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

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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 (ΔΨ) in a manner expected from fatty acid cycling linked II⁺ reuptake. The half-maximal stimulation by linoleic acid was reached at 8.1 ± 0.4 μM. Bovine serum albumin (fatty acid-free), which adsorbs linoleic acid, reversed the respiratory stimulation and correspondingly restored ΔΨ. 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 II⁺ 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 discovery 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 climaxferic 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 (ΔΨ, 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 ΔΨ (14). The apparent Kₘ for LA-induced respiration (i.e. LA concentration that produces 50% stimulation) is about 10 μM (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 ΔΨ. 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.
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 x 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 Stuse et al. (18) 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 µg 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% betamercaptoethanol 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 (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).

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 oxygen consumption and ΔΨ 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 ΔΨ 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 ΔΨ. 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 ΔΨ 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 (S0.5) was reached at 8.1 ± 0.4 µM (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 Rm value (S0.5) for LA in A. castellanii is close to that obtained with isolated tomato fruit mitochondria (10.3 ± 1.1 µM) (11). However, in amoeba mitochondria, S0.5 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
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) have been used to estimate the contribution of AcUCP activity (i.e. part of the cytochrome pathway activity that is finally dissipated into heat, Vcyt diss) and the contribution of ATP synthase activity (i.e. part of the cytochrome pathway activity that is finally conserved into ATP, Vcyt cons) at a given state 3 (V3) respiration rate (plus BHAM).

\[
\text{Vcyt cons} = V3 \times \frac{(\text{ADP/O})_{\text{LA}}}{(\text{ADP/O})_{\text{LA}}}
\]

(Eq. 1)

\[
\text{Vcyt diss} = V3 - \text{Vcyt cons}
\]

(Eq. 2)

Therefore, Vcyt cons represents 57%, and Vcyt diss represents 43% of state 3 respiration at 3.7 μM LA. These calculations are valid only if several requirements are fulfilled. 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 cumulation of both dissipating systems will be discussed. The ADP/O measurements have been used to estimate the contribution of AcUCP activity (i.e. part of the cytochrome pathway activity that is finally dissipated into heat, Vcyt diss) and the contribution of ATP synthase activity (i.e. part of the cytochrome pathway activity that is finally conserved into ATP, Vcyt cons) at a given state 3 (V3) respiration rate (plus BHAM).

![Figure 3. LA-induced respiration versus LA concentration.](image)

![Figure 4. Cyanide (CN)-resistant respiration (with or without 0.6 mM GMP) versus LA concentration.](image)

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

| Condition                  | ADP/O | S.D. | n  |
|----------------------------|-------|------|----|
| With BHAM and LA           | 0.80  | 0.05 | 4  |
| With 3.7 μM LA and BHAM    | 0.96  | 0.04 | 6  |
| With 0.6 μM GMP and BHAM   | 0.96  | 0.06 | 4  |
| With 3.7 μM LA and 0.6 μM GMP | 0.96 | 0.06 | 4  |

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).

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2 W. Jarmuszkiewicz, A. M. Almeida, C. M. Sluse-Goffart, A. E. Vercesi, and F. E. Sluse, manuscript in preparation.
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 euarkytic 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 ΔΨ (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 ΔΨ 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 1) 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 reduct 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 (ΔΨ) either by phosphorylation potential (with oligomycin), by

![Figure 5. Relation between ΔΨ 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 μg/ml of protein oligomycin. State 3 (V3) was reached by the addition of 2 mM ADP. □, state 4 with increasing concentrations of FCCP (0–0.25 μM); ○, state 4 with increasing concentrations of LA (0–33 μM); △, state 3 with increasing concentrations of oligomycin (0–0.5 μg/ml of protein); ●, state 4 plus 5.5 μM LA with increasing concentrations of FCCP (0–0.17 μM); ◆, state 4 plus 9.3 μM LA with increasing concentrations of FCCP (0–0.13 μM). Respiratory rates (V + BHAM) are expressed in nmol of oxygen atom/mg of protein. Membrane potential values (ΔΨ) are presented in mV.](image-url)
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.

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