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Review

Mitochondrial uncoupling proteins in unicellular eukaryotes

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

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

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ABSTRACT

Uncoupling proteins (UCPs) are members of the mitochondrial anion carrier protein family that are present in the mitochondrial inner membrane and mediate free fatty acid (FFA)-activated, purine nucleotide (PN)-inhibited proton conductance. Since 1999, the presence of UCPs has been demonstrated in some non-photosynthesising unicellular eukaryotes, including amoeboid and parasite protists, as well as in non-fermentative yeast and filamentous fungi. In the mitochondria of these organisms, UCP activity is revealed upon FFA-induced, PN-inhibited stimulation of resting respiration and a decrease in membrane potential, which are accompanied by a decrease in membranous ubiquinone (Q) reduction level. UCPs in unicellular eukaryotes are able to divert energy from oxidative phosphorylation and thus compete for a proton electrochemical gradient with ATP synthase. Our recent work indicates that membranous Q is a metabolic sensor that might utilise its redox state to release the PN inhibition of UCP-mediated mitochondrial uncoupling under conditions of phosphorylation and resting respiration. The action of reduced Q (QH_2) could allow higher or complete activation of UCP. As this regulatory feature was demonstrated for microorganism UCPs (*A. castellanii* UCP), plant and mammalian UCP1 analogues, and UCP1 in brown adipose tissue, the process could involve all UCPs. Here, we discuss the functional connection and physiological role of UCP and alternative oxidase, two main energy-dissipating systems in the plant-type mitochondrial respiratory chain of unicellular eukaryotes, including the control of cellular energy balance as well as preventive action against the production of reactive oxygen species.

1. Introduction

The electrochemical proton gradient ($\Delta\mu H^+$) generated by the mitochondrial respiratory chain can be dissipated by proton leak reactions that are catalysed by endogenous proton conductance pathways, implicating the mitochondrial uncoupling protein (UCP) in this process. UCPs are members of the mitochondrial anion carrier protein (MACP) family that are located in the inner mitochondrial membrane and mediate free fatty acid (FFA)-activated, purine nucleotide (PN)-inhibited H^+ re-uptake. Therefore, UCPs modulate the coupling of mitochondrial respiration and ATP synthesis.

The plant-type mitochondrial respiratory chain, found in the mitochondria of some unicellular eukaryotes (protists and fungi), contains two energy-dissipating systems: (i) respiratory chain electron carriers that dissipate redox energy instead of building $\Delta\mu H^+$, i.e., additional, rotenone-insensitive external and internal NAD(P)H dehydrogenases and an alternative ubiquinol cyanide-resistant terminal oxidase (AOX); and (ii) UCP, which directly dissipates energy contained in $\Delta\mu H^+$ [1]. Although these two energy-dissipating systems act at two different levels of the overall energy transduction pathways, they lead to the same final effect, i.e., a decrease in ATP synthesis yield per oxygen consumed.

2. Functional identification of UCPs in unicellular eukaryotes: evolutionary considerations

UCP1 in brown adipocytes was believed to be a late evolutionary acquisition in mammals as a result of adaptive thermogenesis [2] until the discovery of its analogues in various non-thermogenic mammalian tissues [3] but also in other eukaryotes from plants to animals [4,5], as well as in unicellular eukaryotes [1,6]. In 1999, a UCP-like protein from *Acanthamoeba castellanii* (AcUCP), a non-photosynthesising free-living amoeboid protist (Amoebozoa), was the first UCP identified in unicellular eukaryotes, using antibodies raised against plant UCP [7]. The activity of this protein was functionally characterised as FFA-induced PN-sensitive proton conductance. The presence of UCP in *A. castellanii*, which in molecular phylogenesis appears on a branch basal to the divergence points of plants, animals, and fungi [8], allowed the hypothesis that UCPs emerged as specialised proteins for H^+ cycling

Abbreviations: AcUCP, uncoupling protein of *A. castellanii*; AcAOX, alternative oxidase of *A. castellanii*; AOX, alternative oxidase; FCCP, *p*-trifluoromethoxyphenylhydrazone; FFA, free fatty acid; HNE, 4-hydroxy-2-nonenal; PN, purine nucleotide; Q, coenzyme Q, ubiquinone; QH_2, reduced Q, ubiquinol; Q reduction level or Q redox state, QH_2/total Q in the inner mitochondrial membrane; ROS, reactive oxygen species; UCP, uncoupling protein; $\Delta\Psi$, mitochondrial membrane electrical potential; $\Delta\mu H^+$, electrochemical proton gradient

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

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before the major radiation of phenotypic diversity in eukaryotes and maybe even earlier, after the acquisition of mitochondria in Eukarya, and that they could exist in all eukaryotes [7]. The first evidence and characterisation of a UCP-like protein in the parasitic protists (Alveolata) *Plasmodium berghei* [9] and *Plasmodium yoelii yoelii* [10], and in fungi, namely the non-fermentative ascomycetous yeast *Candida parapsilosis* [11], *Candida albinicans* [12], *Yarrowia lipolytica* [13], and the filamentous multicellular fungi *Aspergillus fumigatus* [14] as well as in *Dictyostelium discoideum* [15], a mycetozoan (Amoebozoa), cellular slime mold showing multicellularity and cell differentiation under starvation, support this hypothesis. In the mitochondria of these organisms, UCP activity is revealed upon PN-sensitive stimulation of resting (non-phosphorylating) respiration by FFAs accompanied by decrease in $\Delta\Psi$. Cross-reactivity with antibodies raised against plant or mammalian UCPs indicates the presence of a ~30 kDa mitochondrial protein in the mitochondria of protists and fungi, in which UCP activity has been studied [7,9–15]. Fermentative yeast (such as *Saccharomyces cerevisiae*) seem to be the only exception in eukaryotes not possessing UCP; there are no orthologs of the known members of the UCP family and no evidence for the presence of UCP-like activity stimulated by FFAs and inhibited by PNs [16,17]. While genes coding for UCPs have been identified in plants and animals, evidence for the presence of UCPs in fungi and protists is mainly functional and immunological. Recently, using heterologous expression in *S. cerevisiae*, it has been shown that a gene of yeast *Y. lipolytica* predicted by phylogenetic analysis to be a gene of oxaloacetate carrier encodes protein, which also displays an uncoupling activity stimulated by FFAs and inhibited by PNs [13]. This is the first identified gene of the mitochondrial anion carrier that provides the mitochondria of unicellular eukaryotes with UCP-like activity. It has been hypothesised that the *Y. lipolytica* protein implied by phylogeny to be an oxaloacetate carrier (present in yeast and plant mitochondria but not in animal mitochondria) may have evolved to allow FFA-induced uncoupling activity in contrast to the oxaloacetate carrier of *S. cerevisiae* [13]. It remains unknown if this putative dual function (combined oxaloacetate carrier and UCP activity) and an amino acid sequence resembling the oxaloacetate carrier structure concern other UCPs of unicellular eukaryotes. Interestingly, in recently sequenced genomes of the unicellular green alga *Chlamydomonas reinhardtii* [18] and the amoeboid protist *D. discoideum* (DictyBase), genes encoding UCP(s) were identified by comparative sequence analyses, although so far no functional evidence has proven that these genes in fact encode UCPs. These possible candidates for UCPs seem to be closely related to the oxaloacetate carrier. It must be highlighted that in plant and animal mitochondria, amino acid sequence similarity between UCP1 analogues is rather low. Taken all together, these facts could explain why, even though a large amount of functional information is available, little is known about the genes of unicellular eukaryotes that code for UCPs.

3. Functional characteristics

3.1. Stimulation by FFAs

In the mitochondria of unicellular eukaryotes, in which UCP activity has been studied, low FFA concentrations lead to an increase in mitochondrial resting (state 4) respiration and in simultaneous decrease in $\Delta\Psi$. In the mitochondria of *A. castellanii*, it has been shown that activation of UCP activity by FFAs also results in a PN-sensitive decrease in Q reduction level [19]. The voltage dependence of electron flux (the relationship between $\Delta\Psi$ and the respiration rate) in the mitochondria of protists (*A. castellanii*, *D. discoideum*) and fungi (*C. parapsilosis*) shows that FFA-induced respiration is solely due to proton recycling by UCP, as it corresponds to a pure protonophoretic effect of FFAs that is not distinguishable from the effect of the well-known protonophore carbonyl cyanide *p*-trifluoromethoxy-phenylhydrazone (FCCP) [7,11,15].

The best functionally characterised UCP of unicellular eukaryotes is the UCP of the amoeba *A. castellanii* (AcUCP). In the mitochondria of *A. castellanii*, a profile of the specificity of non-esterified FFAs in mitochondrial uncoupling, i.e., stimulation of resting respiration, reduction of $\Delta\Psi$ and decrease of oxidative phosphorylation yield, has been analysed [20]. Unsaturated (C18–20) FFAs are the most active uncouplers. Uncoupling activity with the best unsaturated FFA (linoleic C18:2) is 2.7-fold higher than with the best saturated FFA (palmitic C16:0). Among saturated FFAs, the potency to reduce $\Delta\Psi$ and to stimulate resting respiration decreases with decreasing carbon chain length (except for stearic acid C18:0, which is slightly active). Palmitic (C16:0) and myristic (C14:0) acids are quite effective as uncouplers, while capric (10:0) and caprylic (8:0) acids are almost inactive. A very similar pattern of FFA specificity in mitochondrial uncoupling (to decrease $\Delta\Psi$) was found in plant (durum wheat) mitochondria [21], which is in contrast to the FFA specificity described for UCP1 proteoliposomes [22,23]. Study of changes in the fatty acid composition of whole cells and purified membrane fractions of *A. castellanii* during growth in batch culture indicates that linoleic (C18:2) and oleic (C18:1) acids, the most effective activators of AcUCP [20], are very abundant (composing 34–54% of the total cellular fatty acids) in amoeba cells [24]. It is very interesting that during the aging of an *A. castellanii* batch culture, the greatest change was evident in the linoleate to oleate ratio (oleate increased) with a concomitant decline in the total level of unsaturation of fatty acids. Moreover, the chilling of amoeba cultures, which leads to an increase in the overall degree of fatty acid unsaturation and a change in the relative proportion of linoleic acid [25], causes an increase in AcUCP protein content and activity (linoleic acid-stimulated, UCP-mediated, carboxyatractyloside-resistant state 4 respiration) [26]. Thus, as in *A. castellanii* cells, FFA efficiency in mitochondrial uncoupling is related to physiological changes in fatty acid composition and unsaturation levels (and thus FFA availability) during amoeba cell growth, it could be a way to regulate the activity of AcUCP and thus the efficiency of oxidative phosphorylation during the life of this eukaryotic unicellular organism.

In contrast to animal cells where the major site for fatty acid β-oxidation is mitochondrion, β-oxidation generally occurs in peroxisomes or glyoxysomes in yeast and protists (as well as in plants) [27–29]. Therefore, the activation of UCP in these unicellular eukaryotes seems not to accelerate fatty acid oxidation as it occurs with UCP1 in mammalian brown adipose tissue. In the ascomycetous yeast *Y. lipolytica*, the FFA-induced and PN-sensitive uncoupling activity seems to be higher in stationary phase (as compared to mid-logarithmic growth phase) [13], when lipid bodies are degraded and the major fatty acids released are linoleic, oleic, stearic and palmitic acids [30]. In the mitochondria of *Y. lipolytica*, all of these FFAs induce mitochondrial uncoupling (increased resting respiration rate) with a decreasing order of activity: linoleic>oleic>palmitic>stearic acid [13], similarly to that described above for the mitochondria of the protist *A. castellanii* [20].

In animal mitochondria, FFA-induced mitochondrial uncoupling can be mediated, at least in part, by several other members of the mitochondrial anion carrier protein family such as the ATP/ADP antiporter, the aspartate/glutamate antiporter and the dicarboxylate carrier [31–33], likely at high $\Delta\Psi$ but unlikely during phosphorylating respiration, when they are mainly employed in the import of their substrates. There is no evidence that FFA-induced UCP-independent uncoupling could occur in the mitochondria of unicellular eukaryotes. Results suggest that neither the ATP/ADP antiporter, the aspartate/glutamate antiporter, nor the dicarboxylate carrier participate in the protonophoric action of FFA in the mitochondria of parasitic protist *P. berghei* [9]. Similarly, the uncoupling effect of fatty acids is insensitive to carboxyatractyloside (an inhibitor of the ATP/ADP antiporter) in the mitochondria of other parasitic protist *P. yoelii* and fungus *A. fumugatus* [10,14].

3.2. Activity in phosphorylating respiration

UCPs are able to share protonmotive force with ATP synthase (H^+ partitioning) during phosphorylating (state 3) respiration and partially uncouple electron transport from ATP synthesis. Most studies on the activation of UCPs have focused on non-phosphorylating respiring mitochondria, i.e., on resting state (state 4) respiration with maximal $\Delta\Psi$ and a high membranous Q redox state. Resting state conditions limit the interpretation of the physiological regulation of UCPs *in vivo*, since mitochondria are ADP-phosphorylating entities, i.e., they work at a lowered $\Delta\Psi$ and Q redox state of phosphorylating respiration. Although state 3 respiration is never increased by the addition of FFAs in studied microorganism mitochondria, this finding does not mean that they cannot induce UCP activity under phosphorylating conditions; rather it suggests that the respiratory chain is at its maximal rate [7,11,15,34]. It has been shown that similarly to mammalian (rat skeletal muscle) and plant (potato tuber) mitochondria [35–39], UCP is able to decrease the efficiency of oxidative phosphorylation (i.e., the amount of ADP phosphorylated per oxygen consumed) during state 3 mitochondrial respiration in a FFA-dependent manner in protist (*A. castellanii*, *D. discoideum*) and yeast (*C. parapsilosis*) mitochondria [7,11,15,31,40]. In the presence of FFA, a decrease in the ADP/O ratio has been observed, while there is no effect on the state 3 respiratory rate and $\Delta\Psi$. The FFA concentration-dependent decrease in the ADP/O ratio clearly indicates the participation of UCP in state 3 respiration. Hence, UCPs of unicellular eukaryotes can be activated when mitochondria are in a phosphorylating state and can compete with ATP synthase for the protonmotive force (H^+ partitioning). The ADP/O method [36] has been utilised to calculate the contributions of UCP activity and ATP synthesis in state 3 respiration of *A. castellanii* mitochondria, using pair measurements of ADP/O ratios in the absence or presence of various concentrations o FFA [34]. The efficiency of AcUCP in mitochondrial uncoupling increases when the state 3 respiratory rate decreases: the AcUCP contribution is constant (at a given FFA concentration), while the ATP synthase contribution decreases with respiratory rate. With higher FFA concentrations, the AcUCP contribution increases at the expense of ADP phosphorylation. These results demonstrate the efficiency of AcUCP diversion of energy from oxidative phosphorylation, especially when state 3 respiration is progressively inhibited.

3.3. Putative activation by ROS and lipid peroxidation products

Interestingly, in the mitochondria of *A. castellanii*, a high uncoupling effect attributed to AcUCP-mediated activity was observed with *all-trans* retinoic acid [20]. In animal mitochondria, products of lipid peroxidation, such as 4-hydroxy-2-nonenal (HNE) and structurally related compounds, *trans*-retinoic acid, *trans*-retinal and other 2-alkenals, specifically induce the uncoupling of mitochondria through the uncoupling proteins UCP1, UCP2 and UCP3 [41]. Similarly, induction of mitochondrial uncoupling by HNE and *trans*-retinal has been observed in plant mitochondria [42]. Moreover, it was shown that exogenously generated superoxide (in the presence of FFAs) activates UCPs in both animals and plants [43,44]. However, the current model (proposed for animal and plant mitochondria, [3,4]) for the activation of UCPs by superoxide through the initiation of lipid peroxidation (the HNE pathway), which leads to feedback down-regulation of mitochondrial ROS production, cannot yet be applied to the UCPs of unicellular eukaryotes. So far, there are no data showing induction of the activity of these proteins by exogenously generated superoxide. However, in the mitochondria of *A. castellanii*, HNE seems to induce PN-inhibited mitochondrial uncoupling (A. Woyda-Ploszczyca and W. Jarmuszkiewicz, unpublished data). In the mitochondria of unicellular eukaryotes, there is no data showing that, like in animal mitochondria [45], superoxide or other lipid derived regulators such as HNE could induce uncoupling through the ATP/ADP antiporter. As mentioned above, a contribution of this carrier in FFA-induced uncoupling has been excluded in several unicellular eukaryote mitochondria [9,10,14].

3.4. Inhibition by PNs: dependence on the redox state of membranous Q

Ubiquinone (coenzyme Q, Q) is a redox intermediate of the respiratory chain that translocates electrons from dehydrogenases (i.e., an electron entry, Q-reducing pathways) to the cytochrome pathway and an alternative oxidase (in plants, protists and some fungi) — the ubiquinol (QH_2)-oxidising pathways. Activation of UCP by FFAs leads to a decrease in the Q reduction level (QH_2/total Q) that can be restored by PN inhibition of UCP-mediated proton conductance [19,38]. In unicellular eukaryotes, FFA-induced mitochondrial uncoupling (UCP activity) is generally inhibited by PNs. However, in some cases, a decrease in membranous Q reduction level is required in order to observe this inhibition. In isolated mitochondria that are respiring in resting non-phosphorylating state (in the absence of cytochrome pathway inhibitors), UCPs of protists (*A. castellanii* and *D. discoideum*) show no detectable effect of 1–2 mM GTP/GDP on FFA-induced state 4 respiration and $\Delta\Psi$ [7,15,19]. Similarly, in phosphorylating (in the absence of cytochrome pathway inhibitors) *A. castellanii* mitochondria, no inhibitory effect of PNs on FFA-induced uncoupling (revealed as a decreased efficiency in oxidative phosphorylation) is observed [34,40]. However, in the mitochondria of the fungi *C. parapsilosis* and *A. fumigatus*, a significant sensitivity to GTP with almost full inhibition of FFA-induced state 4 respiration and restoration of $\Delta\Psi$ is observed [11,14]. These observations could suggest a difference in the sensitivity of fungal and protozoan UCPs to PNs. However, in the mitochondria of *A. castellanii* respiring in phosphorylating (state 3) or non-phosphorylating (state 4) respiration, linoleic acid-induced uncoupling is clearly inhibited by PNs when membranous Q is sufficiently oxidised [19,40]. The dependence of the sensitivity of FFA-induced UCP activity to PNs on Q redox state explains why, in some mitochondria including plant, animal and unicellular eukaryote mitochondria, weak or even lack of inhibition by PNs has been observed in resting respiration (uninhibited by the respiratory chain inhibitors) when the Q reduction level is high [46].

In state 3 measurements, the amplitude of the proton leak induced by FFAs can be determined by pair measurements of ADP/O ratios and respiratory rates in the absence or presence of a given fatty acid concentration when state 3 respiration is decreased (within the range where $\Delta\Psi$ remains constant) [37]. Substrate availability titration or cytochrome pathway activity titration allows the activities of the Q-reducing or ubiquinol (QH_2)-oxidising pathways (decrease or increase of membranous Q reduction level, respectively) to be varied experimentally. The FFA-induced UCP-mediated proton leak can be determined from the relationship between the ADP phosphorylation rate and the state 3 respiration rate. In phosphorylating *A. castellanii* mitochondria, a progressive decrease in the rate of Q-reducing pathways (hence a decrease in the Q reduction level) progressively leads from a non-inhibitory to a full inhibitory effect of GTP [40]. On the other hand, the inhibition of the linoleic acid-induced uncoupling by GTP is not observed in state 3 respiration that is progressively inhibited by inhibitors of complex III or IV, i.e., when the rate of the QH_2-oxidising pathway is decreased (hence the Q reduction level is increased). Moreover, in phosphorylating *A. castellanii* mitochondria isolated from cold-treated cells, where a higher AcUCP activity is observed, inhibition of the linoleic acid-induced proton leak by GTP is revealed for the same low value of the Q reduction level. These observations indicate that in phosphorylating *A. castellanii* mitochondria, the sensitivity of AcUCP activity to PNs depends on the redox state of membranous Q [40], as has been shown for UCP3 (rat skeletal muscle mitochondria) [37], UCP1

(heterologously expressed in yeast) [35] and plant UCP (potato tuber mitochondria) [39].

A common way to determine the activity of UCPs in non-phosphorylating mitochondria is to measure the proton conductance response to the driving force ($\Delta\Psi$), which is expressed as the relationship between the oxygen consumption rate and $\Delta\Psi$ (flux-force relationship) when the potential is varied by titration with respiratory chain inhibitors. In non-phosphorylating *A. castellanii* mitochondria, FFA-induced AcUCP-sustained state 4 respiration is not inhibited (or is weakly inhibited) by the addition of PNs in the absence of respiratory chain inhibitors [19]. However, when endogenous Q is sufficiently oxidised (by decreasing dehydrogenase activity), inhibition by PNs is revealed during a single simultaneous measurement of oxygen consumption and $\Delta\Psi$. This inhibition can be also detected when a flux-force relationship is established. A gradual decrease in the rate of Q-reducing pathways progressively leads to a decrease in the Q redox state and is accompanied by a full inhibitory effect of PN on FFA-induced proton conductance. The lack of the PN inhibitory effect observed when a decrease in respiratory rate is accompanied by an increase in the Q reduction level (titration of QH_2-oxidising pathway by inhibitors of complexes III or IV) proves that the inhibition by nucleotides only occurs for a low Q reduction level. These results demonstrate that in the mitochondria of *A. castellanii*, the redox state of endogenous Q does not affect the basal and FFA-induced UCP-mediated H^+ conductance, but does affect the sensitivity to PNs. This finding was based upon the determination of the membranous Q reduction level was performed when H^+ leak curves were established with inhibitors of the Q-reducing or QH_2-oxidising pathways. It must be noted that in non-phosphorylating *A. castellanii* mitochondria, the transition of the inhibitory effect of PNs on FFA-induced UCP-mediated uncoupling is observed for the same range of the Q reduction level (between 50% and 40%) [19] as that observed previously under phosphorylating conditions [40]. According to our experience, when studying UCP-mediated mitochondrial uncoupling, determination of proton conductance curves (flux-force relationships) should be based on data from separate measurements with different inhibitor concentrations (that are afterwards pooled into common curves) rather than those from sequential additions of inhibitors (sequential titration), in order to avoid possible errors due to non-steady-state conditions in the later case. It concerns all types of mitochondria, not only those in unicellular eukaryotes.

Determination of the membranous Q reduction level when H^+ leak curves (non-phosphorylating mitochondria) or relationships between the ADP phosphorylation rate and the state 3 respiration rate (phosphorylating mitochondria) are established with inhibitors of the Q-reducing or QH_2-oxidising pathways lead to the conclusion that the endogenous Q redox state has no effect on basal and FFA-induced UCP-catalysed H^+ conductance in the absence of PNs, but does affect the sensitivity to inhibition by nucleotides. However, further studies are required to determine whether the regulation observed in protist (*A. castellanii*), plant (potato tuber) and mammalian (rat skeletal and brown adipose tissue) mitochondria [19,37–40] can also be observed under conditions of UCP activation by superoxide. The Q redox state-dependent alleviation of UCP inhibition by PNs may be a general universal feature of all UCPs as it has been observed in the mitochondria of mammals, plants and unicellular eukaryotes [19,37–40]. The Q redox state-dependent sensitivity to PN of UCP in Amoebozoa (amoeboid eukaryote *A. castellanii*), which appear on a branch basal to the divergence points of plants, animals, and fungi in the molecular phylogenetic tree of eukaryotes [8], suggests that this phenomenon occurs in UCPs throughout the whole eukaryotic world. The finding that the redox state of Q modulates the sensitivity of FFA-induced UCP activity to PN (thereby meaning that UCPs are active at a high Q reduction level) provides an explanation for how UCPs could be activated *in vivo* despite the amount of PNs present in the cell (in millimolar concentration) [46]. Moreover, regulation by the Q reduction level explains why a PN-binding site is operational (no Q redox state influence) in reconstituted systems and why inhibition by PNs is weak or undetectable in some mitochondria, in which the Q reduction level is too high. Furthermore, through its redox state, coenzyme Q represents the ideal regulator as it is directly involved in sensing the ATP demand through the redox state of the respiratory chain. When ATP demand is high and an oxidisable substrate availability is low, PN inhibition could be turned on through a low reduced state of Q, leading to an inactivation of UCP and efficient ATP synthesis (Fig. 1). On the other hand, at low ATP demand and high oxidisable substrate availability, the Q reduced state is high and active UCP could work as a safety valve to avoid overload in reducing power and phosphate potential, allowing the Krebs cycle carbon flux and other catabolic pathways to proceed. Altogether, these observations strongly support the idea that UCPs control the energy balance of the cell.

The current model for the activation of UCPs by superoxide (which has not yet been shown for UCPs of unicellular eukaryotes) through initiation of lipid peroxidation [47,48] assumes that superoxide generated within mitochondria and a high membranous Q reduction level (as required for superoxide formation) work indirectly to activate UCP by generating carbon-centred radicals on the polyunsaturated fatty acid chains of phospholipids in the mitochondrial inner membrane. However, in our opinion, these indirect effects could be a late response of UCPs; flux-force studies with isolated mitochondria (of any type, plant or animal) do not reveal any effect of endogenously generated superoxide or the endogenous Q redox state on the basal or FFA-induced activity of UCP1 or its analogues in the absence of PNs. To observe indirect activation of UCPs by superoxide (or a high Q reduction level), lipid peroxidation products (such a HNE) or an exogenous system that generates superoxide (xanthine plus xanthine oxidase) must be applied. Our results obtained for *A. castellanii* mitochondria, as well as those for UCP1-containing yeast and BAT mitochondria and those for UCP1 analogues [19,37–40] indicate that the quick response through the endogenous Q redox state could directly regulate UCP activity. Thus, in our model of UCP activation [19,38], a high endogenous Q reduction level activates UCP by relieving inhibition from PNs, and this quick response does not involve superoxide formation resulting in lipid peroxidation products. At a given FFA concentration, alleviation of PN inhibition is dependent on the endogenous Q redox state. Certainly, further studies are necessary to elucidate the kinetic mechanism of this regulation. However, a model describing the function of reduced ubiquinone (QH_2) in modulating PN inhibition of UCPs can be proposed (Fig. 2). At a given FFA concentration, an increased concentration of membranous QH_2 could lead to a decreased binding affinity of PN, thereby alleviating inhibition of UCP. QH_2 may play a role as a negative regulator of PN binding to UCP. Conversely, at a lower QH_2 concentration, PN may bind to UCP, inhibiting UCP-mediated proton conductance.

3.5. Functional connection with alternative oxidase

In some unicellular eukaryotes, UCP coexists with an alternative oxidase (AOX), an enzyme specialised in redox free energy dissipation that is not present in animal mitochondria. Both energy-dissipating systems, AOX and UCP lead to the same final effect, i.e., a decrease in ATP synthesis yield. The alternative cyanide- and antimycin-resistant ubiquinol oxidase (AOX) consumes mitochondrial reducing power without energy conservation in $\Delta\mu H^+$. The activity of plant and non-plant AOXs depends on the Q redox state (QH_2 being a substrate) [49,50]. While the activity of AOX is stimulated by α-keto acids and is regulated by the redox state of an intermolecular disulphide bond (with the reduced state being more active) in plant mitochondria, these regulatory mechanisms do not apply to AOX in the mitochondria of protists and some primitive fungi [49,51]. In the mitochondria of these unicellular eukaryotes, cyanide-resistant respiration (AOX activity) is

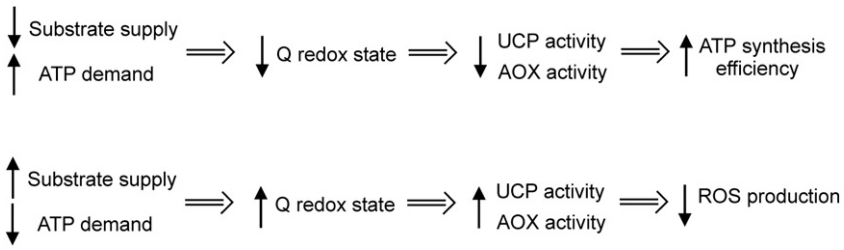

Fig. 1. Co-regulation of two energy-dissipating systems, UCP and AOX, in unicellular eukaryotes.

stimulated by purine nucleotides (guanosine or adenosine nucleoside mono-, di- or triphosphates) with the exception of ATP, which has an inhibitory effect [51]. So far, the simultaneous occurrence of these two energy-dissipating systems, UCP and AOX, has been reported in the amoeboid protists *A. castellanii* [7] and *D. discoideum* [15], the non-fermentative yeast *C. parapsilosis* [11,52], *C. albinicans* [12] and *Y. lipolytica* [13,53] and the filamentous multicellular fungi *A. fumigatus* [14]. Among protozoan parasites, the malaria parasite *P. yoelii yoelii* shows UCP but not AOX activity [10], while the nagana parasite *Trypanosoma brucei brucei* possesses AOX but not UCP [54,55]. Little is known about the physiological implications and regulation resulting from the interaction of AOX and UCP in unicellular eukaryotes. In plant mitochondria, FFAs that activate UCP inhibits the cyanide-resistant AOX-sustained respiration [56]. Contrary to plant mitochondria, the two energy-dissipating systems in the mitochondria of the amoeboid eukaryotes *A. castellanii* and *D. discoideum* are not co-regulated through FFAs, as shown by the insensitivity of AOX activity to inhibition by FFAs and the cumulative effect of the AOX and UCP activities on the efficiency of oxidative phosphorylation [7,15]. In the mitochondria of these organisms, the lowest ADP/O ratio was observed when both AOX and UCP were activated (by GMP and FFAs, respectively), indicating simultaneous action of both energy-dissipating systems *in vivo*. However, in the mitochondria of the yeast *C. parapsilosis*, AOX is inhibited by FFAs (although less efficiently than in plants), and as in plant mitochondria, AOX and UCP do not work together at their maximal capacities [11]. Inhibition of AOX with a high concentration of FFAs was also found in another fungus, *Hansenula anomala* [57]. Thus, it seems that the FFA-regulated interaction between UCP and AOX has evolved from cooperative energy dissipation in amoeboid protists, through a weak inhibition of AOX by UCP activators (FFAs) in fungi, to a strong inhibition in plants that excludes the simultaneous action of AOX and UCP at their maximal capacities [1,6,58–60].

Besides FFAs, PN are common regulators of both energy-dissipating systems in the mitochondria of unicellular eukaryotes. In free-living protists (*A. castellanii* and *D. discoideum*), FFA-insensitive PN (except for ATP)-stimulated AOX coexists with FFA-activated PN-inhibited UCP [7,15,51], while in non-fermentative yeast (*C. parapsilosis* and *C. maltosa*) FFA-inhibited PN (except for ATP)-stimulated AOX coexists with FFA-activated PN-inhibited UCP [51,52]. Recently it has been shown that fungal and protozoan AOXs are regulated by mutual exclusion of PNs, i.e. mutual exclusion of a negative allosteric effector (ATP) and positive allosteric effectors (GMP, GDP, GTP, AMP, and ADP) [51]. ATP, which inhibits both AOX and UCP in protists and fungi, seems to play a special role in the regulation of energy-dissipating systems and thereby in overall energy metabolism in unicellular eukaryotes. This regulation is likely related to respiratory control (through the ratio of the relative concentrations of ATP versus other nucleotides) and can considerably influence the yield of oxidative phosphorylation. This regulation could account for the commonly observed (also in microorganism mitochondria) enhancement of AOX-mediated respiration as well as that of UCP activity under conditions of stress, when the cytochrome pathway is impaired (and ATP production is limited). However, under physiological conditions where ATP production is not impaired in the mitochondria of unicellular eukaryotes, changes in the ATP concentration relative to the concentrations of other PNs (mainly guanine nucleotides) could allow for the rebalancing of the activities of the AOX, UCP and cytochrome pathway, depending on the energy and metabolic status of the cell (Fig. 1). AOX-sustained non-phosphorylating respiration and UCP-mediated mitochondrial uncoupling are likely inhibited by ATP when the oxidation of reduced equivalents by these energy-dissipating systems is not strongly required (e.g., during the late

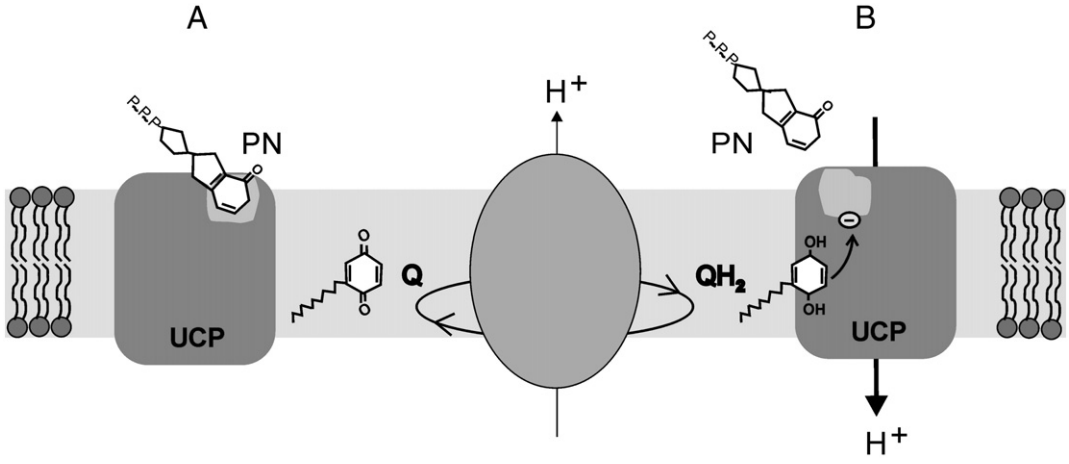

Fig. 2. Model for the regulation of UCPs (including UCPs of unicellular eukaryotes) by the membranous Q redox state. A high endogenous Q reduction level activates UCP by relieving the inhibition caused by PNs. At a given fatty acid concentration, (A) at a low QH_2 concentration, negative regulation of PN-binding site does not occur, and proton conductance through UCP is inhibited by PN. (B) On the contrary, an increased concentration of membranous QH_2 leads to a decrease in the binding affinity of PN (GTP is shown) thereby alleviating the inhibition of UCP activity by the nucleotide. As a result, UCP-mediated H^+ re-uptake is enhanced.

stationary phase of growth when cell division slows). In contrast, at low ATP concentrations, overcoming concentrations of other PNs (mainly GMP and AMP, which are good activators of AOX but poor inhibitors of UCP in unicellular eukaryotes) could relieve the inhibition by ATP, leading to the activation of AOX and UCP in order to prevent the formation of ROS by the mitochondria and/or to decrease the reducing power in the cell. These conditions can occur in intensively dividing and metabolising cells, during the exponential growth phase for example. Studies with *A. castellanii* confirm these considerations. In particular, it has been shown that during the growth of *A. castellanii* in batch culture, ATP content increases exponentially in amoeba cells (approximately 20-fold by stationary phase) [61]. This increase is accompanied by a decrease in cyanide-resistant (unstimulated and GMP-stimulated) AcAOX-mediated respiration and FFA-stimulated AcUCP activity as well as the amount of both proteins observed in amoeba mitochondria [58]. Interestingly, in the mitochondria of *A. castellanii*, ATP also inhibits the activity of another mitochondrial energy-dissipating system, i.e., the mitochondrial ATP-regulated potassium channel (mitoK_{ATP}) [59]. So far, there is little information about potassium transport systems in the mitochondria of unicellular eukaryotes. *A. castellanii* is the only microorganism in which the mitochondrial ATP-regulated potassium channel, described to a greater extent in animal and plant mitochondria, has been characterised [63,64].

4. Physiological role

4.1. No heat production

Heat production is an obligatory side effect event of free energy dissipation, but is not useless when linked to an increase in temperature (thermogenesis). The widespread presence of UCPs in eukaryotes, i.e., in non-thermogenic tissues of animals and plants and in unicellular organisms implies that these proteins may elicit functions other than thermogenesis, which is characteristic for UCP1 in mammalian brown adipocytes. However, the physiological functions of UCP1 analogues, including UCPs of unicellular eukaryotes, are still under debate. An increase in the activity and expression of UCP observed in *A. castellanii* mitochondria after the growth of the amoeba cells at low temperature (6 °C) resembling events in mammalian brown fat during cold adaptation cannot be related to thermogenesis [26]. Undoubtedly, a thermoregulatory role of UCP in unicellular eukaryotes can be excluded because of their microscopic size, which prevents any thermal gradient between the cell and the external environment [6,59]. It can be reasoned that no steady-state local heating could occur in the cytosol of unicellular organisms as heat diffusion is too fast due to the thermal conductivity of the surrounding medium. Moreover, the contribution of UCP to thermogenesis (an increase in the temperature of an organ/organism) also depends on the net increase of the overall steady-state mitochondrial oxygen uptake (i.e. the rate of the oxidative reactions) due to an increase in the amount of protein (translational up-regulation) or post-translational activation of proteins [6,58]. For example, in the mitochondria of *A. castellanii*, maximal phosphorylating (state 3) respiration, uncoupled respiration (in the presence of the uncoupler, protonophore FCCP) and maximally FFA-stimulated UCP-mediated respiration are the same, both in control and cold-treated cells (with increased UCP protein level and activity) [7,26]. It signifies that the overall oxygen uptake is kinetically limited by the respiratory chain capacity, which could also limit thermogenesis *in vivo* when a shift occurs from fully phosphorylating respiration to respiration fully uncoupled by UCP. Therefore, the increase in activity and expression of *A. castellanii* UCP (but also the cyanide-resistant AOX [65]) after cold stress cannot be related to thermogenesis. Thus, in unicellular eukaryotes, heat production by energy-dissipating systems (UCP and AOX) may only be a minor side event.

4.2. Metabolic and energy balance

By modulation of the mitochondrial $\Delta\mu H^+$, microorganism UCPs can modulate the tightness of coupling between mitochondrial respiration and ATP synthesis, thereby maintaining a balance between energy supply and demand in the cell. In amoeba *A. castellanii* cells, UCP could be considered as a response protein to cold exposure (translational regulation), allowing an increase in oxygen consumption (and thereby a decrease in oxidative phosphorylation coupling), and leading to improved biosynthesis and growth at a low external temperature but at the expense of the oxidative phosphorylation yield [26]. Amoeba UCP could have a subtle role (which is more pronounced during chilling) in energy metabolism control, working as a safety valve when overloads occur in redox potential and/or phosphate potential [6,58]. Operation of *A. castellanii* UCP, which is increased during low temperature exposure, could diminish phosphate potential as well as reducing power by diverting energy from oxidative phosphorylation and increasing the electron flux in the respiratory chain, which is released from $\Delta\mu H^+$ control. Thus, the increased *A. castellanii* UCP activity (resulting from increased protein level) could possibly play a more pronounced role during chilling, for instance in maintaining the metabolic and energy balance of the cell by modulation of NAD(P)H reoxidation under conditions of low ATP demand as well as in defence against the production of toxic ROS.

4.3. Defence against ROS production

Conditions that decrease the reduction level of mitochondrial electron carriers lead to a decrease in damaging ROS production [66] as shown for UCP1 analogues in mammalian and plant mitochondria [3,4]. In unicellular eukaryotes, two energy-dissipating activities decrease the mitochondrial production of ROS: AOX, through the oxidation of ubiquinol, and UCP, through a decrease in $\Delta\mu H^+$, thus accelerating the respiratory chain. It has been shown that the mitochondria of unicellular organisms, such as the amoeba *A. castellanii* can prevent ROS formation by FFA-uncoupled (AcUCP-mediated) respiration as well as by non-coupled (AcAOX-sustained) respiration [67]. In the mitochondria of *A. castellanii*, the inhibition of AcUCP (by GDP) and/or AcAOX (by benzohydroxamate) increased ROS production, while the activation of UCP (by FFAs) and/or AOX (by GMP) decreased ROS levels. Therefore, it seems that protection against mitochondrial ROS production may be a physiological role of UCP (and AOX) in all of the eukaryotes that possess these proteins, including protists and fungi. This role could become especially important when the ROS level increases under stress conditions. For instance, it has been reported that the activity and protein level of both AcUCP and AcAOX is enhanced by growth of *A. castellanii* cells at low temperature that is likely to be accompanied by a higher level of cellular ROS generation [25]. Thus, in unicellular eukaryotes, UCP (and AOX) are response proteins that can act as antioxidant systems to prevent damage to the cell at the level of energy production but at the expense of oxidative phosphorylation yield. In the mitochondria of *A. castellanii*, the two energy-dissipating systems lead to the same final energetic effect (i.e., a decrease in ATP synthesis) [7,34,40,68] as well as lower ROS production [62,67]. Moreover, they can cumulate both effects [7,62,67]; it therefore seems that their metabolic usefulness might lie in complementing these functions. Indeed, in the mitochondria of *A. castellanii*, a decrease in the activity and protein level of both AcAOX and AcUCP was observed when the cells shifted from the exponential growth phase to the stationary phase [62]. It is likely that depending on their expression level, AcAOX and AcUCP in a given phase of batch culture of amoeba may be engaged to different extents in the maintenance of cell energy and/or in the limitation of mitochondrial ROS production. In the latter case, at each phase of growth, activation of the two energy-dissipating systems led to a decrease in mitochondrial H_2O_2 formation [62]. Surprisingly, it

became apparent that compared to AcAOX, AcUCP is more efficient *in vitro* in decreasing H_2O_2 formation. However, when AcAOX and AcUCP were simultaneously and maximally stimulated (by GMP and FFAs, respectively), a stable level of H_2O_2 formation was observed. This finding indicates that the contribution of both energy-dissipating systems in the prevention of mitochondrial ROS generation *in vivo* could lead to its constant level throughout the growth cycle of *A. castellanii* batch culture and could insure survival of high quality cysts.

In the case of the opportunistic pathogen fungus *C. albinicans*, it has been suggested that the joint presence of an alternative respiratory chain and UCP results in an increased adaptability to adverse host environments [69]. *C. albinicans* seems to depend on both AOX and UCP to increase its resistance to ROS and enhance its invasiveness.

5. Conclusion

The present overview describes the last 10 years of studies on UCPs in the mitochondria of unicellular eukaryotes. The physiological roles of fungal and protozoan UCPs fall into two categories: (1) control of ROS production by mitochondria, which is related to stress response, and (2) regulation of energy metabolism. In each case, the role of UCPs of unicellular eukaryotes is related to both the capacity of mitochondrial uncoupling (dependent on the UCP expression level and the level of UCP regulators) and the metabolic status of the cell (ATP demand or availability). As a consequence, at a given stage in the cell growth cycle of unicellular organisms, interplay between the availability of non-esterified FFAs (as activators) and PNs (as inhibitors) and the redox state of membranous Q (a modulator of PN inhibition) determines the activity of UCP. It is worth noting that the same regulators determine the activity of cyanide-resistant ubiquinol oxidase (AOX) in unicellular eukaryotes. Non-esterified FFAs are inhibitors of fungal AOX (but not protozoan AOX); PNs (except for ATP, which acts as inhibitor) are activators of AOX and QH_2 is its substrate in the mitochondria of unicellular eukaryotes.

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