

Identification and Characterization of a Protozoan Uncoupling Protein in *Acanthamoeba castellanii**

(Received for publication, April 21, 1999)

Wiesława Jarmuszkiewicz[‡]¶, Claudine M. Sluse-Goffart[§], Lilla Hryniewiecka[‡],
and Francis E. Sluse[‡]

From the [‡]Department of Bioenergetics, Adam Mickiewicz University, Fredry 10, 61-701 Poznań, Poland and the
[§]Laboratory of Bioenergetics, Centre of Oxygen Biochemistry, University of Liege, Sart-Tilman, B-4000 Liege, Belgium

An uncoupling protein (UCP) has been identified in mitochondria from *Acanthamoeba castellanii*, a non-photosynthetic soil amoeboid protozoon that, in molecular phylogenesis, appears on a branch basal to the divergence points of plants, animals, and fungi. The existence of UCP in *A. castellanii* (AcUCP) has been revealed using antibodies raised against plant UCP. Its molecular mass (32,000 Da) was similar to those of plant and mammalian UCPs. The activity of AcUCP has been investigated in mitochondria depleted of free fatty acids. Additions of linoleic acid stimulated state 4 respiration and decreased transmembrane electrical potential ($\Delta\Psi$) in a manner expected from fatty acid cycling-linked H^+ reuptake. The half-maximal stimulation by linoleic acid was reached at $8.1 \pm 0.4 \mu M$. Bovine serum albumin (fatty acid-free), which adsorbs linoleic acid, reversed the respiratory stimulation and correspondingly restored $\Delta\Psi$. AcUCP was only weakly inhibited by purine nucleotides like UCP in plants. A single force-flow relationship has been observed for state 4 respiration with increasing concentration of linoleic acid or of an uncoupler and for state 3 respiration with increasing concentration of oligomycin, indicating that linoleic acid has a pure protonophoric effect. The activity of AcUCP in state 3 has been evidenced by ADP/oxygen atom determination. The discovery of AcUCP indicates that UCPs emerged, as specialized proteins for H^+ cycling, early during phylogenesis before the major radiation of phenotypic diversity in eukaryotes and could occur in the whole eukaryotic world.

Until the discovery of plant uncoupling mitochondrial protein (PUMP)¹ in potato tubers by Vercesi *et al.* (1), the mammalian uncoupling protein (UCP) present in brown adipose tissue (UCP1) was believed to be a late evolutionary acquisition required especially for transient thermogenesis in newborn, cold-acclimated and hibernating mammals (2). The recent dis-

covery of several novel uncoupling proteins (UCP2, UCP3, and UCP4) in various mammalian tissues showed that UCP is more widespread in tissues of higher animals than previously believed and could have various physiological roles (3–5). PUMP has also been shown to be rather ubiquitous in plants. It has been isolated from potato and tomato (6) and immunologically detected in several climacteric fruits (*i.e.* those that exhibit a respiratory burst during ripening) (7). Characterization and expression of UCP-like genes in plants has also been addressed for instance in various tissues of potato (8) and in *Arabidopsis thaliana* (9). The presence of uncoupling protein in plants seems to be widespread, which may suggest various physiological roles for instance in fruit ripening (10, 11) and in oxidative damage protection against oxygen free radicals (12).

UCPs are located in the inner mitochondrial membrane, and their activity dissipates the proton electrochemical gradient built up by respiration and produces heat instead of ATP. The action of UCPs is to mediate free fatty acid (FFA) cycling (13) by exporting anionic FFA outside the mitochondria, where it becomes protonated (neutral) and rapidly recrosses the membrane with its proton. This FFA-cycling H^+ reuptake is driven by transmembrane electrical potential ($\Delta\Psi$, negative inside) and pH difference acidic outside. UCPs are activated by FFA (substrate activation by anionic FFA) and are allosterically inhibited by purine nucleotides (7). For instance, in tomato mitochondria, the addition of linoleic acid (LA) results in mitochondrial uncoupling, as revealed by an increase in cyanide-sensitive state 4 respiration and a decrease in $\Delta\Psi$ (14). The apparent K_m for LA-induced respiration (*i.e.* LA concentration that produces 50% stimulation) is about $10 \mu M$ (11). On the other hand, the addition of GTP, which inhibits PUMP, and bovine serum albumin (BSA, free of fatty acids), which removes FFA, cancels LA-induced respiration and restores $\Delta\Psi$. It has been shown that LA-induced PUMP activity is present during phosphorylating respiration and is able to divert energy from oxidative phosphorylation (14). Moreover, LA-induced stimulation of respiration was due to a pure protonophoric activity without any direct effect on the electron transport chain (14).

The presence of uncoupling proteins in plant and mammalian mitochondria has suggested that the ancestral gene has probably evolved prior to the divergence into animals and plants. In this work, we have identified immunologically and characterized functionally an uncoupling protein in mitochondria from *Acanthamoeba castellanii*, a nonphotosynthetic soil amoeboid protozoon, which in molecular phylogenesis appears on a branch basal to the divergence points of plants, animals, and fungi (15, 16). This discovery proves that UCPs are much more widespread than previously believed and could occur in the whole eukaryotic world.

* This work was supported by the Polish-Belgian Joint Research Program. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¶ To whom correspondence should be addressed: Dept. of Bioenergetics, Adam Mickiewicz University, Fredry 10, 61-701 Poznań, Poland. Tel.: 48-61-8522061; Fax: 48-61-8523615; E-mail: wiesiaj@main.amu.edu.pl.

¹ The abbreviations used are: PUMP, plant uncoupling mitochondrial protein; UCP, uncoupling protein; UCP1, uncoupling protein of brown adipose tissue mitochondria; AcUCP, uncoupling protein of *A. castellanii*; FFA, free fatty acids; $\Delta\Psi$, mitochondrial transmembrane electrical potential; LA, linoleic acid; BSA, bovine serum albumin; BHAM, benzohydroxamate; AOX, alternative oxidase; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; ADP/O, ADP/oxygen atom.

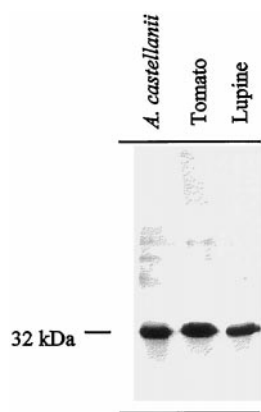


FIG. 1. Immunological identification of AcUCP protein in amoeba *A. castellanii* mitochondria. Detection of PUMP in mitochondria isolated from yellow lupine cotyledons and green tomato fruit is also shown. Primary antibodies were raised against potato uncoupling protein. In all cases, 100 μ g of protein was loaded per lane.

MATERIALS AND METHODS

Cell Culture and Mitochondrial Isolation—Soil amoeba *A. castellanii*, strain Neff, was cultured as described by Jarmuszkiewicz *et al.* (17). Trophozoites of amoeba were collected 22–24 h following inoculation at the middle exponential phase (at a density of about $2-4 \times 10^6$ cells/ml). Mitochondria were isolated and purified on a self-generating Percoll gradient (31%) as described before (17). Mitochondria of green tomato fruits and yellow lupine cotyledons were isolated and purified according to Sluse *et al.* (11) and Tomaszewska *et al.* (18), respectively. Mitochondrial protein concentration was determined by the biuret method (19).

SDS-Polyacrylamide Gel Electrophoresis and Immunoblotting of Uncoupling Protein—Up to 100 μ g of mitochondrial protein was solubilized in the sample buffer containing 1% (w/v) SDS, 0.1 M Tris-HCl, pH 6.8, 10% glycerol, 0.005% (v/v) bromphenol blue, and 0.5% β -mercaptoethanol and boiled for 4–5 min. SDS-polyacrylamide gel electrophoresis was carried out in a manner similar to that of Laemmli (20) using a 5% polyacrylamide stacking gel and a 12% polyacrylamide resolving gel containing 4.5 M urea, followed by Western blotting according to Almeida *et al.* (10). Antibodies against PUMP protein of potato (*Solanum tuberosum*) (generously supplied by Dr. P. Arruda) were used at a dilution of 1:1000. The bands were detected by chemiluminescence (Amersham Pharmacia Biotech ECL system).

Oxygen Uptake and Membrane Potential—Oxygen uptake was measured polarographically using a Rank Brothers (Cambridge, United Kingdom) oxygen electrode in 2.7 ml of standard incubation medium (25 °C) containing 120 mM KCl, 20 mM Tris-HCl, pH 7.4, 3 mM KH_2PO_4 , and 8 mM MgCl_2 , with 1.5–1.7 mg of mitochondrial protein. The membrane potential of mitochondria was measured simultaneously with oxygen uptake using a tetraphenylphosphonium-specific electrode according to Kamo *et al.* (21). For calculation of $\Delta\Psi$ value, the matrix volume of amoeba mitochondria was assumed as $2.0 \mu\text{l} \times \text{mg}^{-1}$ protein. All measurements were made in the presence of 10 mM succinate, 1.5 mM benzohydroxamate (BHAM), 170 μM ATP, and 15 μM rotenone.

The ADP/O ratio was determined by an ADP (130 μM) 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. Measurements of $\Delta\Psi$ allowed the fine control of the duration of state 3 respiration.

Chemicals—Tetraphenylphosphonium was obtained from Fluka. All other chemicals were purchased from Sigma.

RESULTS

Immunological Detection of *A. castellanii* Uncoupling Protein—Immunoblotting of the total mitochondrial protein allowed immunological detection of *Acanthamoeba castellanii* UCP (AcUCP) with antibodies developed against potato PUMP protein. A single protein band with a molecular mass of approximately 32,000 Da is revealed in *A. castellanii*, in green tomato fruit, and in yellow lupine cotyledons, indicating cross-reaction of plant antibodies with amoeba protein (Fig. 1).

Respiratory Rate and Membrane Potential Measurements in *A. castellanii* Mitochondria—Simultaneous measurements of

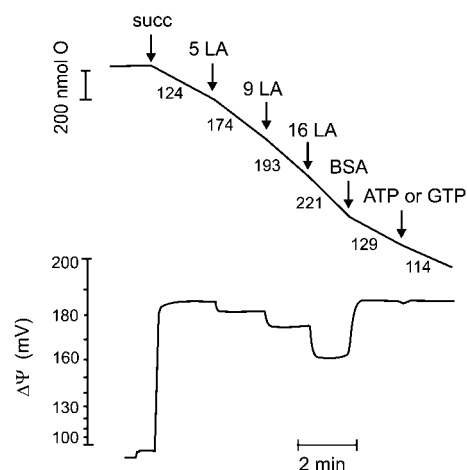


FIG. 2. Influence of LA and purine nucleotides on respiratory rate and transmembrane potential ($\Delta\Psi$) of *A. castellanii* mitochondria depleted of free fatty acids. Mitochondria (1.5 mg of protein) were incubated in the presence of 1.5 mM BHAM, 170 μM ATP, 15 μM rotenone, and 2 $\mu\text{g}/\text{mg}$ protein oligomycin. 10 mM succinate (succ); 5, 9, or 16 μM LA; 0.5% BSA; and 1 mM GTP or ATP was added where indicated. Numbers on the trace refer to oxygen consumption rates in nmol of oxygen atom/min/mg of protein. The membrane potential changes are shown in mV.

oxygen consumption and $\Delta\Psi$ with succinate (plus rotenone) as oxidizable substrate were performed with amoeba mitochondria depleted of FFA by isolation and purification in the presence of BSA. Since the respiratory chain of *A. castellanii*, like that of plant mitochondria, possesses a nonelectrogenic cyanide- and antimycin-resistant alternative oxidase (AOX) (22), its inhibitor (*i.e.* BHAM) has been added to the incubation medium. State 4 (resting) respiration (plus oligomycin, plus BHAM) was stimulated by the successive addition of LA (+80% at 16 μM LA) with a parallel decrease in $\Delta\Psi$ from 185 to 161 mV (-24 mV, -13%) (Fig. 2). The addition of BSA (fatty acid-free), which adsorbs FFA, reversed the respiratory stimulation and correspondingly restored $\Delta\Psi$. Like PUMP (7), and in contrast to the behavior of UCP1, AcUCP was only weakly inhibited by purine nucleotides. Thus, when ATP or GTP was added in the absence of BSA, almost no change in respiratory rate or $\Delta\Psi$ was observed (not shown). LA-induced stimulation of state 4 respiration suggests the existence of energy-dissipating FFA cycling-linked H^+ reuptake in *A. castellanii* mitochondria.

Concentration Dependence of the LA-induced Stimulation of State 4 Respiration—LA-induced BHAM-resistant respiratory rate (*i.e.* the difference between respiratory rate in the presence of LA minus respiratory rate after the addition of BSA) should represent part of total AcUCP activity (*i.e.* part of FFA cycling-linked H^+ reuptake). This activity was measured for various LA concentrations (from 0 to 24 μM) in FFA-depleted amoeba mitochondria (Fig. 3). LA-induced respiratory rate increased with the increasing concentration of LA, and 50% of maximal stimulation by LA ($S_{0.5}$) was reached at $8.1 \pm 0.4 \mu\text{M}$ (Fig. 3, inset). The apparent maximal rate of LA-induced respiration calculated from the linear regression was 129 ± 5 nmol of oxygen atom/min/mg of protein. The apparent K_m value ($S_{0.5}$) for LA in *A. castellanii* is close to that obtained with isolated tomato fruit mitochondria ($10.3 \pm 1.1 \mu\text{M}$) (11). However, in amoeba mitochondria, $S_{0.5}$ expressed per mg of mitochondrial protein is lower (14.6 nmol/mg of protein) compared with tomato mitochondria (26.8 nmol/mg of protein). These quantitative functional measurements are in accordance with the proposal of a FFA cycling-linked H^+ reuptake catalyzed by a UCP-like protein in *A. castellanii* mitochondria. The features of

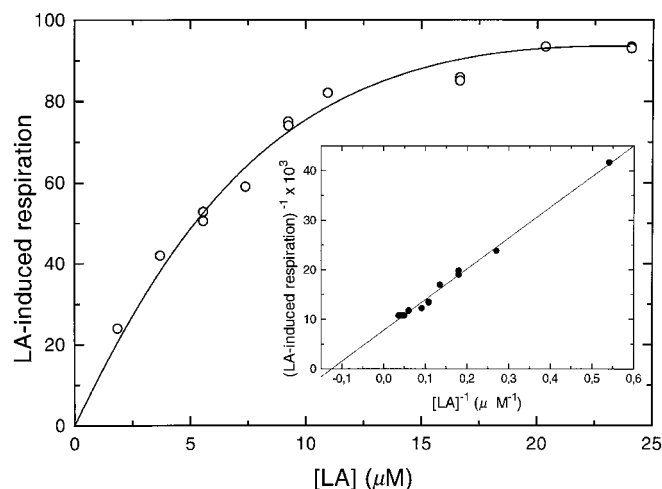


FIG. 3. **LA-induced respiration versus LA concentration.** Amoeba mitochondria fully depleted of free fatty acids were incubated with 10 mM succinate, 1.5 mM BHAM, 170 μM ATP, 15 μM rotenone, and 2 μg of oligomycin/mg of protein. Increasing concentrations of LA (1.3–24 μM) were obtained by successive additions when the steady-state respiration rate was achieved. Several oxygen traces were needed to cover the full investigated range of LA concentration. Values of respiratory rates are expressed in nmol of oxygen atom/min/mg of mitochondrial protein. *Inset*, double reciprocal plot of LA-induced respiration versus LA concentration. The linear regression was made ($y = A + BX$), where $r = 0.996$, $A = 7.74 \pm 0.29$, and $B = 62.38 \pm 1.54$. A^{-1} gives the apparent V_{\max} (129 \pm 5 nmol of oxygen atom/min/mg of protein), and B/A gives $S_{0.5}$ for LA-induced respiration (*i.e.* LA concentration that produces 50% stimulation) and corresponds to 8.1 ± 0.4 μM .

AcUCP are very similar to those of PUMP in plant mitochondria.

Coupling State of Phosphorylating Mitochondria—In *A. castellanii* mitochondria, both AcUCP and AOX dissipate energy and therefore must lead to the same final effect *i.e.* decrease in oxidative phosphorylation efficiency. ADP/O ratio, the best parameter to estimate oxidative phosphorylation efficiency, has been measured under four conditions in fully FFA-depleted amoeba mitochondria (Table I): 1) in the presence of BHAM and BSA, when AOX and AcUCP are inactive (control); 2) in the presence of BHAM and LA, when only AcUCP is active; 3) in the presence of GMP, the allosteric activator of AOX in amoeba mitochondria (22) and BSA, when only AOX is active; and 4) in the presence of GMP and LA, when both AcUCP and AOX are active. Under control conditions, the ADP/O ratio measured with succinate plus rotenone was 1.40 ± 0.03 (S.D., $n = 6$), thereby in accordance with previous results observed in *A. castellanii* mitochondria (23). When AcUCP was activated with a low LA concentration (3.7 μM), ADP/O decreased to 0.80 ± 0.05 (S.D., $n = 4$). When AOX was activated with 0.6 mM GMP, the ADP/O decreased to 0.96 ± 0.04 (S.D., $n = 6$) as already observed in Ref. 23. When both dissipating systems were activated, the ADP/O dropped to 0.63 ± 0.06 (S.D., $n = 4$) indicating a cumulative effect of both dissipating pathways on the oxidative phosphorylation efficiency. Such a cumulative effect was not observed in tomato mitochondria, where LA was shown to inhibit AOX activity (11). Accordingly, the effect of LA has been checked on the cyanide-resistant state 4 (plus oligomycin) respiration (in the presence and absence of GMP) in amoeba mitochondria. As shown in Fig. 4, increasing LA concentration within the 0–22 μM range did not inhibit the AOX activity. This important difference between amoeba and tomato will be discussed. The ADP/O measurements have been used to determine the contribution of each dissipating pathway in state 3 respiration according to the method used to calculate the AOX contribution in amoeba mitochondria (23). The ADP/O ratios with 3.7 μM LA (0.80) and without LA (1.40) (Table I)

TABLE I

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

Assay conditions were as described under “Materials and Methods.” The concentrations used were 1.5 mM BHAM, 0.5% BSA, 3.7 μM LA, and 0.6 mM GMP (where indicated).

	With BSA and BHAM	With 3.7 μM LA and BHAM	With 0.6 mM GMP and BSA	With 3.7 μM LA and 0.6 mM GMP
ADP/O	1.40	0.80	0.96	0.63
S.D.	0.03	0.05	0.04	0.06
<i>n</i>	6	4	6	4

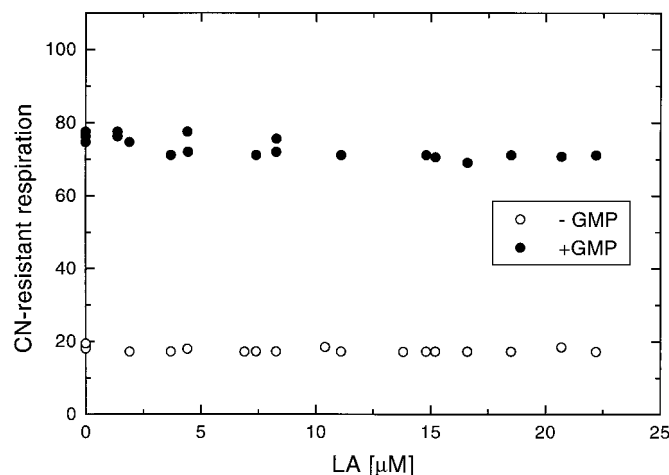


FIG. 4. **Cyanide (CN)-resistant respiration (with or without 0.6 mM GMP) versus LA concentration.** All measurements were made in the presence of 10 mM succinate, 15 μM rotenone, 170 μM ATP, and 2 μg /mg of protein oligomycin. Cyanide-resistant respiration was measured in the presence of 1.5 mM KCN, with or without 0.6 mM GMP. Increasing concentrations of LA (0–22 μM) were obtained by successive additions in several oxygen traces (see legend of Fig. 3). Values of respiratory rates are expressed in nmol of oxygen atom/min/mg of protein.

have been used to estimate the contribution of AcUCP activity (*i.e.* part of the cytochrome pathway activity that is finally dissipated into heat, $V_{\text{cyt diss}}$) and the contribution of ATP synthase activity (*i.e.* part of the cytochrome pathway activity that is finally conserved into ATP, $V_{\text{cyt cons}}$) at a given state 3 (V_3) respiration rate (plus BHAM).

$$V_{\text{cyt cons}} = V_3 \times \frac{(\text{ADP/O})_{+\text{LA}}}{(\text{ADP/O})_{-\text{LA}}} \quad (\text{Eq. 1})$$

and

$$V_{\text{cyt diss}} = V_3 - V_{\text{cyt cons}} \quad (\text{Eq. 2})$$

Therefore, $V_{\text{cyt cons}}$ represents 57%, and $V_{\text{cyt diss}}$ represents 43% of state 3 respiration at 3.7 μM LA. These calculations are valid only if several requirements are fulfilled.² Nevertheless, they show how change in the cellular FFA concentration can decrease the efficiency of oxidative phosphorylation through an activation of AcUCP. The same type of calculations for AOX and cytochrome pathway contributions in state 3 respiration (plus BSA) can be made as described and validated previously (23). Taking the ADP/O ratio with GMP (0.96) (Table I), the contribution of the cytochrome pathway represents 69%, and the contribution of AOX represents 31% of state 3 respiration. Since, in contrast to tomato mitochondria (11), LA did not inhibit AOX activity in amoeba mitochondria (Fig. 3), the cu-

² W. Jarmuszkiewicz, A. M. Almeida, C. M. Sluse-Goffart, A. E. Vercesi, and F. E. Sluse, manuscript in preparation.

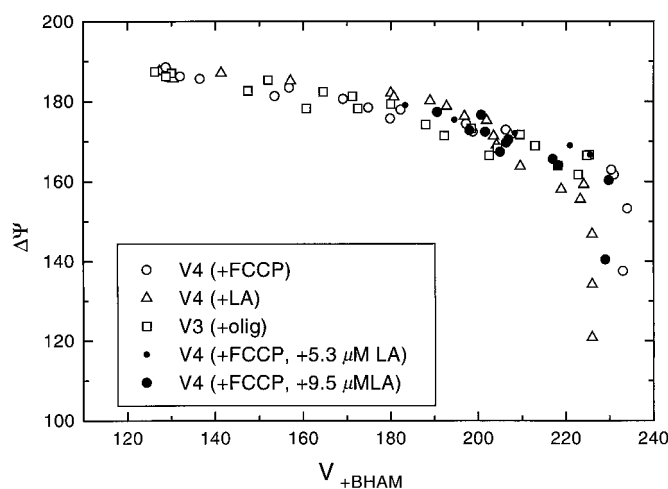


FIG. 5. Relation between $\Delta\Psi$ and mitochondrial respiration rate in the presence of BHAM. All measurements were made in the presence of 10 mM succinate, 5 μM rotenone, 1.5 mM BHAM, and 170 μM ATP. State 4 (V4) was measured in the presence of 2 $\mu\text{g}/\text{mg}$ of protein oligomycin. State 3 (V3) was reached by the addition of 2 mM ADP. \circ , state 4 with increasing concentrations of FCCP (0–0.25 μM); \triangle , state 4 with increasing concentrations of LA (0–33 μM); \square , state 3 with increasing concentrations of oligomycin (0–0.5 $\mu\text{g}/\text{mg}$ of protein); \bullet , state 4 plus 5.3 μM LA with increasing concentrations of FCCP (0–0.17 μM); \bullet , state 4 plus 9.3 μM LA with increasing concentrations of FCCP (0–0.13 μM). Respiratory rates ($V_{+\text{BHAM}}$) are expressed in nmol of oxygen atom/min/mg of protein. Membrane potential values ($\Delta\Psi$) are presented in mV.

mulative effect of both dissipating pathways on oxidative phosphorylation can be observed but not distinguished. Taking the ADP/O value in the presence of LA and GMP (0.63), V_{cyt} cons represents 45% and the total dissipating pathway-sustained respiration represents 55% of state 3 respiration. Thus, 55% of redox energy can be dissipated into heat when both dissipating pathways are activated even for such a low LA concentration (3.7 μM).

Voltage Dependence of Electron Flux in the Respiratory Chain—If LA-induced respiration is only due to a proton recycling by AcUCP, it must correspond to a pure protonophoric effect of LA not distinguishable from the effect of other well known protonophores like carbonyl cyanide *p*-trifluoromethoxyphenylhydrazine (FCCP). Fig. 5 reports a relation between couples of $\Delta\Psi$ and respiration rate measurements in the presence of BHAM under five conditions: 1) in state 4 with increasing concentrations of FCCP (\circ); 2) in state 4 with increasing concentrations of LA (\triangle); 3) in state 3 with increasing concentrations of oligomycin (\square); 4) in state 4 with 5.3 μM LA with increasing concentrations of FCCP (\bullet); and 5) in state 4 with 9.5 μM LA with increasing concentrations of FCCP (\bullet). All state 4 measurements were made in the presence of 2 $\mu\text{g}/\text{mg}$ of protein oligomycin. The maximal respiration in the uncoupled state (at ≥ 0.15 μM FCCP) was 233 nmol of oxygen atom/min/mg of protein. The state 3 respiratory rate was 218 ± 6 nmol of oxygen atom/min/mg of protein (S.D., $n = 7$), and the mean $\Delta\Psi$ value was 164 ± 4 mV (S.D., $n = 7$). The maximal respiration in state 4 with 22–33 μM LA was about 227 nmol of oxygen atom/min/mg of protein. The state 4 respiratory rate was 127 ± 4 nmol of oxygen atom/min/mg of protein (S.D., $n = 12$), and the corresponding mean $\Delta\Psi$ value was 187 ± 2 mV (S.D., $n = 12$). A set of conditions constituted a single force-flow relationship, indicating that LA did not interact with the respiratory electron transport chain in amoeba mitochondria and had a pure protonophoric effect. Moreover, respiratory rates measured with saturating LA and FCCP were almost the same, indicating that maximal electron flux was reached by activation of AcUCP.

DISCUSSION

The recent discovery of an uncoupling protein in the plant phylum together with its well known presence in animal phylum has suggested that its acquisition in eukaryotic cells must have occurred prior to the divergence of the two phyla. In this work for the first time (to our knowledge) we show the presence and we describe the functional properties of a UCP-like protein in mitochondria from protist *A. castellanii*, which has a particularly interesting position in molecular phylogeny (15, 16). The existence of AcUCP has been revealed using antibodies raised against PUMP protein from potato mitochondria. The relative molecular mass of AcUCP (32,000 Da) is similar to those of plant and mammalian UCPs. This and cross-reactivity indicate close molecular relationship with the other members of UCP family.

The results reported here support the hypothesis that AcUCP possesses uncoupling properties similar to those of PUMP and mammalian UCPs. Garlid *et al.* (13) have provided the mechanistic basis of the carrier-mediated uncoupling by the H^+ circuit. They have demonstrated that anionic FFA are electrophoretically exported by UCP and that this efflux is coupled with a fast return of protonated FFA across the phospholipid bilayer leading to a H^+ circuit, which uncouples mitochondria. Accordingly, in amoeba mitochondria, the addition of LA, which can strongly activate H^+ cycling, increased the respiratory rate in the absence of energy-requiring processes and induced a drop in $\Delta\Psi$ (Fig. 2). The LA concentration dependence of LA-induced AcUCP-sustained respiration (Fig. 3) is similar to the LA dependence of PUMP-sustained respiration in tomato mitochondria (11) except that the maximal rate of LA-induced respiration was 5 times higher in green tomato mitochondria. Nevertheless, as in tomato (14), the drop in $\Delta\Psi$ of state 4 respiration due to LA (with both ATPase and AOX activities blocked) resulted in a respiration rate almost equal to the fully uncoupled state (Fig. 5). These two rates were a little higher than the state 3 (plus BHAM) respiratory rate. This close equality means that the three systems (*i.e.* ATP synthesis, AcUCP, and uncoupling by FCCP) have the same ability to consume H^+ electrochemical gradient and to lead to the maximal electron flux in the cytochrome pathway of the respiratory chain. These observations strongly support the view that in amoeba mitochondria LA did not only induce a mild uncoupling through other carriers nonspecialized in FFA-induced H^+ recycling (30) but also that a UCP-like carrier is actually implied in the LA effect. The close equality of LA-stimulated respiration and state 3 respiration also means that LA cannot induce important acceleration of state 3 respiration, as shown in tomato mitochondria (14), but it does not mean that AcUCP activity is not induced by the LA addition. Indeed, if H^+ electrochemical gradient built by state 3 respiration in the presence of LA can be shared between oxidative phosphorylation and AcUCP activity, ADP/O must decrease in the presence of LA. Such decrease has been observed (Table I) with as low LA concentration as 3.7 μM and has been used to determine the contribution of AcUCP activity in state 3 respiration. Contributions of AOX and of AOX plus AcUCP have been determined too. These calculations show that AOX can share electron flux with the cytochrome pathway during phosphorylating respiration and that in our conditions both dissipating pathways can dissipate more than 50% of redox energy.

A single force-flow relationship has been observed for state 4 with increasing concentrations of LA or FCCP (with or without 5.3 or 9.3 μM LA) and for state 3 with increasing concentrations of oligomycin with succinate (plus rotenone) as oxidizable substrate in the presence of BHAM (Fig. 5). Modulation of the force ($\Delta\Psi$) either by phosphorylation potential (with oligomycin), by

H⁺ permeability (with FCCP) or by LA addition leads to the same modification of the flow (respiratory rate). These observations indicate that LA has only a protonophoric effect and does not act directly on the activity of the respiratory chain. They are also in favor of a delocalized H⁺ circuit, since FCCP acts on the "bulk" $\Delta\mu\text{H}^+$, while oligomycin acts on a specific site of ATP synthase, which might be suspected to form a localized H⁺ circuit with respiratory complexes (31). It can be concluded that the presence of LA gives rise to a proton recycling only and that AcUCP activity can efficiently divert energy from oxidative phosphorylation in state 3.

The two energy-dissipating systems, a cyanide-resistant alternative oxidase and an uncoupling protein, were known to exist in plant mitochondria. However, the opposite effects of LA, an abundant FFA in plants, which activates PUMP and inhibits AOX (11), have led to the proposal that these dissipating systems could work sequentially during the life of the plant cell, since a high level of FFA, which induces PUMP activity, switched off AOX activity. Moreover, these results suggested that the respective metabolic roles of each dissipating system are different (10, 11). On the contrary, in amoeba mitochondria the two dissipating systems are not co-regulated through FFA as shown by the insensitivity of AOX to LA (Fig. 4) and their cumulative effect on ADP/O (Table I). This observation does not mean that they are not co-regulated at all. Indeed, considering regulation, AOX of amoeba appears to be a quite different enzyme from plant AOX. While in plant mitochondria, the activity of AOX is stimulated by α -keto acids and regulated by the redox state of the enzyme (32), these regulations do not concern AOX in amoeba mitochondria (17), which, however, is strongly stimulated by purine mononucleotides (like GMP) (22). Moreover, since both dissipating systems, AOX and AcUCP, lead to the same final energetic effect (*i.e.* a decrease in ATP synthesis), their metabolic usefulness can only be found in different physiological roles, and this point imposes a co-regulation perhaps through GMP/GDP/GTP ratios.

The discovery of UCP in protist was more or less logically foreseen after the discovery of PUMP in plants. However, the presence of AcUCP in *A. castellanii* is most significant because of the position of this protist in the molecular phylogenetic tree of eukaryotes (15, 16). Indeed, *A. castellanii* appears on a branch basal to the divergence points of plants, animals, and fungi. Thus, its "bioenergetic" properties should be close to those of a primitive common ancestor of the whole eukaryotic world. *A. castellanii*, a nonphotosynthetic amoeboid protozoan, and higher plants share many common features at the level of respiratory chain like the presence of the nonprotonophoric inner and outer NADH dehydrogenases in addition to the nonprotonophoric AOX. The properties of AcUCP described here are closer to those of PUMP compared with mammalian UCPs. These and other phylogenetic features suggest that *A. castellanii* is closer to the plant phylum than to animal and fungi phyla. However, regulation of AOX by purine mononucleosides like GMP is shared with some primitive fungi (*e.g.* *Hansenula anomala* (24), *Neurospora crassa* (25), *Moniliella tomentosa* (26)), bringing amoeba closer to the fungi phylum. However, this feature of AOX is also found in other protists like photosynthetic *Euglena gracillis* (27) and ciliate *Paramecium tetraurelia* (28). The possibility exists that *A. castellanii* possesses all of the bioenergetic potentialities of the eukaryotic world and that some of them have been lost in the evolution of some phyla.

UCPs belong to the family of mitochondrial anion carriers (29) from which several members can participate in FFA-linked H⁺ recycling as a side function (30) mainly in a high energy

state of mitochondria. The discovery of AcUCP in *A. castellanii* indicates that UCPs, as specialized proteins for FFA-linked H⁺ recycling, emerged very early during phylogenesis and before the major radiation of phenotypic diversity in eukaryotes more than a thousand million years ago, and maybe even earlier after the acquisition of mitochondria in Eukarya. Thus, the discoveries of multiple members of the UCP family in mammals, of PUMP in plants, and now of protozoan UCP in *A. castellanii* indicate that UCPs are very much more widespread than previously believed and could occur in the whole eukaryotic world. The presence of both UCP and AOX in *A. castellanii* also demonstrates that the appearance of intracellular organellar mitochondrial oxidative phosphorylation coupling was accompanied very soon by the emergence not only of a protein carrier specialized in FFA-mediated H⁺ recycling but also of an energy-dissipating oxidase that can modulate the tightness of coupling between respiration and ATP synthesis, thereby maintaining a balance between energy supply and demand in the cell.

REFERENCES

- Vercesi, A. E., Martins, I. S., Silva, M. A. P., Leite, H. M. F., Cuccovia, I. M., and Chaimovich, H. (1995) *Nature* **375**, 24
- Klingenberg, M. (1990) *Trends Biochem. Sci.* **15**, 108–112
- Fleury, C., Neverova, M., Collins, S., Raimbault, S., Champigny, O., Levi-Meyreuis, C., Bouillaud, F., Seldin, M. F., Surwit, R. S., Ricquier, D., and Warden, C. H. (1997) *Nat. Genet.* **15**, 269–272
- Boss, O., Samec, S., Paoloni-Giacobino, A., Rossier, C., Dullo, A., Seydoux, J., Muzzin, P., and Giacobino, J.-P. (1997) *FEBS Lett.* **408**, 39–42
- Mao, W., Yu, X. X., Zhong, A., Li, W., Brush, J., Sherwood, S. W., Adams, S. H., and Pan, G. (1999) *FEBS Lett.* **443**, 326–330
- Ježek, P., Costa, A. D. T., and Vercesi, A. E. (1997) *J. Biol. Chem.* **272**, 24272–24278
- Ježek, P., Engstova, H., Zackova, M., Vercesi, A. E., Costa, A. D. T., Arruda, P., and Garlid, K. D. (1998) *Biochim. Biophys. Acta* **1365**, 319–327
- Laloi, M., Klein, M., Riesmeier, J. W., Muller-Rober, B., Fleury, C., Bouillaud, F., and Ricquier, D. (1997) *Nature* **389**, 135–136
- Maia, I. G., Benedetti, C. E., Leite, A., Turcinelli, S. R., Vercesi, A. E., and Arruda, P. (1998) *FEBS Lett.* **429**, 403–406
- Almeida, A. M., Jarmuszkiewicz, W., Khomsi, H., Arruda, P., Vercesi, A. E., and Sluse, F. E. (1999) *Plant Physiol.* **119**, 1–7
- Sluse, F. E., Almeida, A. M., Jarmuszkiewicz, W., and Vercesi, A. E. (1998) *FEBS Lett.* **433**, 237–240
- Kowaltowski, A. J., Costa, A. D., and Vercesi, A. E. (1998) *FEBS Lett.* **425**, 213–216
- Garlid, K. D., Orosz, D. E., Modriansky, M., Vassanelli, P., and Ježek, P. (1996) *J. Biol. Chem.* **271**, 2615–2620
- Jarmuszkiewicz, W., Almeida, A. M., Sluse-Goffart, C. M., Sluse, F. E., and Vercesi, A. E. (1998) *J. Biol. Chem.* **273**, 34882–34886
- Wainright, P. O., Hinkle, G., Sogin, M. L., and Stickle, S. K. (1993) *Science* **260**, 340–342
- Gray, M. W., Burger, G., and Lang, B. F. (1999) *Science* **283**, 1476–1481
- Jarmuszkiewicz, W., Wagner, A. M., Wagner, M. J., and Hryniewiecka, L. (1997) *FEBS Lett.* **411**, 110–114
- Tomaszewska, B., Jarmuszkiewicz, W., and Schramm, R. W. (1992) *Plant Physiol. Biochem.* **29**, 489–496
- Gornall, A. G., Bardawill, C. J., and David, M. M. (1949) *J. Biol. Chem.* **177**, 751–766
- Laemmli, U. K. (1970) *Nature* **227**, 680–685
- Kamo, N., Muratsugu, N., Hongoh, R., and Kobatake, Y. (1979) *J. Membr. Biol.* **49**, 105–121
- Hryniewiecka, L., Jenek, J., and Michejda, J. (1978) in *Plant Mitochondria* (Ducet, G., and Lance, C., eds) pp. 307–314, Elsevier Science Publishers B.V., Amsterdam
- Jarmuszkiewicz, W., Sluse-Goffart, C. M., Hryniewiecka, L., Michejda, J., and Sluse, F. E. (1998) *J. Biol. Chem.* **273**, 10174–10180
- Sakajo, S., Minagawa, N., and Yoshimoto, Y. (1997) *Biosci. Biotech. Biochem.* **61**, 397–399
- Vanderleyden, J., Peeters, C., Verachtert, H., and Bertrand, H. (1980) *Biochem. J.* **188**, 141–144
- Vanderleyden, J., Van den Eynde, E., and Verachtert, H. (1980) *Biochem. J.* **186**, 309–316
- Sharpless, T. K., and Butow, R. A. (1970) *J. Biol. Chem.* **245**, 58–70
- Doussiere, J., and Vignais, P. V. (1984) *Biochem. J.* **220**, 787–794
- el Mualij, B., Duyckaerts, C., Lamotte-Brasseur, J., and Sluse, F. E. (1997) *Yeast* **13**, 573–581
- Skulachev, V. P. (1998) *Biochim. Biophys. Acta* **1363**, 100–124
- Westerhoff, H. V., Malandri, B. A., Venturoli, G., Azzone, G. F., and Kell, D. B. (1984) *Biochim. Biophys. Acta* **768**, 257–292
- Vanlerberghe, G. C., and McIntosh, L. (1997) *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **48**, 703–734