Uncoupling protein and alternative oxidase of *Dictyostelium discoideum*: occurrence, properties and protein expression during vegetative life and starvation-induced early development

Wiesława Jarmuszkiewicz[a], Maciej Behrendt[a], Rachel Navet[b], Francis E. Sluse[b,*]

[a] *Department of Bioenergetics, Institute of Molecular Biology and Biotechnology, Adam Mickiewicz University, Fredry 10, 61-701 Poznan, Poland*
[b] *Laboratory of Bioenergetics, Department of Life Sciences, Institute of Chemistry B6, University of Liège, Sart Tilman, B-4000 Liège, Belgium*

Received 11 October 2002; revised 11 November 2002; accepted 15 November 2002

First published online 28 November 2002

Edited by Vladimir Skulachev

Abstract In this study we show that mitochondria of *Dictyostelium discoideum* contain both alternative oxidase (AOX) and uncoupling protein (UCP). AOX was stimulated by purine mononucleoside and was monomeric. UCP was stimulated by free fatty acids and was poorly sensitive to GTP. Both proteins collaborated in energy dissipation when activated together. AOX expression in free-living ameboid cells decreased strongly from exponential to stationary phase of growth but much less during starvation-induced aggregation. In contrast, UCP expression was constant in all conditions indicating permanent need. Our results suggest that AOX could play a role in cell differentiation, mainly by protecting prespore cells from programmed cell death.

Key words: Mitochondrion; Alternative oxidase; Uncoupling protein; Development; *Dictyostelium discoideum*

1. Introduction

Mycetozoa (slime molds) have been used as model systems for studying the origin of several characteristics of higher eukaryotes such as movement, cell differentiation and morphogenesis. Opinions on their phylogenetic affinity have varied widely over time as they have been classified as plants, animals, fungi or protists. Molecular phylogenies of rRNA genes show the Mycetozoa to be polyphyletic and dictyostelid (one subgroup) and to appear as one of the earliest branches of mitochondrial eukaryotes [1–3]. However, protein sequence data analysis supports the Mycetozoa as being a monophyletic group and as late-emerging eukaryotes (in the Eukaryotic Crown Group) more closely related to the 'super' animal–fungal clade than are green plants [4] which would have emerged earlier as an independent lineage.

Cells of *Dictyostelium discoideum* multiply as ameboid cells pinocytosing external nutrients but under starvation they aggregate by chemotaxis to form multicellular slugs. The slugs move and undergo zonal differentiation before constructing a fruiting body with a mass of spores and a supporting stalk made of sterile dead cells. It is striking that this differentiation is predetermined in the slug [5,6]. Developmental cell death which produces stalk cells is associated with the release of apoptosis-inducing pathway factor from the mitochondria that is involved in a caspase-independent cell death pathway [7].

A cyanide-resistant ubiquinol alternative oxidase (AOX) present in all plants, many algae, fungi, some protozoa and other eukaryotic microorganisms seems to be widespread in eukaryotes except in the animal clade [8,9]. The plant oxidase is dimeric and can be stimulated by α-keto acids. The 'non-plant-type', fungal and protozoan AOX generally exists as a monomer and is not subject to organic acid stimulation but can be stimulated by purine nucleotides. Uncoupling protein (UCP), which mediates free fatty acid (FFA)-activated, purine nucleotide-inhibited H^+ re-uptake, seems to occur in the whole eukaryotic world except in fermentative yeast such as *Saccharomyces cerevisiae* [10]. These two proteins (AOX and UCP) are free energy-dissipating systems which both divert energy from ATP synthesis: AOX can consume the reducing power provided by substrates without energy conservation into an H^+ electrochemical gradient (energy source for ATP synthesis) and UCP can dissipate the H^+ gradient built by the respiratory chain. In specialized tissues, AOX is responsible for thermogenesis in plants and UCP in mammals. One important role of AOX and UCP in non-thermogenic tissues is to decrease the formation of reactive species of oxygen (ROS) [11–13] that are initiators of apoptosis [14].

It has been shown that application of inhibitors of the cyanide-resistant ubiquinol AOX to starved *D. discoideum* cells induced the formation of cell aggregates in which cells differentiated mainly into stalk-like sterile cells [15]. Moreover, expression of prestalk-specific genes and prespore-specific genes was enhanced or inhibited, respectively, suggesting a particular involvement of cyanide-resistant respiration in cell type differentiation during the *D. discoideum* early development.

In contrast, nothing is known about UCP in *D. discoideum*. UCPs are a subfamily of the mitochondrial anion carrier pro-

*Corresponding author. Fax: (32)-4-366 2878.
E-mail address: f.sluse@ulg.ac.be (F.E. Sluse).

Abbreviations: AOX, alternative oxidase; BHAM, benzohydroxamate; BSA, bovine serum albumin; CAT, carboxyatractyloside; DdAOX, alternative oxidase of *Dictyostelium discoideum*; DdUCP, uncoupling protein of *Dictyostelium discoideum*; DTT, dithiothreitol; EGS, ethylene glycol-bis-(succinimidylsuccinate); FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; FFA, free fatty acids; LA, linoleic acid; ROS, reactive oxygen species; state 3, phosphorylating respiration in the presence of added ADP; state 4, resting respiration in the absence of added ADP; UCP, uncoupling protein; $\Delta\Psi$, mitochondrial transmembrane electrical potential

0014-5793/02/$22.00
PII: S0014-5793(02)03734-1

tein (MACP) family, which contains homologous proteins with a threefold sequence repeat and a MACP sequence signature. Sequence analysis of the UCP subfamily reveals sequence motifs called UCP signatures that have allowed a UCP phylogenesis description including UCP of *D. discoideum* [16].

The aim of this study was to show the simultaneous presence in *D. discoideum* mitochondria of two free energy-dissipating systems, namely AOX and UCP, to characterize their activity and regulatory properties, and to compare their protein expression during the ameboid vegetative life and the early development of multicellularity. It was found that during transformation into aggregates, the expression of AOX was maintained at a high level compared to the level in stationary free-living cell culture. Contrarily, the expression of UCP remained constant.

2. Materials and methods

2.1. Cell culture conditions

Ameboid cells of *D. discoideum* AX-2 strain (ATCC 24397) were grown axenically at 20°C in a liquid medium containing: 1.43% (w/v) proteose peptone (Difco), 0.7% (w/v) yeast extract (Difco), 1.8% (w/v) maltose, 3.6 mM Na_2HPO_4 and 3.6 mM KH_2PO_4. Small stock cultures (60 ml) supplemented with streptomycin (0.25 g in 1 l) were used at the late exponential phase for inoculation into 900 ml medium. Generation time in continuously agitated small and large cultures was about 12 h. After approximately 70 h of exponential growth, cells reached the stationary phase with a density of $12–16\times10^6$ cells/ml. Free-living ameboid cells were harvested from cultures at two different phases of growth, i.e. 30 h (the middle exponential phase) and 70 h (the stationary phase) after inoculation.

2.2. Aggregation conditions

Ameboid cells from the middle exponential phase of growth were pelleted, washed and resuspended (to a density around 6×10^6 cells/ml) in a medium containing 20 mM phosphate buffer (pH 6.4), 10 mM KCl and 1 mM $MgCl_2$. Development was initiated by transferring approximately 3×10^8 cells on 15 large Petri dishes (530 cm^2) with 2% agar. Subsequently cells were incubated at 20°C. Under these starving conditions, free-living cells aggregated by chemotaxis towards cAMP diffusing from centrally located cells that results in transformation into a differentiated multicellular organism (slug, pseudopodium) forming a fruiting body [17,18]. For our study, we collected cells from three aggregation stages preceding slug formation and differentiation, i.e. the small aggregated group (12 h), large aggregated group (24 h) and large aggregated concentric group (36 h) stages.

2.3. Mitochondrial isolation

Free-living cells and cell aggregates were pelleted by centrifugation for 5 min at $600\times g$ and washed twice in A medium containing 15 mM KH_2PO_4 and 2 mM Na_2HPO_4 (pH 6.0). Cells were homogenized with a Dounce homogenizer (40 ml volume) by six fast strokes. Homogenate (15%, w/v) prepared in B isolation medium (0.38 M sucrose, 15 mM Tris–HCl, pH 7.6, 0.5 mM EDTA, 1% bovine serum albumin (BSA), defatted) was centrifuged for 5 min at $800\times g$ and the resulting supernatant centrifuged for 10 min at $8600\times g$. The pellet was resuspended in C isolation medium (0.38 M sucrose, 15 mM Tris–HCl, pH 7.2, 0.5 mM EDTA, 0.5% BSA). Mitochondria were purified on a self-generating Percoll gradient (25%). The presence of defatted BSA during isolation and purification depleted the mitochondria of endogenous FFA. After centrifugation for 40 min at $40\,000\times g$, mitochondria were collected and washed twice in medium D (0.38 M sucrose, 15 mM Tris–HCl, pH 7.2).

2.4. Oxygen uptake and membrane potential

Oxygen uptake and mitochondrial membrane potential ($\Delta\Psi$) measurements were performed with mitochondria isolated from ameboid cells from the middle exponential phase of growth. O_2 uptake was measured polarographically with a Rank Bros. (Cambridge, UK) oxygen electrode in 2.7 ml of the medium (25°C) containing 120 mM KCl, 20 mM Tris–HCl, pH 7.0, 3 mM KH_2PO_4, 8 mM $MgCl_2$, plus or minus 0.5% BSA, with 2 mg of mitochondrial protein. When sensitivity of UCP from *D. discoideum* (DdUCP) to GTP was tested, $MgCl_2$ was omitted from the incubation medium. The $\Delta\Psi$ was measured simultaneously with oxygen uptake using a tetraphenylphosphonium-specific electrode according to Kamo et al. [19]. Measurements of $\Delta\Psi$ were performed in the presence of 6 μM tetraphenylphosphonium. For calculation of the $\Delta\Psi$ value the matrix volume of mitochondria was assumed to be 2.0 μl/mg protein. Oxidizable substrates were 10 mM malate or 10 mM succinate, the latter in the presence of 170 μM ATP and 5 μM rotenone. Cyanide (1.5 mM) and benzohydroxymate (BHAM, 1.5 mM) were used as inhibitors of the cytochrome pathway and AOX, respectively. Measurements were performed in the absence (state 4) or presence (state 3) of 0.17 mM (pulse) or 1.5 mM ADP. The ADP/O ratio was determined by an ADP pulse method with succinate (plus rotenone) as oxidizable substrate. The total amount of oxygen consumed during state 3 respiration was used for calculation of the ratio. Values of O_2 uptake are presented in nmol O/min/mg protein. Details of measurements are included in the legends of figures.

2.5. SDS–PAGE and immunoblotting of AOX and UCP proteins

Mitochondria isolated from ameboid cells from the middle exponential phase of growth were solubilized in the sample buffer (1% (w/v) SDS, 60 mM Tris–HCl, pH 6.8, 10% glycerol, 0.004% bromophenol blue, with or without reducing agent, 1 mM dithiothreitol (DTT) or 0.5% β-mercaptoethanol) and boiled for 5 min. Electrophoresis (SDS–PAGE) was carried out using 5% polyacrylamide stacking gel and 12% polyacrylamide resolving gel, followed by Western blotting as described earlier [20]. For AOX immunoblotting analysis, the procedure of mitochondria treatment with diamide and DTT (which induce disulfide bond formation and reduction, respectively) as well as cross-linking with ethylene glycol-bis-(succinimidylsuccinate) (EGS, a Lys-Lys-specific cross-linker) was performed as described by Umbach and Siedow [21,22]. Bio-Rad prestained low molecular mass markers were used. Antibodies raised against the *Sauromatum guttatum* AOX (generously supplied by Dr. T.E. Elthon) and antibodies raised against *Arabidopsis thaliana* UCP (generously supplied by Dr. P. Arruda) were used at the same dilution (1:1000). The bands were visualized using the Amersham ECL system.

3. Results and discussion

3.1. Alternative oxidase in D. discoideum (DdAOX) is 'non-plant-type' and is efficient in free energy dissipation

As shown in Fig. 1A, when *D. discoideum* mitochondria isolated from ameboid cells of the exponential phase of growth respire with malate as a reducing substrate, cyanide did not completely inhibit respiration. Cyanide-resistant respiration was further stimulated by GMP, very partially inhibited by rotenone (an inhibitor of complex I) and almost totally inhibited by BHAM (an inhibitor of AOX). These observations indicate that DdAOX is a 'non-plant-type' AOX as it is stimulated by purine mononucleotides (GMP was the most efficient compared to GDP and GTP, not shown) and that an internal NADH dehydrogenase insensitive to rotenone (complex I bypass) can provide electrons from malate to AOX. Measurements of $\Delta\Psi$ changes in state 4 respiration showed a high H^+ pumping activity with malate ($\Delta\Psi\approx185$ mV) that collapsed in the presence of cyanide (no $\Delta\Psi$) and reincreased when AOX was activated by GMP due to complex I activity driven by AOX ($\Delta\Psi\approx150$ mV) as proven by its cancellation by rotenone. Thus, DdAOX provides a fully dissipating pathway from matrix NADH when its activity is connected to a rotenone-insensitive inner NADH dehydrogenase.

Fig. 1C shows the immunoblotting of the DdAOX protein in various conditions, i.e. in the absence or presence of reagents: reductant (20 mM DTT), oxidant (3 mM diamide) or cross-linker (0.5 mM EGS). All conditions led to a single

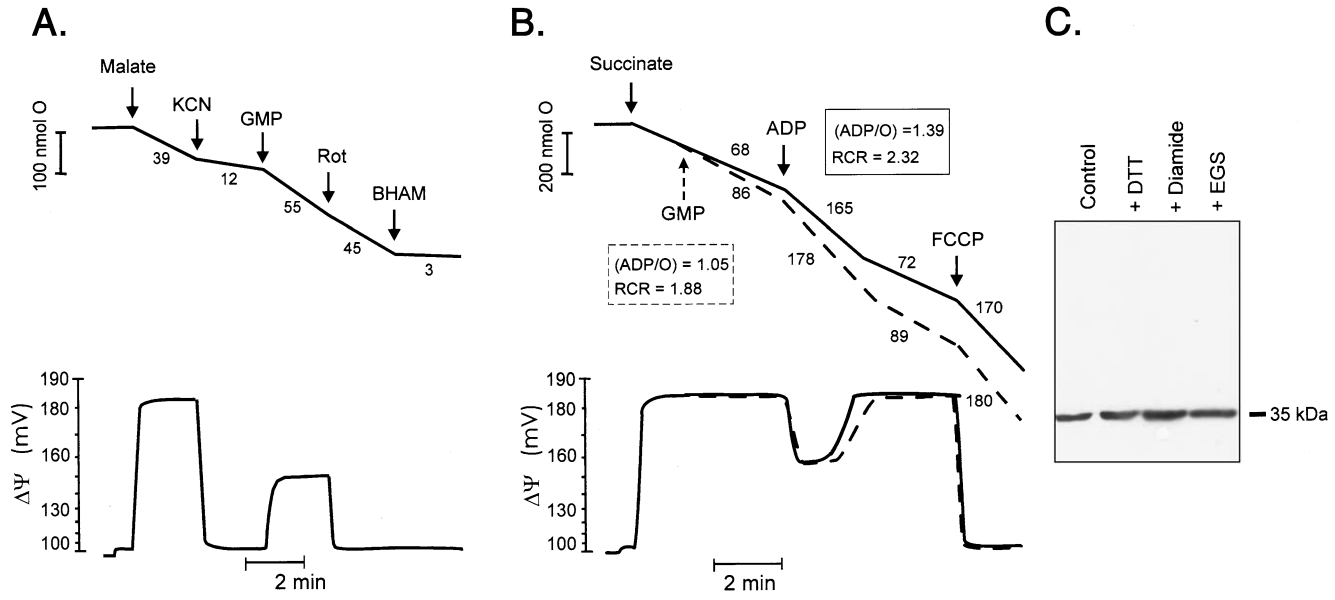

Fig. 1. A,B: Influence of GMP on cyanide-resistant respiration, coupling parameters and $\Delta\Psi$ of *D. discoideum* mitochondria. Mitochondria were incubated in the presence of 0.5% BSA. Additions: A: 10 mM malate, 1.5 mM KCN, 0.6 mM GMP, 5 μM rotenone (Rot), 1.5 mM BHAM; B: 10 mM succinate (plus 5 mM rotenone and 0.17 mM ATP), 0.5 mM GMP (dotted lines), 0.18 mM ADP, 1 μM FCCP. Numbers on the traces refer to O_2 consumption rates in nmol O/min/mg protein. Membrane potential changes are shown in mV. C: Immunological analysis of DdAOX protein in control (no additions), reducing (+20 mM DTT), oxidizing (+3 mM diamide), and cross-linking (0.5 mM EGS) conditions. The amount of protein loaded for all lanes was 100 μg.

~35 kDa band indicating that DdAOX cannot be covalently linked by diamide and thus reduced by DTT, and it cannot be cross-linked by a Lys-specific cross-linker. Thus, DdAOX is not a dimeric and disulfide-linked protein. These results are also in accordance with the monomeric properties of 'non-plant-type' AOX found in some fungi and protozoa [9,23].

The efficiency of DdAOX in diverting energy from oxidative phosphorylation can be assessed by the ADP/O method [24]. Fig. 1B shows the determination of ADP/O ratios after the pulse of ADP in the presence or absence of GMP with succinate as an oxidizable substrate. The transient state 3 respiration (phosphorylating respiration) is also visualized by a transient drop in $\Delta\Psi$. The ADP/O ratio was decreased from 1.39 (no GMP) to 1.05 (plus 0.6 mM GMP). Taking into account these values, contributions of AOX (V_{alt}) and the cytochrome pathway (V_{cyt}) in state 3 respiration can be calcu-

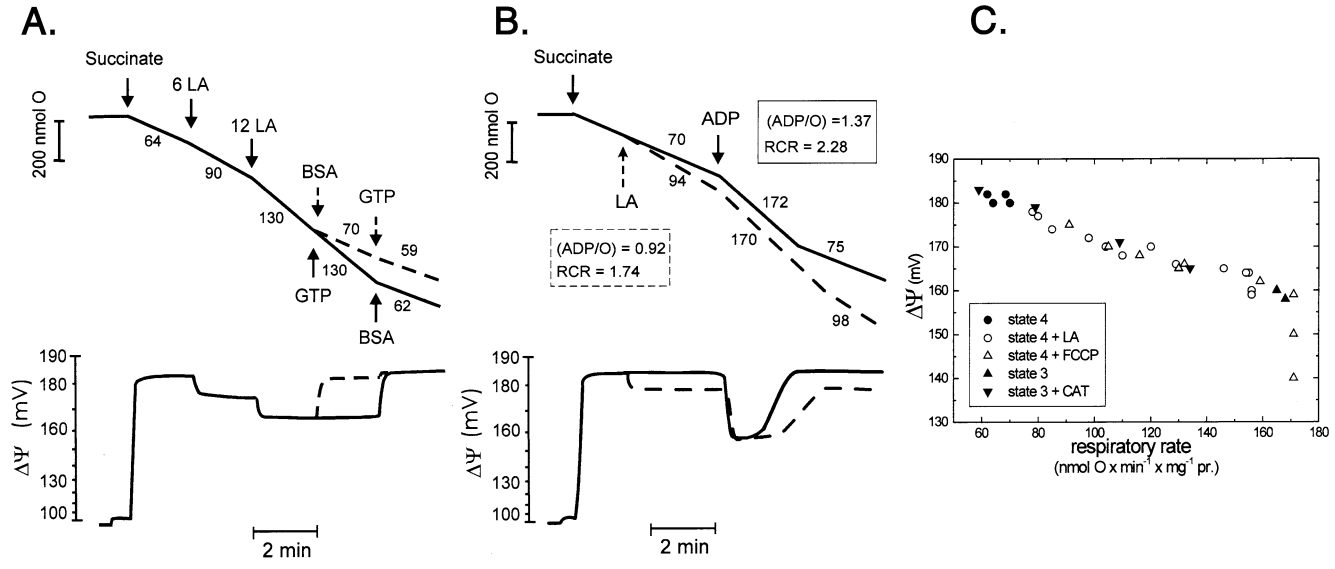

Fig. 2. A,B: Influence of LA on respiratory rates, coupling parameters and $\Delta\Psi$ of *D. discoideum* mitochondria. Mitochondria were incubated in the presence of 1.5 mM BHAM, 5 μM rotenone, 0.17 ATP and the presence (A) or absence (B) of 1 μM CAT. In A, $MgCl_2$ was omitted from the incubation medium. Additions: A: 10 mM succinate, 6 or 12 μM LA, 0.5% BSA, 2 mM GTP; B: 10 mM succinate, 4 μM LA (dotted line), 0.18 mM ADP. C: Relation between $\Delta\Psi$ and mitochondrial respiration in the presence of BHAM. All measurements were made in the presence of 10 mM succinate, 5 μM rotenone, 0.17 mM ATP and 1.5 mM BHAM. State 4 was measured in the presence of 1 μM CAT, state 3 in the presence of 1.5 mM ADP. (●) State 4, (○) state 4 with increasing concentration of LA (2–26 μM), (△) state 4 with increasing concentration of FCCP (0.005–0.05 μM), (▲) state 3, and (▼) state 3 with increasing concentration of CAT (0.08–1.2 μg/mg protein).

Table 1
Influence of various conditions on the ADP/O ratio with succinate (plus rotenone) as oxidizable substrate

	+BSA +BHAM	+LA +BHAM	+BSA +GMP	+LA +GMP	−LA, −BSA −GMP, −BHAM
ADP/O	1.38 ± 0.04	0.98 ± 0.05	1.10 ± 0.03	0.78 ± 0.04	1.34 ± 0.07

Assay conditions were as described in Section 2. The concentrations used were: 1.5 mM BHAM, 0.5% BSA, 4 μM LA, and 0.6 mM GMP (where indicated). The data are presented as the means ± S.D. from four independent experiments.

lated according to the ADP/O method [24]: $V_{cyt} = 135$ nmol O/min/mg protein, $V_{alt} = 43$ nmol O/min/mg protein. These results indicate that when AOX is activated by GMP it can efficiently divert energy from oxidative phosphorylation during state 3 respiration when both AOX and the cytochrome pathway work simultaneously.

3.2. *DdUCP is stimulated by free fatty acids and is efficient in H^+ electrochemical gradient dissipation*

DdUCP was immunologically identified with plant antibodies that cross-reacted with a single band at around 32 kDa (Fig. 3, lower part). The results presented in Fig. 3 show that the gene of DdUCP [16] is present and translated during the single-celled ameboid life of *D. discoideum* as well as during the development of multicellularity.

In order to check the FFA activation and the GTP inhibition sensitivity of DdUCP we measured the respiration in state 4 with succinate (plus rotenone) as a reducing substrate and the effect of successive additions of linoleic acid (LA) on respiratory rate and $\Delta\Psi$ with FFA-depleted mitochondria (Fig. 2). These measurements were made with mitochondria isolated from the free-living ameboid cells from the exponential phase of growth. Fig. 2A shows that state 4 respiration is accelerated by a low concentration of LA. The concentration of LA that leads to a half maximum stimulation of UCP-sustained state 4 respiration was around 10 μM (not shown).

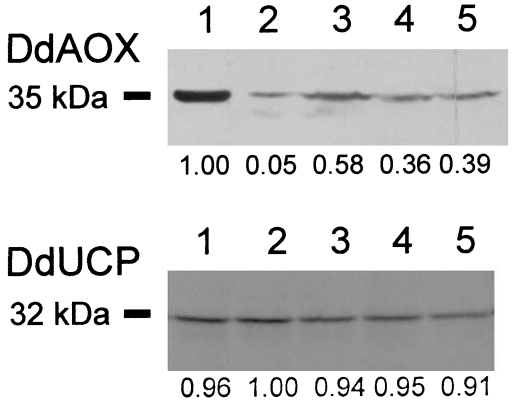

Fig. 3. Immunodetection of AOX and UCP proteins during single-cell life and during aggregation phases in *D. discoideum* mitochondria. The total mitochondrial protein load was 100 μg for AOX detection and 50 μg for UCP detection. Lanes: 1,2: free-living ameboid cells from the middle exponential (lane 1) and stationary phase (lane 2) of growth, i.e. 30 h and 70 h after inoculation, respectively; 3–5: starvation-induced aggregation phases, i.e. 12 h starved small aggregated group (lane 3), 24 h starved large aggregated group (lane 4), 36 h starved large aggregated concentric group (lane 5). The protein bands were visualized by chemiluminescence and quantitated digitally. The highest intensity band of the profile was set at 1 and others calculated relative to that value. Relative intensity is given below each band.

The effects of LA additions on $\Delta\Psi$ are also shown in Fig. 2A. It appears that at low concentration LA induced a mild uncoupling in state 4 ($\Delta\Psi$ decreased from 183 mV to 175 mV upon addition of 6 μM LA). GTP had no effect on the LA-stimulated state 4 respiration and $\Delta\Psi$ in the absence of BSA and had little recoupling effect on the respiratory rate in the presence of BSA which chelated FFA. This assay was performed in the absence of $MgCl_2$ in the incubation medium. Results in Fig. 2A indicate that FFA, at low concentrations, accelerate state 4 respiration through an uncoupling and that this effect of FFA could be due to a stimulation of UCP activity (the UCP-sustained respiration) as GTP had little inhibitory effect in state 4 respiration in the presence of BSA and as the measurement was done in the presence of ATP (and also carboxyatractyloside (CAT)), succinate, and inorganic phosphate that avoids the participation of their respective carriers in the uncoupling [10].

The proton electrochemical gradient should be partitioned between UCP and ATP synthesis during state 3 respiration as both proteins participate in H^+ re-uptake. In order to assess how efficient DdUCP is in the H^+ diversion from ATP synthesis, we measured the ADP/O ratio (with succinate as reducing substrate) in the presence and absence of 4 μM LA, when AOX was blocked by BHAM. As shown in Fig. 2B, LA addition increased state 4 respiration but not state 3 respiration. Moreover, it decreased $\Delta\Psi$ of state 4 but not $\Delta\Psi$ of state 3. However, the ADP/O ratio was clearly lowered in the presence of LA indicating that in state 3, even if the respiratory rate was not modified, activation of UCP by LA diverts energy from ATP synthesis. The contribution of UCP (V_{UCP}) and ATP synthase ($V_{cyt\ cons}$) in H^+ re-uptake during state 3 respiration can be calculated [25] from the ADP/O values in the absence or presence of LA (1.37 and 0.92, respectively). When UCP was activated by 4 μM LA, the contribution of UCP was 51 nmol O/min/mg protein and the contribution of ATP synthase was 115 nmol O/min/mg protein. Thus, activation of UCP by LA at low concentration can efficiently divert energy from oxidative phosphorylation. Other carriers that could participate in uncoupling induced by LA in state 4 (at least in animal mitochondria), namely the ADP/ATP carrier [26], the dicarboxylate carrier [27], the phosphate carrier [28], and the aspartate/glutamate carrier [29,30], are mainly employed in the import of their substrates during state 3 respiration (except the last one in our conditions). Thus, it is unlikely that mitochondrial uncoupling mediated by these carriers occurs during phosphorylating respiration and is responsible for the decrease in the ADP/O ratio observed here.

It was important to check if in *D. discoideum* mitochondria, the LA-induced increase in state 4 respiration was only due to a proton recycling by UCP and thus if it corresponds to a pure protonophoric effect of LA. The results shown in Fig. 2C report relations between couples of $\Delta\Psi$ and respiratory rate measurements in state 4 and state 3. State 4 respiration was

titrated in two conditions, i.e. with increasing concentrations of LA or carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP). State 3 respiration was titrated with increasing concentrations of CAT. The maximal coupled (state 3) and uncoupled respiration were the same and the LA maximally stimulated respiration was a little lower. Nevertheless, a set of conditions constituted a single force-flow relationship indicating that LA exerts the same control of the flow (oxygen consumption rate) as the protonophore FCCP. These results indicate that LA has a pure protonophoric effect and does not interact directly with the respiratory chain, therefore does not induce an intrinsic uncoupling (slip).

3.3. Collaboration of DdAOX and DdUCP in energy dissipation

As in plant and yeast *Candida parapsilosis* mitochondria, LA inhibits AOX and activates UCP, the two energy-dissipating systems cannot work together at their maximal rate [31,32]. As shown in Table 1, in FFA-depleted *D. discoideum* mitochondria, the activities of AOX and UCP have an additive effect on the yield of oxidative phosphorylation. When DdUCP was activated by a low concentration of LA (4 μM) in the presence of BHAM (exclusive effect of UCP) or when DdAOX was activated by 0.6 mM GMP in the presence of BSA (exclusive effect of AOX), the ADP/O decreased compared to the control value measured with both energy-dissipating systems blocked. However, the lowest ADP/O value was observed when both proteins, AOX and UCP, were activated indicating a cumulative effect of both dissipating pathways on the oxidative phosphorylation efficiency. This collaboration of AOX and UCP, observed also in mitochondria of the ameba *Acanthamoeba castellanii* [33], results from insensitivity of cyanide-resistant respiration (i.e. AOX activity) to LA inhibition. In *D. discoideum* mitochondria, increasing the LA concentration within the 0–26 μM range did not inhibit AOX activity (not shown). Thus, it seems that FFA-regulated interaction between AOX and UCP changed with evolution, not being present in Mycetozoa and *A. castellanii*, less dramatic in fungi and strong in plants [10].

3.4. DdAOX and DdUCP protein expression

Changes in the expression of AOX and UCP proteins during single-celled life and aggregation phases of *D. discoideum* are shown in Fig. 3. When free-living ameboid cells were harvested in the middle exponential phase of growth, a large amount of AOX was immunodetected (Fig. 3, lane 1). When cells were harvested in the stationary phase of growth, the amount of immunodetected DdAOX was much lower (lane 2), indicating a strong down-regulation of expression (around 20-fold). In contrast, when cells from the exponential phase of growth were starved to induce aggregation, the expression of DdAOX was maintained at around a 50% level for the three stages of aggregation i.e. after 12 h, 24 h and 36 h of starvation (lanes 3–5). This indicates that during the aggregation phases, which precede the cell differentiation, the expression of DdAOX is maintained at a quite high level. Results from free-living cells (not starved) of *D. discoideum* match with those obtained with *A. castellanii* cells, where the amount of AOX protein drops significantly in the stationary phase of growth [23]. In *D. discoideum* mitochondria, the evolution profile of DdUCP protein showed a constancy in its expression in all conditions, both in free-living exponential and in stationary cell phases (lanes 1,2) and aggregating cell phases (lanes 3–5). These observations favor the idea of a constant need of UCP in *D. discoideum* cells whatever the age or level of cellularity (uni- or pluri-). The stability of the amount of protein was also observed for the mitochondrial adenine nucleotide translocator in *D. discoideum* cells from the vegetative stage throughout development, even if mRNA levels decreased rapidly after starvation [34].

3.5. Conclusion

The results of the present study can be summarized as follows.

D. discoideum mitochondria contain simultaneously two free energy-dissipating systems, AOX and UCP. DdAOX is a monomeric, GMP-stimulated, FFA-insensitive 'non-plant-type' AOX that is efficient in free energy dissipation by decreasing the yield of oxidative phosphorylation during state 3 respiration. DdUCP is stimulated by FFA and almost insensitive to GTP. The sensitivity of UCP to GTP could be an evolutionary acquisition that appeared only in fungal and animal clades [10]. The FFA-induced increase in state 4 respiration is a pure protonophoric effect of LA that could be due to a proton recycling by DdUCP. FFA did not stimulate state 3 respiration but decreased the ADP/O ratio indicating that DdUCP activation by FFA can divert energy from oxidative phosphorylation due to an H^+ electrochemical gradient partitioning between DdUCP and ATP synthase. Thus, DdAOX and DdUCP can collaborate in free energy dissipation and thereby weaken oxidative phosphorylation together.

DdAOX and DdUCP proteins are expressed throughout the life of *D. discoideum*, i.e. in vegetative free-living cells and during development of multicellularity. However, while DdUCP protein remains at a constant level, like adenine nucleotide transporter [34], indicating a permanent need of both, the expression of AOX protein is down-regulated at the stationary phase of growth in non-starved cell culture. Conversely, during starvation-induced development, the level of AOX protein remains at least 10 times higher compared to the stationary phase cells. This indicates an essential role of AOX in *D. discoideum* development and differentiation. It can be proposed that DdAOX could play a key role in cell differentiation in relation to the ability of energy-dissipating systems to decrease ROS production in vivo and in vitro [10–13,35]. Indeed, ROS are considered to be early inducers in programmed cell death [14]. As transformation of *D. discoideum* aggregates into slug and finally into the fruiting body involves the differentiation of cells in 80% in viable spores and in 20% in dead stalk cells (resulting from developmental cell death) [36], it can be proposed that signals which control this 80:20 ratio could down-regulate expression of DdAOX in prestalk cells and up-regulate its expression in prespore cells. Thus, by decreasing ROS production, DdAOX could play an important role in supporting the viability of prespore cells, while its down-expression could allow developmental cell death of prestalk cells. This proposal also explains the effect of AOX inhibitor which induces the formation of mainly stalk-like cells during differentiation [15].

Acknowledgements: This work was supported by the Polish Committee of Scientific Research (KBN 6 P04A 005 18), the Belgian National Funds for Scientific Research (FRFC 2.4517.00), and 'la présente publication a été rendue possible grâce à l'accord qui lie la Communauté Wallonie-Bruxelles et la Pologne'.

References

[1] Douglas, S.E., Murphy, C.A., Spencer, D.F. and Gray, M.W. (1991) Nature 350, 148–151.
[2] Knoll, A.H. (1992) Science 256, 622–627.
[3] Wainright, P.O., Hinkle, G., Sogin, M.L. and Stickel, S.K. (1993) Science 260, 340–342.
[4] Baldauf, S.L. and Doolittle, W.F. (1997) Proc. Natl. Acad. Sci. USA 94, 12007–12012.
[5] Gross, J.D. (1994) Microbiol. Rev. 58, 330–351.
[6] Kay, R.R. (1994) Curr. Opin. Genet. Dev. 4, 637–641.
[7] Arnould, D., Tatischeff, I., Estaquier, J., Girard, M., Sureau, F., Tisser, J.P., Grodet, A., Dellinger, M., Traincard, F., Kahn, A., Ameisen, J.-C. and Petit, P.X. (2001) Mol. Biol. Cell 12, 3016–3030.
[8] Sluse, F.E. and Jarmuszkiewicz, W. (1998) Braz. J. Med. Biol. Res. 31, 733–747.
[9] Siedow, J.N. and Umbach, A.L. (2001) Bichim. Biophys. Acta 1459, 432–439.
[10] Sluse, F.E. and Jarmuszkiewicz, W. (2002) FEBS Lett. 510, 117–120.
[11] Popov, V.N., Simonian, R.A., Skulachev, V.P. and Starkov, A.A. (1997) FEBS Lett. 415, 87–90.
[12] Kowaltowski, A.J., Costa, A.D. and Vercesi, A.N. (1998) FEBS Lett. 425, 213–216.
[13] Maxwell, D.P., Wang, Y. and McIntosh, L. (1999) Proc. Natl. Acad. Sci. USA 96, 8271–8276.
[14] Skulachev, V.P. (1996) FEBS Lett. 397, 7–10.
[15] Matsuyama, S.-I. and Maeda, Y. (1995) Dev. Biol. 172, 182–191.
[16] Hanak, P. and Ježek, P. (2001) FEBS Lett. 495, 137–141.
[17] Konijin, T.M., Van De Meene, J.G.C., Bonner, J.T. and Barkley, D.S. (1967) Proc. Natl. Acad. Sci. USA 58, 1152–1154.
[18] Bonner, J.T., Barkley, D.S., Hall, E.M., Konijin, T.M., Mason, J.W., O'Keefe, G. and Wolfe, P.B. (1969) Dev. Biol. 20, 72–87.
[19] Kamo, N., Muratsugu, M., Hongoh, R. and Kobatake, Y. (1979) J. Membr. Biol. 49, 105–121.
[20] Almeida, M.A., Jarmuszkiewicz, W., Khomsi, H., Arruda, P., Vercesi, A.E. and Sluse, F.E. (1998) Plant Physiol. 119, 1323–1329.
[21] Umbach, A.L. and Siedow, J.N. (1993) Plant Physiol. 103, 845–854.
[22] Umbach, A.L. and Siedow, J.N. (2000) Arch. Biochem. Biophys. 378, 234–245.
[23] Jarmuszkiewicz, W., Wagner, A.M., Wagner, M.J. and Hryniewiecka, L. (1997) FEBS Lett. 411, 110–114.
[24] Jarmuszkiewicz, W., Sluse-Goffart, C.M., Hryniewiecka, L., Michejda, J. and Sluse, F.E. (1998) J. Biol. Chem. 273, 10174–10180.
[25] Jarmuszkiewicz, W., Almeida, A.M., Vercesi, A.E., Sluse, F.E. and Sluse-Goffart, C.M. (2000) J. Biol. Chem. 275, 13315–13320.
[26] Andreyev, A.Y., Bondareva, T.O., Dedukhova, V.I., Mokhova, E.N., Skulachev, V.P., Tsofina, L.M., Volkov, N.L. and Vygodina, T.V. (1989) Eur. J. Biochem. 182, 585–592.
[27] Wieckowski, M. and Wojtczak, L. (1997) Biochem. Biophys. Res. Commun. 232, 414–417.
[28] Žačková, M., Krämer, R. and Ježek, P. (2000) Int. J. Biochem. Cell Biol. 32, 499–508.
[29] Samartsev, V.N., Mokhova, E.N. and Skulachev, V.P. (1997) FEBS Lett. 412, 251–257.
[30] Skulachev, V.P. (1998) Biochim. Biophys. Acta 1363, 100–124.
[31] Sluse, F.E., Almeida, A.M., Jarmuszkiewicz, W. and Vercesi, A.E. (1998) FEBS Lett. 433, 237–240.
[32] Jarmuszkiewicz, W., Milani, G., Fortes, F., Schreiber, A.Z., Sluse, F.E. and Vercesi, A.E. (2000) FEBS Lett. 467, 145–149.
[33] Jarmuszkiewicz, W., Sluse-Goffart, C.M., Hryniewiecka, L. and Sluse, F.E. (1999) J. Biol. Chem. 274, 23198–23202.
[34] Bof, M., Brandolin, G., Satre, M. and Klein, G. (1999) Eur. J. Biochem. 259, 795–800.
[35] Skulachev, V.P. (1996) Q. Rev. Biophys. 29, 169–202.
[36] Kessin, R.H. (2000) Nature 408, 917–919.