Minireview

Uncoupling proteins outside the animal and plant kingdoms: functional and evolutionary aspects

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Abstract The appearance of intracellular oxidative phosphorylation at the time of acquisition of mitochondria in Eukarya was very soon accompanied by the emergence of uncoupling protein, a carrier specialized in free fatty acid-mediated H^+ recycling that can modulate the tightness of coupling between mitochondrial respiration and ATP synthesis, thereby maintaining a balance between energy supply and demand in the cell and defending cells against damaging reactive oxygen species production when electron carriers of the respiratory chain become overreduced. The simultaneous occurrence of redox free energy-dissipating oxidase, which has the same final effect, could be related to the functional interactions between both dissipative systems.

Key words: Mitochondrion; Uncoupling protein; Alternative oxidase; *Acanthamoeba castellanii*; *Candida parapsilosis*; *Dictyostelium discoideum*

1. Introduction

Uncoupling proteins (UCPs) form a subfamily within the mitochondrial anion carrier protein (MACP) family. They catalyze a protonophoretic cycle activated by free fatty acids (FFA) and dissipate a H^+ electrochemical gradient ($\Delta\tilde{\mu}H^+$) built up by the mitochondrial respiratory chain thereby uncoupling respiration from phosphorylation. Thus, a direct consequence of their activity is a decrease in ATP synthesis per oxygen consumed (yield of oxidative phosphorylation). Before 1995, the uncoupling protein of mammalian brown adipose tissue (UCP1) was believed to be a late evolutionary acquisition required for non-shivering transient thermogenesis and restricted to newborn, cold-acclimated and hibernating mammals [1]. Discovery of plant uncoupling protein (PUMP) in potato tubers in 1995 by Vercesi et al. [2], confirmed by the cloning of its cDNA [3], has suggested that the ancestral gene of UCP probably evolved prior to the divergence into metazoan and plant clades. More recent the discovery of several novel UCPs in various mammalian tissues showed that UCP is more widespread in tissues of higher animals than previously believed and that it could have various physiological roles [4–8]. PUMP is also rather ubiquitous in plants and widespread in various plant tissues [9]. Moreover, the discovery of UCP in *Acanthamoeba castellanii* [10], a non-photosynthetic soil amoeboid protozoan, in *Candida parapsilosis* [11], a parasitic non-fermentative yeast, and in *Dictyostelium discoideum* (W. Jarmuszkiewicz, unpublished data), a mycetozoan (cellular slime mold) undergoing multicellularity and cell differentiation upon starvation, indicates that UCPs, as specialized proteins for FFA-linked H^+ recycling, emerged very early during phylogenesis before the major radiation of phenotypic diversity in eukaryotes and could occur in the whole eukaryotic world. Except for *D. discoideum* [12], indications that UCP is present in the mentioned unicellulars are mainly based on functional studies (see below) and cross-reactivity of around 30 kDa mitochondrial protein with polyclonal antibodies developed against plant UCPs. Genes of *A. castellanii* and *C. parapsilosis* UCPs have not yet been isolated and sequenced.

2. Phylogenetic positions of *A. castellanii* and *D. discoideum*

Molecular phylogenies are far from fully reliable in building phylogenetic trees as different trees can be obtained according to the used sequence comparison. Trees based on ribosomal RNA sequence comparison [13,14] and on protein sequence comparison (EF-1α [15], actin [16,17], ADP-ATP carrier [18]) lead to quite confusing conclusions not only concerning *D. discoideum* but also grouping of the three main clades (plants, fungi and animals). In ribosomal RNA tree, *A. castellanii* appears on a branch basal to the divergence points of plants, animals and fungi, and *D. discoideum* appears much earlier in the tree, soon after the acquisition of mitochondria in Eukarya. In the actin and EF-1α trees, plants separate first from the 'trunk' before the 'super-clade' including animal clade, fungi clade and mycetozoa group with *D. discoideum* and *A. castellanii*. The ADP-ATP carrier tree provides another view of the eukaryotic world with the plant–fungal 'super-clade' excluding metazoa and *D. discoideum*. Thus, in protein phylogenies *A. castellanii* and/or *D. discoideum* are placed as the immediate

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Abbreviations: AOX, alternative oxidase; EF-1α, protein synthesis elongation factor; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; FFA, free fatty acids; LA, linoleic acid; MACP, mitochondrial anion carrier protein; PUMP, plant uncoupling mitochondrial protein; ROS, reactive oxygen species; state 3, phosphorylating respiration in the presence of ADP; state 4, resting respiration in the absence of added ADP; UCP, uncoupling protein; UCP1, uncoupling protein of brown adipose tissue; $\Delta\tilde{\mu}H^+$, proton electrochemical gradient

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outgroup to either the animal–fungal clade (actin and EF-1α trees) or to the animal clade (ADP-ATP tree).

3. Functional properties

The action of UCP is to mediate FFA-activated, purine nucleotide-inhibited H^+ re-uptake driven by membrane potential and pH (both constituting $\Delta\tilde{\mu}H^+$). Stimulation of UCP by FFA results in an increase in mitochondrial resting respiration (state 4) and in a decrease in membrane potential. The concentration of linoleic acid (LA) that leads to half maximal stimulation of UCP-sustained state 4 respiration is the same (8–10 μM) for *A. castellanii* [10], *C. parapsilosis* [11] and *D. discoideum* (W. Jarmuszkiewicz, unpublished data) and also when compared to plants (10 μM) [19,20]. The voltage dependence of electron flux in the mitochondria of *A. castellanii*, *C. parapsilosis* and *D. discoideum* shows that LA-induced respiration is only due to proton recycling by UCP as it corresponds to a pure protonophoretic effect of LA not distinguishable from the effect of a well known protonophore, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP). Couples of membrane potential and respiratory rate measurements in the presence of benzohydroxamate (an inhibitor of alternative oxidase), in state 4 with increasing concentrations of FCCP, in state 4 with increasing concentrations of LA and in phosphorylating respiration (state 3) with increasing concentrations of oligomycin or carboxyatractyloside (inhibitors of ATP synthesis) constitute a single force-flow relationship indicating that LA does not interact with the respiratory chain.

In animal mitochondria, FFA-induced mitochondrial uncoupling can be mediated, at least in part, by several other members of the MACP family like the ATP/ADP antiporter, the aspartate/glutamate antiporter, the dicarboxylate carrier, and the phosphate carrier [21–26]. This uncoupling is exclusively observed in resting respiration of oligomycin-treated mitochondria (thus at high $\Delta\tilde{\mu}H^+$) and can be reversed to some extent by millimolar purine nucleotides (in some tissues). However, it has never been shown that this UCP-independent FFA-induced uncoupling occurs during state 3 respiration and is able to decrease efficiency of oxidative phosphorylation (ADP/O). Moreover, translocated substrates or specific inhibitors of the implicated carriers inhibit this uncoupling. Thus, it is unlikely that mitochondrial uncoupling mediated by these carriers occurs during phosphorylating respiration (at lower $\Delta\tilde{\mu}H^+$), when they are mainly employed in import of ADP, dicarboxylates or phosphate. Although like in plant mitochondria [20], state 3 respiration in *A. castellanii* [10], *C. parapsilosis* [11] and *D. discoideum* (W. Jarmuszkiewicz, unpublished data) mitochondria is never increased by FFA addition, this does not mean that FFA cannot induce UCP activity under phosphorylating conditions in these mitochondria but that the respiratory chain is at its maximal rate (limiting step of the whole process). Indeed, we have shown that $\Delta\tilde{\mu}H^+$ is shared between oxidative phosphorylation and UCP in state 3 after addition of LA. The LA concentration-dependent decrease in the ADP/O ratio clearly indicates a participation of UCP in state 3 respiration leading to a strong decrease in the efficiency of oxidative phosphorylation for a low LA concentration [10,11,27]. Thus UCP activity can divert energy from oxidative phosphorylation in state 3 respiration.

Concerning the sensitivity of UCP to purine nucleotides in isolated respiring mitochondria, UCPs of *A. castellanii* [10] and *D. discoideum* (W. Jarmuszkiewicz, unpublished data) seem to be close to PUMP with no detectable effect of 1–2 mM GTP or ATP, if added before addition of bovine serum albumin (which chelates FFA). However, in mitochondria of *C. parapsilosis*, UCP reveals a significant sensitivity with an almost full inhibition of FFA-induced state 4 respiration and a membrane potential restoration at 2 mM GTP in the absence of albumin [11]. This means that the sensitivity of UCP to purine nucleotides could be a progressive evolutionary acquisition which appeared in fungal-type UCP and improved in animal UCP, and that UCPs of *A. castellanii*, *D. discoideum* and plants kept similar regulatory properties.

4. Functional interaction with alternative oxidase

Animal mitochondria have no enzyme specialized in redox free energy dissipation compared to mitochondria of plants, many fungi and some protists where such enzymes exist (i.e. ubiquinol cyanide-resistant alternative oxidase, AOX, and rotenone-insensitive NADH dehydrogenases) [28]. The simultaneous occurrence in mitochondria of *A. castellanii*, *D. discoideum*, *C. parapsilosis* and plants of two energy-dissipating systems, UCP which dissipates $\Delta\tilde{\mu}H^+$ and AOX which dissipates redox energy instead of building $\Delta\tilde{\mu}H^+$, is quite amazing, especially the fact that both lead to the same final effect (i.e. decrease in the yield of ATP synthesis). In plant mitochondria, it has been shown that the AOX and UCP activations do not have an additive effect on the yield of oxidative phosphorylation [20]. This observation has been explained by the discovery of AOX inhibition by LA ($I_{0.5} = 4$ μM) [19]. On the other hand, in *A. castellanii* [10] and *D. discoideum* (W. Jarmuszkiewicz, unpublished data), the activities of AOX and UCP have an additive effect on the yield of mitochondrial oxidative phosphorylation. This results from the lack of inhibition of AOX by LA in mitochondria of these organisms allowing the two energy-dissipating systems to work together to divert energy from oxidative phosphorylation. In *C. parapsilosis* mitochondria, AOX is inhibited by FFA ($I_{0.5} = 33$ μM, so less efficiently than in plants) and like in plant mitochondria AOX and UCP do not work together at their maximal capacities [11]. Inhibition of AOX by high concentration of FFA was also found in another fungus, *Hansenula anomala* [29]. Thus, it seems that FFA-regulated interaction between AOX and UCP has also evolved: strong inhibition of AOX in the presence of activator of UCP in plants, less dramatic in fungi and cooperation in energy dissipation between AOX and UCP in mycetozoa and *A. castellanii*. Thus, for this property *D. discoideum* and *A. castellanii* are more distant from plants compared to fungi.

5. Putative role

5.1. Heat production

Heat production is a side event of free energy dissipation but it is not useless when linked to an increase in temperature (thermogenesis), which provides better intracellular thermal surroundings in poikilotherms. This idea is related to cold stress responses in plants involving an increase in expression of both proteins, AOX and UCP [3,30–32]. However, this thermal response to environmental thermal pressure does

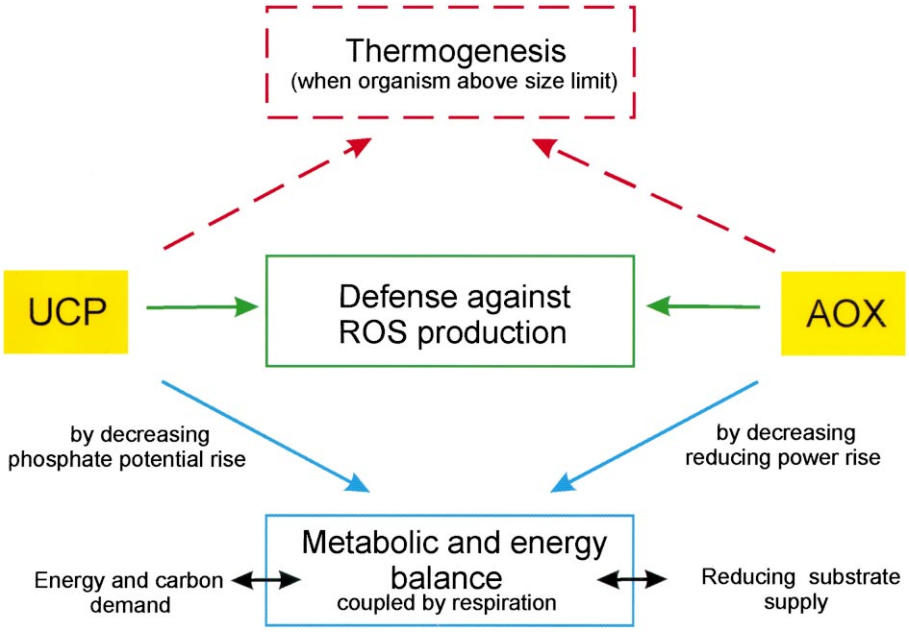

Fig. 1. Roles of UCP and AOX.

not apply to unicellulars because of their size which prevents any thermal gradient as a fast heat diffusion in surrounding medium occurs. Moreover, the effect of AOX or UCP activation on heat production (increase) in a given constant metabolic steady state in vivo implies that different oxidative pathways have large differences in enthalpy changes, i.e. more exothermic for AOX- and UCP-sustained respiration compared to ATP synthesis-sustained respiration. Combined calorimetric and respirometric data invalidate this idea [33]. Therefore, every increase in heat production must come from an overall increase in rate of oxidative reactions (respiration) due to an increase in protein amount (translational upregulation) or to posttranslational activation of enzymes. Thus, thermogenesis through UCP and AOX is subject to a lower-size limit and depends on the net increase in overall steady-state oxygen uptake (Fig. 1). Therefore, for instance, the increase in activity and expression of AOX observed in *A. castellanii* after cold stress [34] cannot be related to thermogenesis.

5.2. Protection against oxygen free radicals

Aerobic cell energy is generated mostly by the mitochondrial respiratory chain where a four-electron reduction of oxygen occurs. However, reactive oxygen species (ROS) arise from the partial reduction of molecular oxygen at the level of respiratory complexes utilizing or producing ubiquinol [35,36]. ROS can damage, as first targets, mitochondrial proteins, DNA and functions [37,38]. Conditions that increase or decrease the reduction level of mitochondrial electron carriers lead to an increase or decrease in damaging ROS production, respectively [39]. Energy-dissipating systems like AOX, which oxidizes ubiquinol in a way insensitive to the phosphate potential back pressure, and like UCP, which dissipates $\Delta\tilde{\mu}H^+$ allowing an increase in electron flux at the expense of ubiquinol, have been shown to decrease ROS production in vitro [40,41] and in vivo [42]. Therefore, UCP and AOX can be considered additional endogenous antioxidant systems preventing damage of the cell at the level of energy production but at the expense of oxidative phosphorylation yield (Fig. 1).

5.3. Metabolic and energy balance

Energy-dissipating systems could have a subtle role in energy metabolism working as safety valves when overloads occur in reducing power or in phosphate potential (Fig. 1) [43]. These overloads are consequences of imbalance between reducing substrate supply and energy plus carbon demand for biosynthesis, both being coupled by the respiratory chain activity. UCPs consume H^+ gradient when activated by FFA and consequently decrease phosphate potential as well as reducing power by diverting energy from oxidative phosphorylation and by increasing the electron flux in the respiratory chain freed from $\Delta\tilde{\mu}H^+$ control, respectively. Activation of AOX, which is not controlled by energy status of the cell, directly decreases the reducing power.

6. Are these UCPs 'true UCPs'?

The question is not trivial and it surpasses, in our opinion, a simple semantic problem. As properly pointed out by Bouillaund et al. [44], it is an over-simplification to consider the uncoupling of mitochondria solely in terms of heat production as the widespread occurrence of UCPs questions the role of such proteins in thermogenesis. As mentioned above, first, uncoupling is not synonymous to heat production which in turn is not synonymous to thermogenesis, and second, partial uncoupling may have several roles in the cell, like protection against ROS production and maintaining metabolic balance. Thus, 'to be or not to be true UCP' is not linked to the ability to lead to thermogenesis but depends on precise molecular and functional properties.

MACPs, to which UCPs belong as a subfamily, are homologous proteins with a threefold sequence repeat of about 100 residues forming six transmembrane α-helices and with a MACP signature sequence [45]. Sequence analysis of the UCP subfamily revealed their common specific sequence mo-

tifs, called UCP signatures, which do not exist in the other MACPs [46]. The presence of these signatures allowed an attempt at UCP phylogenesis description including UCP of *D. discoideum* [12].

Thus, in our opinion, a safe functional definition of 'true UCPs' would be the following: UCPs are able, in the presence of low FFA concentrations, to divert energy from ATP synthesis in state 3 respiration, with a decrease in ADP/O ratio, without necessarily an increase in respiration and decrease in membrane potential. In state 4 respiration, in the presence of carboxyatractyloside, high phosphate and dicarboxylate concentrations (to avoid participation of the ADP-ATP, phosphate and dicarboxylate carriers in uncoupling) and in the presence of a low FFA concentration, UCPs are able to stimulate reversibly oxygen uptake and to decrease membrane potential. Therefore, accordingly UCPs of *A. castellanii*, *D. discoideum* and *C. parapsilosis* are true UCPs. Of course, molecular mechanism and three-dimensional structure are needed for a better understanding of structure–function relationships in the UCP subfamily.

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