{-3}\pm2.09\cdot10^{-5}$, $r=0.999$, $n=10$; for pH 6.8 with 0.1 mM GMP, $A=8.79\cdot10^{-3}\pm1.66\cdot10^{-4}$, $B=5.49\cdot10^{-3}\pm2.26\cdot10^{-5}$, $r=0.999$, $n=10$; for pH 6.8 with 0.2 mM GMP, $A=8.33\cdot10^{-3}\pm2.38\cdot10^{-4}$, $B=7.68\cdot10^{-3}\pm5.73\cdot10^{-5}$, $r=0.999$, $n=8$; for pH 7.4 with no GMP, $A=9.04\cdot10^{-3}\pm8.26\cdot10^{-5}$, $B=4.25\cdot10^{-3}\pm1.12\cdot10^{-5}$, $r=0.999$, $n=10$.

Table 3
The effect of GMP and ATP on the activity of solubilised AcAOX

Additions	Activity of AcAOX solubilised in the presence of GMP	Activity of AcAOX solubilised in the absence of GMP
No PNs	3.2±0.3	2.0±0.3
+ATP 4 mM	1.9±0.1	1.9±0.2
+GMP 2 mM	2.9±0.3	3.0±0.3

The rate of DQH_2-dependent cyanide-insensitive respiration (in nmol $O \times min^{-1} \times mg^{-1}$) of AcAOX solubilised by 0.1% Triton X-100 (±1 mM GMP) was measured with 1 mM DQH_2 as the respiratory substrate, in the presence of cyanide (1.5 mM) and with 0.5 mg of solubilised mitochondria. The data are presented as the mean±S.D. from three experiments.

results clearly indicate that the affinity of ATP for the nucleotide-binding site of AcAOX depends on pH. At pH 6.8, in the presence of two different concentrations of GMP (0.1 mM and 0.2 mM), the slope of the double-reciprocal plot increased, and the apparent $N_{0.5}$ values for ATP were higher (Fig. 5B). When the concentration of GMP was increased, the apparent value of $N_{0.5}$ increased (from 0.33 mM to 0.63 and 0.92 mM, for 0.1 mM and 0.2 mM GMP, respectively), but the maximal rate did not change. This same feature was also observed at pH 7.4 (data not shown), indicating the mutual exclusion of GMP and ATP at AcAOX's binding site.

The results presented in Figs. 4 and 5 clearly indicate that GMP and ATP occupy the same, or at least overlapping, binding sites on the enzyme and thus their binding is mutually exclusive. Therefore, inhibition by ATP can be overcome by sufficiently high concentrations of GMP and, conversely, stimulation by GMP can be overcome by sufficiently high concentrations of ATP. Moreover, the $N_{0.5}$ value for ATP calculated from the plot of the reciprocal of the $N_{0.5\ ATP}$ values (obtained at concentrations of 0, 0.1 and 0.2 mM GMP) against GMP concentration (not shown) is 0.34 mM. This result is similar to the $N_{0.5}$ value for ATP calculated in the absence of the positive effector (0.33 mM) (Fig. 5B). Similarly, the $P_{0.5}$ value for GMP calculated from the graph of the reciprocal of the $P_{0.5\ GMP}$ values (obtained at concentrations 0, 0.5 and 1 mM ATP) against ATP concentration is 0.12 mM (not shown), which is the same as the $P_{0.5}$ value for GMP calculated in the absence of the negative effector (Fig. 4B). This confirms that much lower concentrations (around one-third the concentration) of GMP than of ATP give a half-maximal effect on AcAOX activity, indicating a higher binding affinity for the positive allosteric effector at the same nucleotide binding site on AcAOX.

In order to ensure that the effect of the PNs during the cyanide-resistant respiration of isolated *A. castellanii* mitochondria could be attributed to AcAOX and not to an NADH- (or other substrate)-Q-complex, the effect of GMP and ATP on mitochondria oxidising an artificial respiratory substrate, DQH_2 (1 mM), was studied. Compared to the NADH-supported AcAOX activity, the DQH_2-dependent AcAOX activity (mainly GMP-stimulated) was lower, but the inhibitory effect of ATP and the mutual exclusion of ATP and GMP on the enzyme's binding site were still observed (Table 4).

3.4. A. castellanii alternative oxidase activity in solubilised mitochondria

In order to study the effect of PNs on AcAOX activity in solubilised mitochondria, different detergents were used. For example, Table 3 shows rates of DQH_2-dependent cyanide-resistant activity measured with AcAOX solubilised in 0.1% Triton X-100. Independent of the detergent used, we found that the solubilisation of the AcAOX resulted in an enormous (more than 90%) loss of the DQH_2-dependent cyanide-resistant activity measured relative to intact mitochondria. Moreover, after solubilisation, the binding of the allosteric modulators (GMP and ATP) to AcAOX is much weakened. The presence of added GMP during solubilisation did not lead to the stabilisation of the solubilised AcAOX. Unlike plant AOXs which are regulated by covalent modifications (thiohemiacetal and disulphide bond formations), the investigation of non-plant allosterically regulated enzymes seems to be more difficult after solubilisation, since their activity is greatly reduced. In the only published attempt to study solubilised non-plant AOX (of *Moniliella tomentosa* mitochondria) to date, AOX-mediated quinol (menadiol) oxidation was not affected by the addition of AMP, which was contrary to the results with intact mitochondria [27]. Moreover, the activity of the solubilised *M. tomentosa* AOX was inhibited by different detergents, at least at certain detergent:protein ratios [27]. Our results show that solubilised AcAOX was only weakly sensitive to PNs (Table 3) as compared to the sensitivity observed with intact mitochondria (Table 4). Therefore, stimulation by GMP and inhibition by ATP, although observable, were very difficult to investigate under these conditions, likely due to the allosteric nature of the enzyme. Nonetheless, the results obtained indicate a direct interaction of PNs with AcAOX and confirm their opposite effects on the enzyme's activity.

3.5. The inhibition of alternative oxidase activity by ATP in D. discoideum and C. maltosa mitochondria

To establish the possible universality of the inhibition observed with ATP, in what could be a feature common to all non-plant PN-regulated AOXs, we tested the ATP sensitivity of the DQH_2-dependent (unstimulated and GMP-stimulated) cyanide-resistant respiration in mitochondria isolated from two other sources. Mitochondria from *D. discoideum*, a protist that exhibits an unusual life cycle characterised by a unicellular stage and a facultative multicellular stage, and mitochondria from an asporogenic yeast *C. maltosa* were studied. Using DQH_2 and NADH as respiratory substrates, a BHAM-insensitive cytochrome pathway-mediated respiration was evaluated (data not shown). The coupling parameters (ADP/O and respiratory control ratio) of *D. discoideum* and *C. maltosa* mitochondria were comparable to those of *A. castellanii* mitochondria (Table 2). With regard to cyanide-insensitive AOX-mediated respiration, we found that like in *A. castellanii* mitochondria, in another protozoan *D. discoideum* mitochondria and fungal *C. maltosa* mitochondria, GMP stimulates DQH_2-dependent AOX activity, while ATP inhibits unstimulated and GMP-stimulated DQH_2-dependent AOX activity (Table 4). Similar effects were observed with mitochondria oxidising external NADH

Table 4
The inhibitory effect of ATP on DQH_2-dependent cyanide-insensitive respiration in isolated *A. castellanii*, *D. discoideum* and *C. maltosa* mitochondria

Type of mitochondria	Rate of DQH_2-supported cyanide-resistant respiration (nmol $O \times min^{-1} \times mg^{-1}$ protein)					
	No PNs	+GMP 1.5 mM	+ATP 1.5 mM	+GMP 1.5 mM +ATP 0.5 mM	+GMP 1.5 mM +ATP 1.5 mM	+GMP 1.5 mM +ATP 4 mM
A. castellanii	64±8	167±15	16±2	119±10	88±7	20±2
D. discoideum	12±1	29±2	9±1	24±2	19±2	8±1
C. maltosa	83±9	109±9	30±3	97±9	82±7	34±3

Respiration was measured with 1 mM DQH_2 as the respiratory substrate, in the presence of cyanide (1.5 mM for *A. castellanii* and *D. discoideum* mitochondria or 10 mM for *C. maltosa* mitochondria), and 1 mg of mitochondrial protein. In all three types of mitochondria, DQH_2-dependent cyanide-insensitive respiration was fully inhibited by 1.5 mM BHAM. The pH values of the reaction media were 6.8 (*A. castellanii* mitochondria) and 7.0 (*D. discoideum* and *C. maltosa* mitochondria). The data are presented as the mean±S.D. from three experiments.

(data not shown). Table 4 reveals a clear competition between ATP and GMP, which denotes the mutual exclusion of these two molecules from the enzyme's binding site(s).

4. Discussion

The results presented above show the effect of different PNs on cyanide-resistant respiration (AcAOX activity) in *A. castellanii* mitochondria. We found that guanine nucleotides activate AcAOX to a greater degree than adenine nucleotides, and that nucleoside monophosphates are more efficient activators than nucleoside di- or triphosphates. The strength of the PN's influence on AcAOX is dependent on the pH of the medium, and it is more pronounced at pH 6.8, which is optimal for AcAOX activity, than at pH 7.4, which is optimal for the cytochrome pathway [23]. Moreover, we have shown for the first time that ATP has an opposite, inhibitory effect on AOX activity. It has been previously demonstrated that cyanide-resistant AOX activity in amoeba mitochondria is stimulated by purine nucleoside 5′-monophosphates [11,12]. This study widens the list of AcAOX activators to include adenine and guanine nucleoside diphosphates and GTP, but ATP has been demonstrated to be an inhibitor. The stimulatory effect of PNs (mainly purine mononucleotides) on the cyanide-resistant alternative pathway was also observed in other protists and some primitive fungi [13–21]. However, neither the inhibitory effect of ATP on AOX nor any stimulatory effects have been found thus far in mitochondria of *Neurospora crassa*, *Paramecium tetraurelia*, *Hansenula anomala*, *Moniliella tomentosa* or *Yarrowia lipolytica* [15–17,27,28]. In our opinion, based on the results presented in this work, in order to observe the inhibition of cyanide-resistant AOX-mediated respiration by ATP in microorganism mitochondria, it is crucial (i) to use a respiratory substrate that does not react with ATP-stimulated dehydrogenase (e.g., external NADH dehydrogenase), (ii) to supplement the reaction medium with oligomycin- and carboxyatractyloside in order to inhibit the action of ATP synthase (synthesis or hydrolysis), and (iii) to use a sufficiently high ATP concentration so that it overcomes any possible stimulation from the endogenous levels of other PNs. Thus, we propose that AOX is regulated by the energetic status of the cell, specially by the relative concentrations of ATP and the guanine nucleotides by means of mutually exclusive binding to AOX, which could occur in all non-plant AOXs (i.e., fungal and protozoan oxidases that are regulated by PNs). This proposal is strongly supported by the results obtained with the mitochondria from the amoeboid *D. discoideum* and the yeast *C. maltosa* that reveal a similar PN regulation of AOX, which we characterised more precisely in *A. castellanii* mitochondria.

Although the PN (mainly monophosphate)-dependent stimulation of AOX (the cyanide-insensitive respiration) is a well-established phenomenon in mitochondria from an array of eukaryotic microorganisms, its mechanism has remained elusive. Therefore, our finding that ATP has an inhibitory effect on AcAOX activity, in contrast to the stimulatory effects of other adenine nucleotides and guanine nucleotides, could be very important in elucidating the regulatory mechanism as well as physiological role of non-plant AOXs (i.e., the fungal and protozoan oxidases that are regulated by PNs). Specifically, the mutual exclusion of a negative allosteric effector (ATP) and the positive allosteric effectors (GMP>GDP>GTP>AMP>ADP, in descending order of stimulatory efficiency) at the AcAOX binding site is likely related to a respiratory control (through the ratio of the relative concentrations of ATP versus other nucleotides) and can considerably influence the yield of oxidative phosphorylation (Table 2, Fig. 3). This regulation could account for the commonly observed (also in microorganism mitochondria) enhancement of AOX-mediated respiration under conditions of stress when the cytochrome pathway is impaired (and ATP production is limited). However, under physiological conditions such that ATP production is not impaired in *A. castellanii* mitochondria, changes in the ATP concentration relative to the concentrations of the of other PNs (mainly guanine nucleotides) could allow the rebalancing of the AOX and cytochrome pathway activities depending on the energy and metabolic status of the cell. AcAOX-mediated non-phosphorylating respiration is likely inhibited by ATP when there is no need to limit the production of reactive oxygen species and/or when the oxidation of reduced equivalents by this energy-dissipating system is not strongly required (e.g., during the late stationary phase of growth when *A. castellanii* cell division slows down). In contrast, at low ATP concentrations, overcoming concentrations of the other PNs (mainly guanine nucleotides) would relieve the inhibition by ATP, leading to the activation of the enzyme in order to prevent the formation of reactive oxygen species by the mitochondria and/or to decrease the reducing power in the cell. Such conditions could occur in intensively dividing and metabolising cells, for example, during the exponential growth phase. Therefore, the enormous potential stimulatory effect of PNs (mainly GMP, which could potentially cause an increase of about 30-fold in capacity of AcAOX, Table 1) observed in isolated *A. castellanii* mitochondria could reflect the potential range of *in vivo* AcAOX activity, depending on the energetic and metabolic status of the cell and the relative intracellular concentrations of the PNs (mainly ATP versus guanine nucleotides). Previous studies confirm the present findings. In particular, it has been shown that during the growth of *A. castellanii* in batch culture, ATP content increases exponentially in amoeba cells [11]. This increase is accompanied by a decrease in cyanide-resistant unstimulated and GMP-stimulated AcAOX-mediated respiration as well as the amount of AcAOX protein observed in amoeba mitochondria [6]. Thus, during the exponential growth phase, the largest amount of AcAOX is present, and the enzymatic capacity is accompanied by a low concentration of ATP in the cell (approximately 20 times lower than during stationary phase). Attributing the inhibitory effect on AcAOX to ATP is important for elucidating the physiological role of the energy-dissipating systems in unicellular organisms, especially since in *A. castelanii* mitochondria, ATP also inhibits the activities of the uncoupling protein and the ATP-regulated potassium channel [29,30].

The mechanism by which PNs regulate non-plant AOX have not yet been well characterised. In our previous work, we proposed a model to explain the pH dependence of GMP's stimulation of AcAOX, which implicates a protonation/deprotonation process of the ligand (at one hydroxyl of GMP) and of protein (likely at two conserved histidines in the N-terminal domain of GMP-dependent oxidases) with an optimum pH of 6.8 [23]. In the present study, we have shown that for AcAOX, an increase in the number of phosphate groups that the ligand contains decreases its ability to activate AcAOX in both the guanine (GMP>GDP>GTP) and adenine (AMP>ADP) nucleotides. Additionally, the presence of adenine clearly diminishes the efficiency

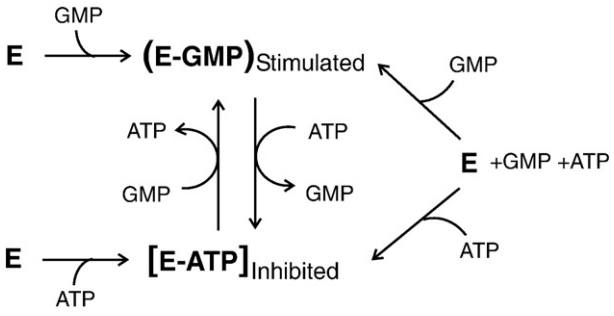

Fig. 6. A proposed model explaining the regulation of non-plant AOX activity by the mutual exclusion of ATP (a negative allosteric effector) and GMP (a positive allosteric effector) from a binding site on the enzyme. The enzyme (E) converts from a GMP-stimulated state to an ATP-inhibited state and vice versa depending on the availability (concentration) of the allosteric modulators. In the figure, GMP represents all the PNs that activate AOX. ATP's inhibition can be overcome by a sufficiently high concentration of GMP and conversely, the stimulatory effect of GMP can be overcome by a sufficiently high concentration of ATP.

of the nucleotide stimulation (adenine nucleotides activated AcAOX to a lower degree than guanine nucleotides), leading to an inhibitory effect in the case of ATP. Three hydroxyl groups are not sufficient to bring about inhibition, since GTP is a quite efficient activator, but in combination with an adenine base, they give rise to allosteric inhibition of the enzyme. Thus, it seems that in this regulatory mechanism, the type of base is primary in relation to the degree of phosphorylation of the PN. This is confirmed by the higher binding affinity of GMP as compared to ATP (around three times) for the same, or at least overlapping, nucleotide-binding sites on AcAOX (Figs. 4, 5). The domain of non-plant AOXs that interacts with PNs remains to be determined, although sequence analyses suggest some possibilities, such as a unique loop region at the N-terminus or a short C-terminal sequence, both exposed to the matrix side [18]. In mitochondria from *A. castellanii*, *D. discoideum*, *C. maltosa* (this work), *H. anomala* [17] and *P. tetraurelia* [15], and thus to our knowledge in all non-plant mitochondria in which the effect of carboxyatractyloside has been studied, the adenine nucleotide antiporter inhibitor does not alter the influence of PNs on cyanide-resistant respiration. This observation could indicate interactions of PNs with AOX from the outer surface of the mitochondrial inner membrane that are difficult to understand in light of the current model of the structure of plant and non-plant AOXs. Although no crystallographic structure is currently available, AOX is considered to be a monotopic integral membrane protein, associating with one leaflet of the bilayer exposed to the matrix [31,32]. The model leaves no clear way to understand how PNs on the outer surface of the inner membrane can interact with the enzyme. This is clearly a puzzle that needs to be examined and verified by X-ray crystallography.

In conclusion, given the large regulatory effect that PNs have on non-plant AOXs, such as protozoan and fungal AOXs, the levels of PNs (mainly, GMP on one hand and ATP on the other) found in a cell could provide a powerful regulatory mechanism for the energy-dissipating AOX-sustained pathway in unicellular organisms. This regulatory mechanism is made possible by the mutual exclusion of these nucleotides from the binding site(s) on the enzyme, as kinetically characterised for AcAOX in *A. castellanii* mitochondria and observed for AOXs in *D. discoideum* and *C. maltosa* mitochondria. Whereas plant AOX enzymes are regulated by covalent modifications (thiohemiacetal and disulphide bond formation), non-plant AOXs are allosteric enzymes regulated by allosteric modulators. Our kinetic data show that ATP (acting as negative allosteric effector) and GMP (acting as positive allosteric effector) display competitive effects. Such mutual exclusion between the two molecules for binding to an enzyme may be caused by binding to a common site or by the steric constraints of separate but close sites, which prevent the two molecules from binding simultaneously. Fig. 6 shows a proposed model explaining the allosteric regulation of non-plant AOX activity involving the mutually exclusive binding of ATP and GMP.

Acknowledgement

This work was supported by a grant from the Polish Ministry of Education and Science (3382/B/P01/2007/33).

References

[1] P.O. Wainright, G. Hinkle, M.L. Sogin, S.K. Stickel, Monophyletic origins of the metazoa: an evolutionary link with fungi, Science 260 (1993) 340–342.
[2] W. Jarmuszkiewicz, A.M. Wagner, M.J. Wagner, M.J., L. Hryniewiecka, Immunological identification of the alternative oxidase of *Acanthamoeba castellanii*, FEBS Lett. 411 (1997) 110–114.
[3] W. Jarmuszkiewicz, M. Czarna, F.E. Sluse, Substrate kinetics of the *Acanthamoeba castellanii* alternative oxidase and effects of GMP, Biochim. Biophys. Acta 1708 (2005) 71–78.
[4] W. Jarmuszkiewicz, C.M. Sluse-Goffart, L. Hryniewiecka, J. Michejda, F.E. Sluse, Electron partitioning between the two branching quinol-oxidizing pathways in *Acanthamoeba castellanii* mitochondria during steady-state state 3 respiration, J. Biol. Chem. 273 (1998) 10174–10180.
[5] M. Czarna, W. Jarmuszkiewicz, Activation of alternative oxidase and uncoupling protein lowers hydrogen peroxide formation in amoeba *Acanthamoeba castellanii* mitochondria, FEBS Lett. 579 (2005) 3136–3140.
[6] M. Czarna, F.E. Sluse, W. Jarmuszkiewicz, Mitochondria function plasticity in *Acanthamoeba castellanii* during growth in batch culture, J. Bioenerg. Biomembr. 39 (2007) 149–157.
[7] W. Jarmuszkiewicz, O. Frączyk, L. Hryniewiecka, Effect of growth at low temperature on the alternative pathway respiration in *Acanthamoeba castellanii* mitochondria, Acta Biochim. Pol. 48 (2001) 729–737.
[8] D.A. Day, J. Whelan, A.H. Millar, J.N. Siedow, J.T. Wiskich, Regulation of the alternative oxidase in plants and fungi, Aust. J. Plant. Physiol. 22 (1995) 497–509.
[9] A.L. Moore, M.S. Albury, Further insights into the structure of the alternative oxidase: from plants to parasites, Biochem. Soc. Trans. 36 (2008) 1022–1026.
[10] J.N. Siedow, A.L. Umbach, The mitochondrial cyanide-resistant oxidase: structural conservation and regulatory diversity, Biochim. Biophys. Acta 1459 (2000) 432–439.
[11] S.W. Edwards, D. Lloyd, Changes in oxygen uptake rates, enzyme activities, cytochrome amounts and adenine nucleotide pool levels during growth of *Acanthamoeba castellanii* in batch culture, J. Gen. Microbiol. 102 (1977) 135–144.
[12] L. Hryniewiecka, J. Jenek, J. Michejda, Cyanide resistance in soil amoeba *Acanthamoeba castellanii*, in: G. Ducet, C. Lance (Eds.), Plant Mitochondria, Elsevier Science Publishers B.V., Amsterdam, 1978, pp. 307–314.
[13] T.K. Sharpless, R.A. Butov, An inducible alternative terminal oxidase in *Euglena gracilis* mitochondria, J. Biol. Chem. 245 (1970) 58–70.
[14] L. Hanssens, H. Verachtert, Adenosine 5′-monophosphate stimulates cyanide-insensitive respiration in mitochondria of *Moniliella tomentosa*, J. Bacteriol. 125 (1976) 829–836.
[15] J. Doussiere, P.V. Vignais, AMP-dependence of the cyanide-insensitive pathway in the respiratory chain of *Paramecium tetraurelia*, Biochem. J. 220 (1984) 787–794.
[16] J. Vanderleyden, C. Peeters, H. Verachtert, H. Bertrandt, Stimulation of the alternative oxidase of *Neurospora crassa* by nucleoside phosphates, Biochem. J. 188 (1980) 141–144.
[17] S. Sakajo, N. Minagawa, Y. Yoshimoto, Effects of nucleotides on cyanide-resistant respiratory activity in mitochondria isolated from antimycin A-treated yeast *Hansenula anomala*, Biosci. Biotech. Biochem. 61 (1997) 397–399.
[18] A.L. Umbach, J.N. Siedow, The cyanide-resistant alternative oxidases from the fungi *Pichia stipis* and *Neurospora crassa* are monomeric and lack regulatory features of the plant enzyme, Arch. Biochem. Biophys. 378 (2000) 234–245.
[19] G. Milani, W. Jarmuszkiewicz, C.M. Sluse-Goffart, A.Z. Scheiber, A.E. Vercesi, F.E. Sluse, Respiratory chain network in mitochondria of *Candida parapsilosis*: ADP/O appraisal of the multiple electron pathways, FEBS Lett. 508 (2001) 231–235.
[20] W. Jarmuszkiewicz, M. Behrendt, R. Navet, F.E. Sluse, Uncoupling protein and alternative oxidase of *Dictyostelium discoideum*: occurrence, properties and protein expression during vegetative life and starvation-induced early development, FEBS Lett. 532 (2002) 459–464.
[21] O. Juarez, G. Guerra, I. Velazquez, O. Flores-Herrera, R.E. Rivera-Perez, J.P. Pardo, The physiological role of alternative oxidase in *Ustilago maydis*, FEBS J. 273 (2006) 4603–4615.
[22] W. Jarmuszkiewicz, F.E. Sluse, L. Hryniewiecka, C.M. Sluse-Goffart, Interactions between the cytochrome pathway and the alternative oxidase in isolated *Acanthamoeba castellanii* mitochondria, J. Bioenerg. Biomembr. 34 (2002) 31–40.
[23] W. Jarmuszkiewicz, L. Hryniewiecka, F.E. Sluse, The effect of pH on the alternative oxidase activity in isolated *Acanthamoeba castellanii* mitochondria, J. Bioenerg. Biomembr. 34 (2002) 221–225.
[24] W. Jarmuszkiewicz, G. Milani, F. Fortes, A.Z. Schreiber, F.E. Sluse, A.E. Vercesi, First evidence and characterization of an uncoupling protein in fungi kingdom: CpUCP of *Candida parapsilosis*, FEBS Lett. 467 (2000) 145–149.
[25] N. Kamo, N. Muratsugu, R. Hongoh, Y. Kobatake, Membrane potential of mitochondria measured with an electrode sensitive to tetraphenyl phosphonium and relationship between proton electrochemical and phosphorylation potential, J. Membr. Biol 49 (1979) 105–121.
[26] T.E. Elthon, L. McIntosh, Characterization and solubilization of the alternative oxidase of *Sauromatum guttatum* mitochondria, Plant Physiol. 82 (1986) 1–6.
[27] J. Vanderleyden, E. Van den Eynde, H. Verachtert, Nature of the effect of adenosine 5′-monophosphate on the cyanide-insensitive respiration in mitochondria of *Moniliella tomentosa*, Biochem. J. 186 (1980) 309–316.
[28] A.G. Medentsev, A. Yu. Arinbasarova, N.M. Smirnova, V.K. Akimenko, Activation of the alternative oxidase of *Yarrowia lipolytica* by adenosine monophosphates, Microbiology 73 (2004) 117–123.
[29] W. Jarmuszkiewicz, C.M. Sluse-Goffart, L. Hryniewiecka, F.E. Sluse, Identification and characterization of a protozoa uncoupling protein in *Acanthamoeba castellanii*, J. Biol. Chem. 274 (1999) 23198–23202.
[30] A. Kicinska, A. Swida, P. Bednarczyk, I. Koszela-Piotrowska, K. Choma, K. Dolowy, A. Szewczyk, W. Jarmuszkiewicz, ATP-sensitive potassium channel in mitochondria of the eukaryotic microorganism, *Acanthamoeba castellanii*. J. Biol. Chem. 282 (2007) 17433–17441.
[31] M.E. Andersson, P. Nordlund, A revised model of the active site of alternative oxidase, FEBS Lett. 449 (1999) 17–22.
[32] D.A. Berthold, M.E. Andersson, P. Nordlund, New insight into the structure and function of the alternative oxidase, Biochim. Biophys. Acta 1460 (2000) 241–254.