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Redox State of Quinone Affects Sensitivity of *Acanthamoeba castellanii* Mitochondrial Uncoupling Protein to Purine Nucleotides

Aleksandra SWIDA, Andrzej WOYDA-PLOSZCZYCA and Wieslawa JARMUSZKIEWICZ*

Laboratory of Bioenergetics, Adam Mickiewicz University, Umultowska 89, 61-614 Poznan, Poland

Short title: Ubiquinone redox state modulates mitochondrial uncoupling protein activity

*To whom correspondence should be addressed: Wieslawa Jarmuszkiewicz, Laboratory of Bioenergetics, Adam Mickiewicz University, Umultowska 89, 61-614 Poznan, Poland, Tel +48618295881; Fax +48618295636; E-mail: wiesiaj@amu.edu.pl

Abbreviations used: AcUCP, uncoupling protein of *Acanthamoeba castellanii* mitochondria; BHAM, benzohydroxamate; BSA, bovine serum albumin; CATR, carboxyatractylolide; FCCP, carbonylcyanide 4-(trifluoromethoxy)phenylhydrazone; FFA, free fatty acids; LA, linoleic acid; MX, myxothiazol; PN, purine nucleotides; TPP⁺, tetraphenylphosphonium; UCP, uncoupling protein; UCP1, uncoupling protein of brown adipose tissue; Q, quinone; Q_{ox}, oxidized quinone; Q_r or QH₂ reduced quinone (quinol); Q_t, total endogenous pool of quinone in the inner mitochondrial membrane (Q_{ox} + Q_r); Q_r/Q_t, reduction level of quinone; ΔΨ, mitochondrial membrane electrical potential; ΔμH⁺, proton electrochemical gradient

Key words: *Acanthamoeba castellanii*, mitochondria, uncoupling protein, quinone redox state

Synopsis

We studied free fatty acid-induced uncoupling activity in *A. castellanii* mitochondria in nonphosphorylating state. Either succinate or external NADH was used as a respiratory substrate to determine the proton conductance curves and the respiratory rate versus the quinone reduction level relationships. Our determinations of the membranous quinone reduction level in nonphosphorylating mitochondria show that activation of UCP activity leads to a purine nucleotide-sensitive decrease in quinone redox state. The gradual decrease in the rate of quinone-reducing pathways (titration of dehydrogenases activity) progressively leads to a full inhibitory effect of GDP on linoleic-acid induced proton conductance. This inhibition cannot be attributed to changes in the membrane potential. Indeed, the lack of GDP inhibitory effect observed when decrease in respiratory rate is accompanied by an increase in the quinone reduction level (titration of quinol-oxidizing pathway) proves that the inhibition by nucleotides can be revealed only for a low quinone redox state. It must be underlined that in *A. castellanii* nonphosphorylating mitochondria, the transition of the inhibitory effect of GDP on linoleic acid-induced UCP-mediated uncoupling is observed for the same range of quinone reduction level (between 50% and 40%) as that observed previously for phosphorylating conditions. This observation drawn from the two different metabolic states of mitochondria indicates that quinone could affect UCP activity through sensitivity to purine nucleotides.

INTRODUCTION

Acanthamoeba castellanii is a free-living amoeba found in soil, marine, and freshwater environments and an important predator of bacteria. *A. castellanii* is also an opportunistic pathogen of clinical interest, responsible for several distinct human diseases. Under axenic non-pathogenic conditions, *A. castellanii* has been used frequently as a model organism to study mitochondrial energy-dissipating systems, such as a cyanide-resistant alternative oxidase [1,2], an ATP-sensitive potassium channel [3], and an uncoupling protein (AcUCP) [4-8].

Uncoupling proteins (UCPs) catalyze a proton conductance that dissipates an H^+ electrochemical gradient ($\Delta\mu H^+$) built up by the mitochondrial respiratory chain in animal, plant and some fungal and protist mitochondria [for review see Refs. 9-15]. Uncoupling proteins have been proposed to fulfill a physiological function through a $\Delta\mu H^+$ dissipation by a free fatty acid (FFA)-activated purine nucleotide (PN)-inhibited H^+ cycling process driven by membrane potential ($\Delta\Psi$) and pH (both constituting $\Delta\mu H^+$). In unicellular organisms, as well as in non-thermogenic plant and animal tissues, the physiological role of this energy-dissipating system has not yet been established. Recent discussions imply that UCPs (with the exception of thermogenic UCP1 of mammalian brown adipose tissue) may play a central role in the limitation of the production of mitochondrial reactive oxygen species and in the maintenance of the cell energy metabolism balance related to the regulation of ATP production as well as control of the NADH/NAD⁺ ratio [9-15].

In mitochondria of the amoeboid protozoan *A. castellanii*, the action of AcUCP has been shown to mediate FFA-activated, PN-inhibited H^+ re-uptake driven by $\Delta\mu H^+$ that in state 3 respiration can divert energy from oxidative phosphorylation [4]. The fatty acid efficiency profile in uncoupling of *A. castellanii* mitochondria has been described [8]. It has been also shown that a cold treatment of amoeba culture increases AcUCP activity and protein level, indicating that UCP could be a cold response protein in unicellulars [5]. Moreover, we have shown that UCP may play a role in decreasing reactive oxygen species production in unicellular organisms, such as amoeba *A. castellanii* [6].

As UCPs are specialized proteins for $\Delta\mu\text{H}^+$ dissipation, their activity must be finely regulated. For a long time, an inhibition of H^+ conductance by PN has been considered as a diagnostic of UCP activity. However, for UCP1 homologues conflicting results have been obtained with isolated respiring mitochondria, where the FFA-induced H^+ conductance has been shown to be differently sensitive to PN under non-phosphorylating conditions. Insensitivity to PN has been observed for UCPs of amoeboid eukaryotes (*A. castellanii* and *D. discoideum*), plant UCPs and UCP2 (UCP ubiquitous in mammalian tissues) as well as UCP3 (UCP predominantly specific for skeletal muscle) in mitochondria respiring in non-phosphorylating conditions in the absence of superoxide [4,16-21]. On the other hand, under these conditions inhibition by PN has been observed for UCP in isolated mitochondria of *C. parapsilosis* [22] and potato tuber [23]. In the case of mammalian UCPs, quinone (Q) has been shown to be an obligatory cofactor for their action [24,25]. Furthermore, during non-phosphorylating respiration, stimulation by superoxide has been shown to be necessary to reveal sensitivity to PN in isolated kidney and skeletal muscle mitochondria [21,26-28] as well as in plant mitochondria [20]. However, in the reconstituted system with heterogeneously expressed mammalian UCPs, no superoxide activation has been required to demonstrate the FFA-activated PN-sensitive H^+ translocation and Q has no significant activating effect nor any effect on the inhibition by PN [29,30]. Moreover, taking into account the apparent affinity of reconstituted UCPs for purine nucleotides [25,30] and the concentration of nucleotides *in vivo* (2-15 mM, depending on material), UCPs should be almost fully inhibited under *in vivo* conditions, even in the presence of FFA, unless a regulatory factor or mechanism could modulate (lower) the inhibition by PN [11]. Recently, it has been proposed that the membranous Q redox state could be a metabolic sensor that modulates the PN inhibition of FFA-activated UCPs as observed in isolated skeletal muscle, potato tuber and *A. castellanii* mitochondria respiring in phosphorylating conditions in the absence of endogenous superoxide production [18,7,31]. Therefore, the question arises as to whether the same regulation takes place in the quite different mitochondrial metabolic state, i.e., under nonphosphorylating conditions thereby at higher $\Delta\Psi$ and redox state of Q (and other respiratory chain components) compared to phosphorylating ones.

The aim of the present study was to examine for the first time the influence of endogenous Q redox state on PN-inhibition of the FFA-induced UCP activity in non-phosphorylating mitochondria. This study may shed light on differential results obtained so far for isolated mitochondria from various materials under non-phosphorylating conditions.

EXPERIMENTAL

Cell culture and isolation of mitochondria

The soil amoeba *Acanthamoeba castellanii*, strain Neff, was cultured as described by Jarmuszkiewicz *et al.* [1]. Trophozoites of the amoeba were collected between 44 and 48 h following inoculation at the middle exponential phase (at a density of about $4\text{--}5 \times 10^6$ cells/ml). Mitochondria were isolated and purified on a self-generating Percoll gradient (30%) as described earlier [1]. The presence of 0.4% bovine serum albumin (BSA) in isolation media allowed FFA to be chelated from the mitochondrial suspension and also allowed mitochondria fully depleted of FFA to be obtained. For each mitochondrial preparation, full depletion of FFA was tested by measuring the effect of BSA on LA-induced respiration as described by Jarmuszkiewicz *et al.* [16]. Mitochondrial protein concentration was determined by the biuret method.

Mitochondrial oxygen consumption

Oxygen uptake was measured polarographically with a Clarck type oxygen electrode (Rank Brothers, Cambridge, UK) in 2.8 ml of incubation medium (25°C), with 1 mg of mitochondrial protein. State 3 (phosphorylating) respiration measurements were performed in order to check coupling parameters. Only high quality mitochondria preparations, i.e., with an ADP/O value of around 1.40 and a respiratory control ratio of around 2.5–3.0, were used in all experiments. State 3 respiratory rate was 260 ± 21 nmol O \times min⁻¹ \times mg⁻¹ protein ($n = 15$). Values of O₂ uptake are presented in nmol O \times min⁻¹ \times mg⁻¹ protein.

Mitochondrial membrane potential measurements

The mitochondrial membrane electrical potential ($\Delta\Psi$) was measured using a tetraphenylphosphonium (TPP^+)-specific electrode according to Kamo *et al.* [32]. The TPP^+ -electrode was calibrated with four sequential additions (0.4, 0.4, 0.8, and 1.6 μM) of TPP^+ . After each run, 0.5 mM FCCP was added to release TPP^+ for base-line correction. For calculation of the $\Delta\Psi$ value, the matrix volume of amoeba mitochondria was assumed to be 2.0 $\mu\text{l} \times \text{mg}^{-1}$ protein. Calculation assumes that TPP^+ distribution between mitochondria and medium followed the nernst equation. Corrections were made for the binding of TPP^+ to mitochondrial membranes. Values of $\Delta\Psi$ are presented in mV.

Proton leak measurements

The proton conductance response to its driving force can be expressed as the relationship between the oxygen consumption rate and $\Delta\Psi$ (flux-force relationship) when varying the potential by titration with respiratory chain inhibitors. Respiration rate and $\Delta\Psi$ were measured simultaneously using electrodes sensitive to oxygen and TPP^+ . Proton leak rates can be calculated from respiration rates by multiplying by a H^+/O ratio of 6. Mitochondria (0.36 mg of protein/ml) were incubated in a standard incubation medium (25°C) containing: 120 mM KCl, 20 mM Tris-HCl pH 7.2, 3 mM KH_2PO_4 , 0.8 mM MgCl_2 , and 0.5 mM EGTA. Measurements were performed in the absence of added ADP, i.e., in resting state (state 4). To exclude the activity of an ATP/ADP antiporter and ATP synthase, 1.8 μM carboxyatractylolide (CATR) and 0.5 $\mu\text{g/ml}$ oligomycin were used respectively. Benzohydroximate (BHAM) was used to inhibit alternative oxidase activity. The oxidizable substrates were succinate (5 mM) or external NADH (1 mM or 0.5-0.003 mM) in the presence of rotenone (4 μM) to block electron input from complex I. To activate succinate dehydrogenase 80 μM ATP was used. To induce AcUCP activity-mediated respiration, measurements were made in the presence of 1.2, 2.4 or 4.8 μM LA. To inhibit the LA-induced AcUCP activity, 1 mM GDP was added.

Respiratory rate, $\Delta\Psi$ and Q redox state were varied by modulating the Q-reducing or QH_2 -oxidizing pathways. To decrease the rate of the Q-reducing pathway during state 4 respiration (thereby decreasing a

steady-state resting respiration), titration of a dehydrogenase activity was carried out. To titrate succinate oxidation an increasing concentration of malonate (up to 5 mM), a competitive inhibitor of succinate dehydrogenase, was used. Titration of NADH oxidation was performed as described previously varying the NADH concentration (0.5-0.003 mM) in the presence of an enzymatic regenerating system [33]. To decrease the rate of the QH₂-oxidizing pathway during state 4 respiration, the *bc*₁ complex (complex III) or complex IV was inhibited with myxothiazol (MX) (up to 9 μ M) and cyanide (up to 20 μ M), respectively.

Measurements of Quinone Reduction Level

The redox state of Q in steady-state respiration was determined by an extraction technique followed by HPLC detection according to Van den Bergen *et al.* [34]. As previously found, endogenous quinone in *A. castellanii* mitochondria is Q₉ (34). For calibration of the peaks commercial Q₉ (Sigma) was used. A completely oxidized extract was obtained during incubation in the absence of substrate using the evaporation/ventilation step, a completely reduced extract was obtained upon anaerobiosis and in the presence of substrates (5 mM succinate or 1 mM NADH), 1.5 mM KCN and 1.5 mM BHAM. An inactive Q pool contains quinol that can never be oxidized and quinone that can never be reduced. The presented values of the redox state of Q deal with the active Q pool (the difference between the total Q pool minus the inactive Q pool) in a given mitochondrial preparation.

RESULTS

Fatty acid-stimulated proton leak and redox state of endogenous Q in *A. castellanii* mitochondria

Uncoupling protein of *A. castellanii* (AcUCP) mitochondria is activated by FFA, among which LA is the most efficient [4,8]. So far, to our knowledge, there is no experimental data describing the effect of UCP activity, including FFA-induced UCP activity, on the redox state of endogenous mitochondrial Q in non-phosphorylating conditions. Figure 1 shows the influence of AcUCP activation by addition of increasing LA concentration on membranous Q redox state in a given steady-state of state 4 respiratory rate and $\Delta\Psi$ with succinate as a respiratory substrate. Additions of LA progressively decreased the Q redox state

together with $\Delta\Psi$ and stimulated respiratory rate. Moreover, respiratory rates and Q redox state values measured with saturating LA (maximal AcUCP activity) and protonophore (FCCP, fully uncoupled state) were almost the same, indicating that maximal electron flux through the respiratory chain was reached at 66-68% of Q redox state. Moreover, similar values of Q reduction level were observed in *A. castellanii* mitochondria oxidizing succinate under phosphorylating conditions [7].

Inhibitory effect of GDP on FFA-induced proton leak when the Q redox state is decreased in *A. castellanii* mitochondria

As previously reported [4,5], the FFA-induced AcUCP-sustained state 4 respiration was not inhibited (or weakly) by the addition of PN (Figure 2A). Since in *A. castellanii* mitochondria it has been shown that FFA-induced uncoupling during phosphorylating respiration can be inhibited by PN when endogenous Q is sufficiently oxidized [7], we investigated the effect of 1 mM GDP on the LA-induced uncoupling when the rate of the Q-reducing pathway is decreased in nonphosphorylating conditions. In the presence of malonate, when the rate of succinate oxidation and the corresponding Q redox state were decreased by around 60%, the full inhibition of the LA-induced H^+ leak was observed (Figure 2B). The inhibitory effect of GDP was revealed by inhibition of respiratory rate as well as restoration of $\Delta\Psi$ and Q redox state to values observed before LA addition. Thus, our determinations of the Q reduction level in mitochondria respiring in nonphosphorylating conditions show for the first time that activation of UCP activity leads to a GDP-sensitive decrease in the Q redox state. This conclusion can be extended to other PN, since the same results were obtained with 1 mM ATP or GTP (not shown). In the following experiments, GDP has been used to study the inhibitory effect on FFA-induced proton leak.

To establish a flux-force relationship to which a direct access is given by the simultaneous measurement of oxygen consumption and $\Delta\Psi$, after energization of mitochondria with respiratory substrate (succinate or external NADH), the potential was titrated by the addition of a given concentration of inhibitors (malonate, myxothiazol or cyanide). In order to avoid possible errors due to non-steady-state conditions (when sequential addition of inhibitors is applied) as well as to assess the Q redox state for a

given steady-state, data from separate measurements with different inhibitor concentration (as in Figures 2,4,6) were afterwards pooled on common curves (Figures 3,5,7). Figures 3A, 5A, 7A show the response of the H^+ leak rate to its driving force, $\Delta\Psi$. Under control nonphosphorylating conditions, *A. castellanii* mitochondria exhibited a characteristic non-linear response of H^+ leak to $\Delta\Psi$ as animal [21,28] and plant mitochondria [19,20].

Figure 3A shows that when the rate of the Q-reducing pathway was progressively decreased during succinate oxidation (with increasing concentration of malonate), in the presence of LA, the proton conductance was greater than in the controls, in LA-concentration dependent manner. This is demonstrated by the upward displacement of the H^+ leak curves: the mitochondria required a higher respiration rate to maintain any given $\Delta\Psi$ when incubated with LA. Below approximately 155 mV, the increase in H^+ conductance was diminished and then fully inhibited by addition of GDP. The relationship between respiratory rate and the Q reduction level (Q_r/Q_t) (Figure 3B) revealed the inhibitory effect of GDP on LA-induced AcUCP-sustained respiration for Q redox state below 50%. The full inhibitory effect was reached at approximately 40% of Q reduction level, when points obtained in the presence of LA and GDP reached the control curve. The respiratory rate versus the Q reduction level gave a single relationship for the all sets of experimental conditions (Figure 3C). This single relationship, showing that the Q reduction level declined linearly from 92 to 20%, clearly demonstrates that LA and/or GDP do not affect the activity of the cytochrome pathway during substrate oxidation titration in nonphosphorylating conditions (independently of respiratory substrate, see also Figure 5C).

To establish if the effect of PN on LA-induced activity of AcUCP is observed for the same range of respiratory rate, $\Delta\Psi$ and Q reduction level, independently of applied substrate, external NADH was used as a respiratory substrate. Similarly to succinate oxidation (Figure 2), the LA-induced AcUCP-sustained state 4 respiration with saturating NADH concentration (0.5 mM) was not inhibited by the addition of GDP (Figure 4A). However, when the Q reduction level was decreased (approximately by 70%) by a lower NADH concentration (0.005 mM), the inhibitory effect of GDP was revealed by inhibition of the

respiratory rate as well as the restoration of $\Delta\Psi$ and Q redox state to the values observed before LA addition (Figure 4B).

Figures 5A and 5B show the effect of GDP on the respiration sustained by the LA-induced H^+ leak (AcUCP activity) when the rate of the Q-reducing pathway is gradually decreased in mitochondria oxidizing external NADH or succinate. Determinations of H^+ conductance curves (Figure 5A), the relationships between respiratory rate as well as the $\Delta\Psi$ versus Q reduction level (Figures 5B, 5C) in the presence or absence of 1.2 μM LA and/or 1 mM GDP indicate that there is no difference between the results obtained with NADH and with succinate. With gradually decreased state 4 respiratory rate, LA-induced uncoupling activity was inhibited by GDP at the $\Delta\Psi$ below 160 mV and the Q reduction level below 50%.

No inhibitory effect of GDP on FFA-induced proton leak when the Q redox state is increased in *A. castellanii* mitochondria

The above experiments indicate that in *A. castellanii* mitochondria under nonphosphorylating conditions, sensitivity to PN of the FFA-induced AcUCP-sustained uncoupling is dependent on membranous Q redox state. To exclude the role of respiratory rate and $\Delta\Psi$, we varied (progressively decreased) them with inhibitors of the QH_2 -oxidizing pathway (myxothiazol or cyanide) leading to an increase in the Q reduction level. Figure 6 shows an example experiment in which the respiratory rate, $\Delta\Psi$ and Q redox state were measured for nonphosphorylating succinate oxidation with a given concentration of myxothiazol (5.8 μM), when the effect of PN on the LA-induced mitochondrial uncoupling was studied. In the presence of myxothiazol, when the respiratory rate was decreased (by approximately 50%) and the corresponding Q redox state was increased (by 10%), no inhibition of the LA-induced H^+ leak by 1 mM GDP was observed. This results from the absence of GDP effect on respiratory rate, $\Delta\Psi$ and Q redox state in the presence of LA.

Figure 7 presents determinations of H^+ conductance curves (Figure 7A), the relationships between respiratory rate as well as the $\Delta\Psi$ versus Q reduction level (Figures 7B,7C) in the presence or absence of 1.2 μM LA and/or 1 mM GDP, when the rate of the QH_2 -oxidizing pathway (the cytochrome pathway activity) was gradually decreased by increasing concentrations of myxothiazol or cyanide during succinate oxidation. Comparison of Figures 7A and Fig. 5A indicates that *A. castellanii* mitochondria energized with succinate or external NADH and titrated with inhibitors of the Q-reducing or QH_2 -oxidizing pathways showed the same H^+ leak curves (no GDP, plus or minus FFA conditions). Thus, this clearly indicates that the redox state of endogenous Q does not affect the basal and FFA-induced proton conductance of amoeba mitochondria. Moreover, when the rate of the QH_2 -oxidizing pathway was gradually decreased (during succinate oxidation), the $\Delta\Psi$ versus Q reduction level (Figure 7C) gave a single relationship for all sets of experimental conditions, showing the increase in Q redox state from 83 to 98% during state 4 titration with both the cytochrome pathway inhibitors (myxothiazol and cyanide).

The force-flux relationship established in the presence of 1.2 μM LA to activate AcUCP and in the presence or absence of 1 mM GDP indicates that the nucleotide does not inhibit the LA-induced H^+ conductance when the Q reduction level was gradually increased (Figure 7A). Although titration of succinate oxidation with cyanide or myxothiazol comprised the respiratory rate and $\Delta\Psi$ ranges at which GDP-sensitivity was observed for Q-reducing pathway titration (with malonate, Figures 3A, 5A), no inhibitory effect of GDP was revealed under these conditions. However, the respiratory rate versus the Q reduction level relationship is clearly out of the Q redox state range where sensitivity to PN is revealed under state 4 conditions (Figure 7B). These results clearly indicate that the progressive inhibition of the LA-induced H^+ leak in the presence of GDP cannot be attributed to changes in the $\Delta\Psi$. The lack of any inhibitory effect of GDP observed when decrease in state 4 respiration was accompanied by an increase in the Q reduction level proves that the inhibition by PN can be revealed only for a low Q redox state. Thus, we can conclude that in *A. castellanii* mitochondria the inhibitory effect of GDP on the LA-induced AcUCP-mediated uncoupling during nonphosphorylating respiration depends on the membranous Q

redox state, being the most efficient (the full inhibition) when the Q reduction level reaches approximately 40%.

DISCUSSION

The results presented in this study obtained with nonphosphorylating *A. castellanii* mitochondria confirm our previous studies performed on isolated mitochondria (of rat skeletal muscle, potato tubers and *A. castellanii* cells) respiring in the different metabolic state (phosphorylating conditions), where the ADP/O method has been applied to calculate the rate of ADP phosphorylation [7,18,31]. In this approach, we have determined the efficiency of FFA-induced UCP activity in the uncoupling of oxidative phosphorylation when state 3 respiration, within the range where $\Delta\Psi$ remains constant, is gradually decreased by a lowering rate of the Q-reducing or QH₂-oxidizing pathways. The present work, in which the common approach to study nonphosphorylating mitochondria, i.e. H⁺ conductance curve determinations (when $\Delta\Psi$ is varied with respiratory chain inhibitors) has been applied, confirms that the Q redox state has no effect at the level of FFA-induced UCP activity in the absence of PN and could only regulate this activity through the efficiency of inhibition by PN. It must be underlined that in *A. castellanii* nonphosphorylating mitochondria (Figures 3B,5B), the transition of GDP inhibitory effect (from 0% to 100%) on LA-induced UCP-mediated uncoupling is observed for the same range of Q reduction level (between 50% and 40%) as that observed previously for phosphorylating conditions [7]. This observation drawn from the two different metabolic states of mitochondria and from studies with the application of two different experimental approaches strongly indicates that quinone (Q or QH₂) affects affinity of UCP for GDP. Certainly, further studies are necessary to elucidate the mechanism of such regulation.

In *A. castellanii* mitochondria, for both respiration states, the full inhibitory effect of PN is observed for a Q reduction level approximately below 43%. It seems that the range of the Q redox state when the inhibition by PN occurs could be different for different mitochondria. Indeed, in

phosphorylating mitochondria, full sensitivity to PN has been observed for a Q reduction level below 57% in rat skeletal muscle mitochondria [18] and below 25% in potato tuber mitochondria [31]. Moreover, the regulation of the PN-sensitivity of FFA-induced UCP activity by Q redox state could explain why, in some mitochondria, no or weak inhibition by PN has been observed in nonphosphorylating (state 4) respiration, thereby when the Q reduction level could be high. Therefore, this phenomenon could account for the insensitivity to PN of FFA-induced uncoupling observed for UCPs of amoeboid eukaryotes (*A. castellanii* and *D. discoideum*), some plant UCPs as well as mammalian UCP2 and UCP3 in mitochondria respiring in nonphosphorylating conditions in the absence of superoxide [4,16-21]. Indeed, proton conductance curves established for FFA-induced non-phosphorylating uncoupling have not revealed sensitivity to PN during titration with cyanide when Q redox state is likely increased in potato tuber mitochondria [19,20]. On the other hand, as inhibition by PN has been observed for UCP in isolated mitochondria of *C. parapsilosis* [22] and some varieties of potato tubers [23] it seems that in some organisms (most probably dependently on tissue, physiological state, age, and variety) the Q reduction level of nonphosphorylating state could be sufficiently low to allow inhibition by nucleotides.

Studies with mammalian UCPs have revealed conflicting results concerning the possibility that Q may be an obligatory cofactor for their action. Namely, oxidized Q has been shown to activate PN-sensitive FFA-dependent H⁺ transport through reconstituted UCP1-3 [24,25]. Therefore, it has been proposed that oxidized Q could facilitate H⁺ conductance induced by FFA during UCP action [25]. On the other hand, other studies have shown that Q has no significant activating effect on the FFA-dependent H⁺ translocation nor any effect on the inhibition by PN in reconstituted UCP1-3 [29]. Photoaffinity labeling of purified UCP1 with retinoic acid has indicated that Q increases binding of activator by UCP1 [37]. From flux-force relationship studies performed with isolated kidney mitochondria, it has been concluded that the redox state of endogenous Q does not affect mitochondrial proton conductance [35]. The results on isolated *A. castellanii* mitochondria presented in this study, based on Q redox determinations, evidence that the redox state of endogenous Q does not affect the basal and FFA-induced UCP-mediated H⁺ conductance but its sensitivity to PN. This conclusion can be taken as determination of membranous Q

reduction level was performed when H⁺ leak curves were established with inhibitors of the Q-reducing or QH₂-oxidizing pathways in isolated respiring mitochondria.

It must be underlined that the described regulation of UCP activity by Q redox state so far has been observed only for the FFA-induced UCP-mediated uncoupling. Indeed, from proton conductance curves established in different mitochondria, under conditions when UCPs are activated by superoxide during non-phosphorylating respiration [19,20,25-27, 35,36], it is difficult to univocally estimate if sensitivity to PN depends on Q redox state during titrations of the Q-reducing pathway (with malonate when succinate is oxidized) and the QH₂-oxidizing pathway (with cyanide or myxothiazol). However, this cannot be excluded since determinations of Q reduction level have not been performed in these experiments. Interestingly, addition of reduced coenzyme Q to respiring kidney mitochondria has resulted in an increase in FFA-induced GDP-sensitive proton conductance through production of superoxide [35]. Therefore, further studies should explain if regulation of sensitivity of AcUCP to PN through Q redox state could be also observed under conditions when uncoupling is activated by superoxide. So far, in *A. castellanii* mitochondria there is no evidence that AcUCP activity is induced by reactive oxygen species.

Taking into account the apparent affinity of reconstituted UCPs for PN [21,30] and the concentration of nucleotides in vivo (2-15 mM, depending on material), UCPs should be almost fully inhibited under in vivo conditions, even in the presence of FFA, unless a regulatory factor or process could overcome (lower) the inhibition by PN [14,18]. In the case of UCP1, it has been found that FFA do this in a kinetically simple competitive manner [39]. Moreover, studies with mitochondria isolated from yeast mutant cells lacking Q and expressing mouse UCP1 have shown that FFA-induced GDP-sensitive proton conductance by UCP1 expressed in yeast mitochondria is not dependent on the presence of Q in the mitochondrial membrane [38]. However, there is no data excluding the possibility that a highly reduced membranous Q lead to overcoming PN-inhibition of UCP1 activity in mammalian mitochondria. The present study evidences that at least in *A. castellanii* mitochondria, a metabolic sensor that modulates the PN inhibition of FFA-activated UCP activity is the Q redox state.

In the molecular phylogenetic tree of eukaryotes, *A. castellanii*, the nonphotosynthesizing amoeboid protozoan, appears on a branch basal to the divergence points of plants, animals, and fungi [40,41]. Therefore, the evidence of Q redox state-dependent sensitivity to PN of AcUCP could suggest that this phenomenon occur in UCPs throughout the whole eukaryotic world. However, it seems also probably that in *A. castellanii* mitochondria, like in plant and some fungal mitochondria, where a cyanide-resistant alternative oxidase is present, the mechanism of interaction between Q pool redox state and UCP activity may be a feedback to prevent the Q pool from becoming overly reduced. Such a role would not apply to mammals. Fact that in *A. castellanii* mitochondria, the transition of the inhibitory effect of PN on FFA-induced AcUCP-mediated uncoupling is observed for the same range of Q reduction level in two different metabolic states of mitochondria brings us nearer to elucidate one of mechanisms of UCP activity regulation in protozoan mitochondria.

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Figure legends:

Figure 1 The effect of sequential addition of increasing LA concentration on respiratory rate, membrane potential and Q redox state in *A. castellanii* mitochondria. Mitochondria were incubated in the presence of 1.8 μ M CATR, 3 mM BHAM, 4 μ M rotenone, and 80 μ M ATP. Additions: 5 mM succinate (Succ), 1.2, 1.2, 2.4 μ M LA, 0.5 μ M FCCP. Numbers on the traces refer to O₂ consumption rates in nmol O/min/ mg protein or to $\Delta\Psi$ values in mV. The Q redox state values (in %) corresponding to given experimental steady-state conditions are shown. An example of twelve measurements (using mitochondria from six different preparations) are shown.

Figure 2 The effect of GDP on the change in respiration, membrane potential and Q redox state caused by LA-induced proton leak (AcUCP activity) when the rate of the Q-reducing pathway is decreased in mitochondria oxidizing succinate. Assay conditions and presentation as in Fig. 1. Additions: 5 mM succinate (Succ), 2.5 mM malonate (Mal), 2.4 μ M LA, 1 mM GDP.

Figure 3 The inhibitory effect of GDP on the LA-induced proton leak (AcUCP activity) when the Q-reducing pathway is decreased during state 4 respiration in mitochondria oxidizing succinate in the presence of two different LA concentrations. The relationships between (A) the respiration rate (V_4) and membrane potential (flux-force relationship), (B) V_4 and Q redox state and (C) $\Delta\Psi$ and Q redox state are presented. Succinate (Succ) oxidation was gradually decreased by increasing the concentration of malonate (0-5 mM). Measurements were made (where indicated) in the absence or presence of 1.2 or 2.4 μM LA, in the absence or presence of 1 mM GDP, and in the absence or presence of malonate as shown in Fig. 2. Data deal with one representative experiment of six different mitochondria preparations. The range of the Q redox state with the transition of GDP inhibitory effect (from 0% to 100%) is marked as a grey box.

Figure 4 The effect of GDP on the change in respiration, membrane potential and Q redox state caused by LA-induced proton leak (AcUCP activity) when the rate of the Q-reducing pathway is decreased in mitochondria oxidizing external NADH. Assay conditions and presentation as in Figure 1. Additions: NADH 0.5 mM or 0.005 mM, 1.2 μM LA, 1 mM GDP.

Figure 5 The inhibitory effect of GDP on the LA-induced proton leak (AcUCP activity) when the Q-reducing pathway is decreased during state 4 respiration in mitochondria oxidizing succinate or external NADH. The relationships between (A) the respiration rate (V_4) and membrane potential (proton leak kinetics), (B) V_4 and Q redox state and (C) $\Delta\Psi$ and Q redox state are presented. Substrate oxidation was gradually decreased by increasing the concentration of malonate (0-5 mM) (with succinate, Succ) or by decreasing the concentration (0.5-0.003 mM) of NADH (with NADH). Measurements were made (where indicated) in the absence or presence of 1.2 LA, and in the absence or presence of 1 mM GDP as shown in Figure 4. Data deal with one representative experiment of six different mitochondria

preparations. The range of the Q redox state with the transition of GDP inhibitory effect (from 0% to 100%) is marked as a grey box.

Figure 6 The effect of GDP on the change in respiration, membrane potential and Q redox state caused by an LA-induced proton leak (AcUCP activity) when the rate of the QH₂-oxidizing pathway is decreased. Assay conditions and presentation as in Figure 1. Additions: 5 mM succinate (Succ), 5.8 μ M myxothiazol (MX), 1.2 μ M LA, 1 mM GDP.

Figure 7 No inhibitory effect of GDP on the LA-induced proton leak (AcUCP activity) when the QH₂-oxidizing pathway is decreased during state 4 respiration in mitochondria oxidizing succinate. The relationships between (A) the respiration rate (V₄) and membrane potential (proton leak kinetics), (B) V₄ and Q redox state and (C) $\Delta\Psi$ and Q redox state are presented. To decrease the rate of the QH₂-oxidizing pathway, complex III was inhibited with myxothiazol (MX) (up to 9 μ M) or complex IV was inhibited with cyanide (KCN) (up to 20 μ M). Measurements were made (where indicated) in the absence or presence of 1.2 μ M LA, and in the absence or presence of 1 mM GDP as shown in Figure 5. Data deal with one representative experiment of six different mitochondria preparations. The range of the Q redox state with the transition of GDP inhibitory effect (from 0% to 100%) is marked as a grey box.

Fig. 1

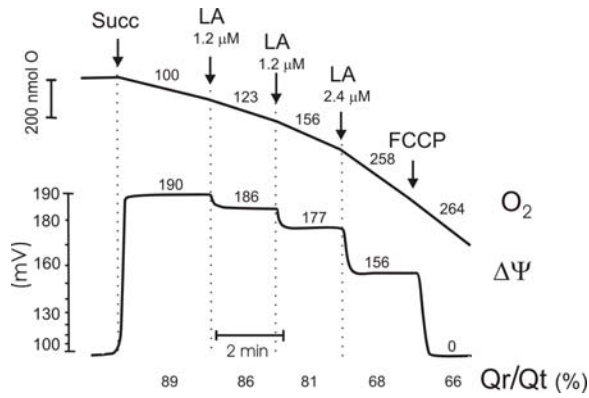
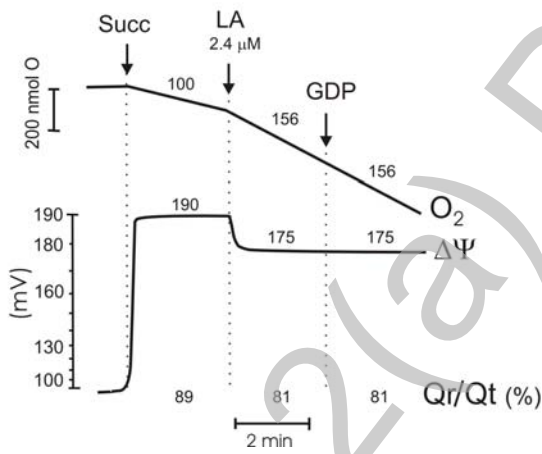
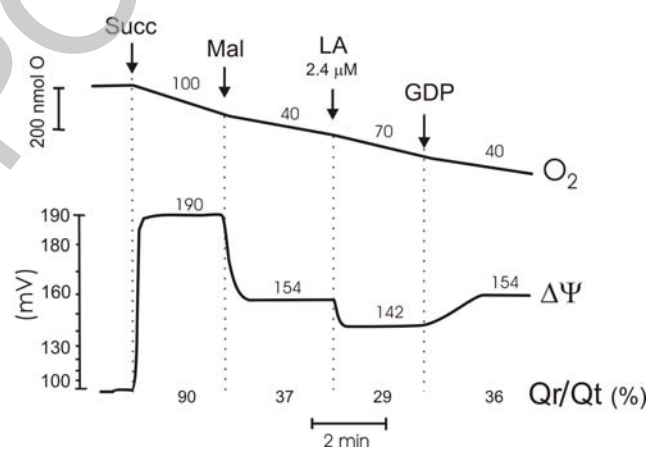


Fig. 2

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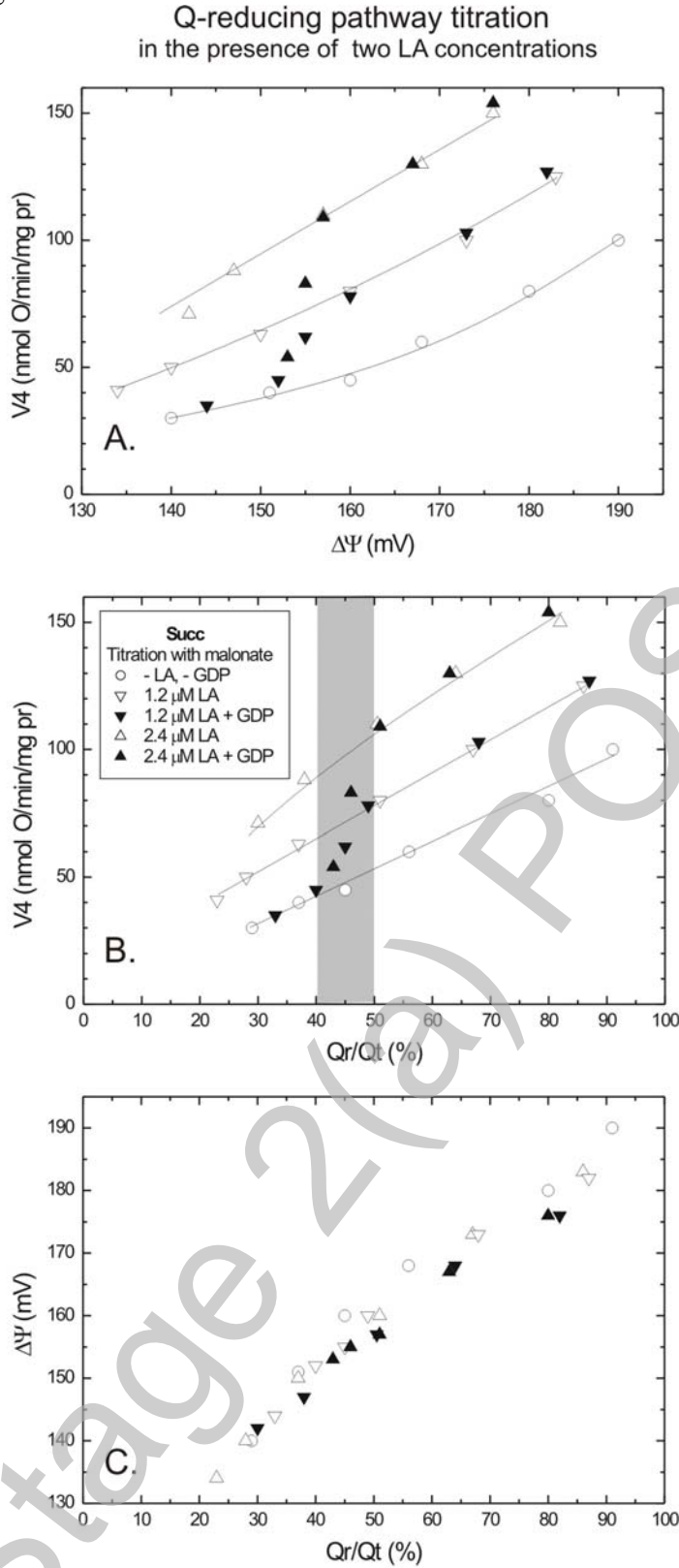


B.



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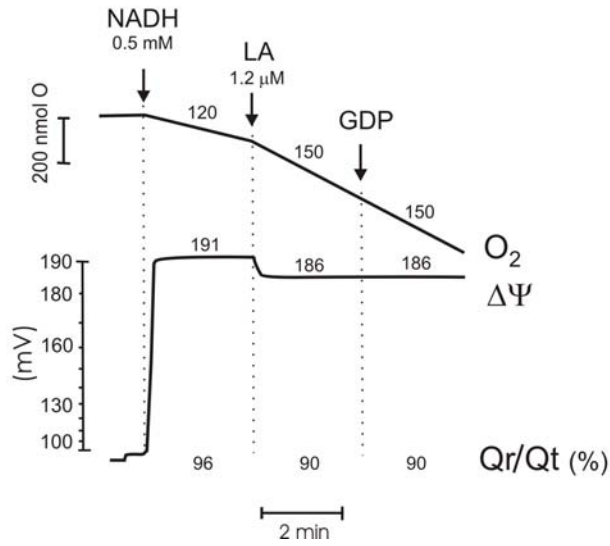
Fig. 3



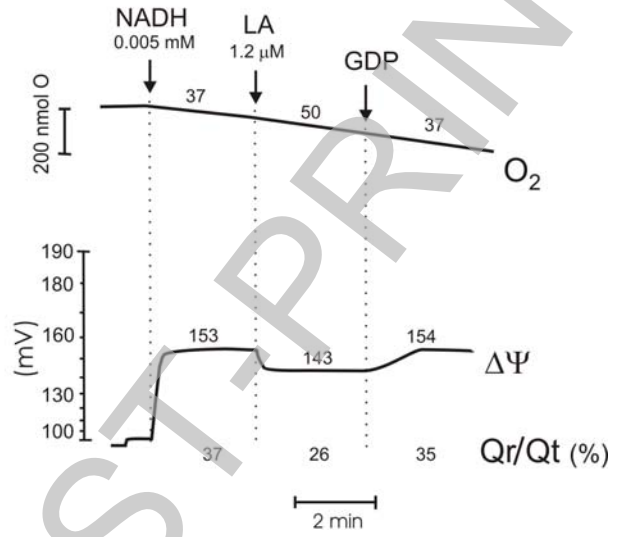
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Fig.4

A.

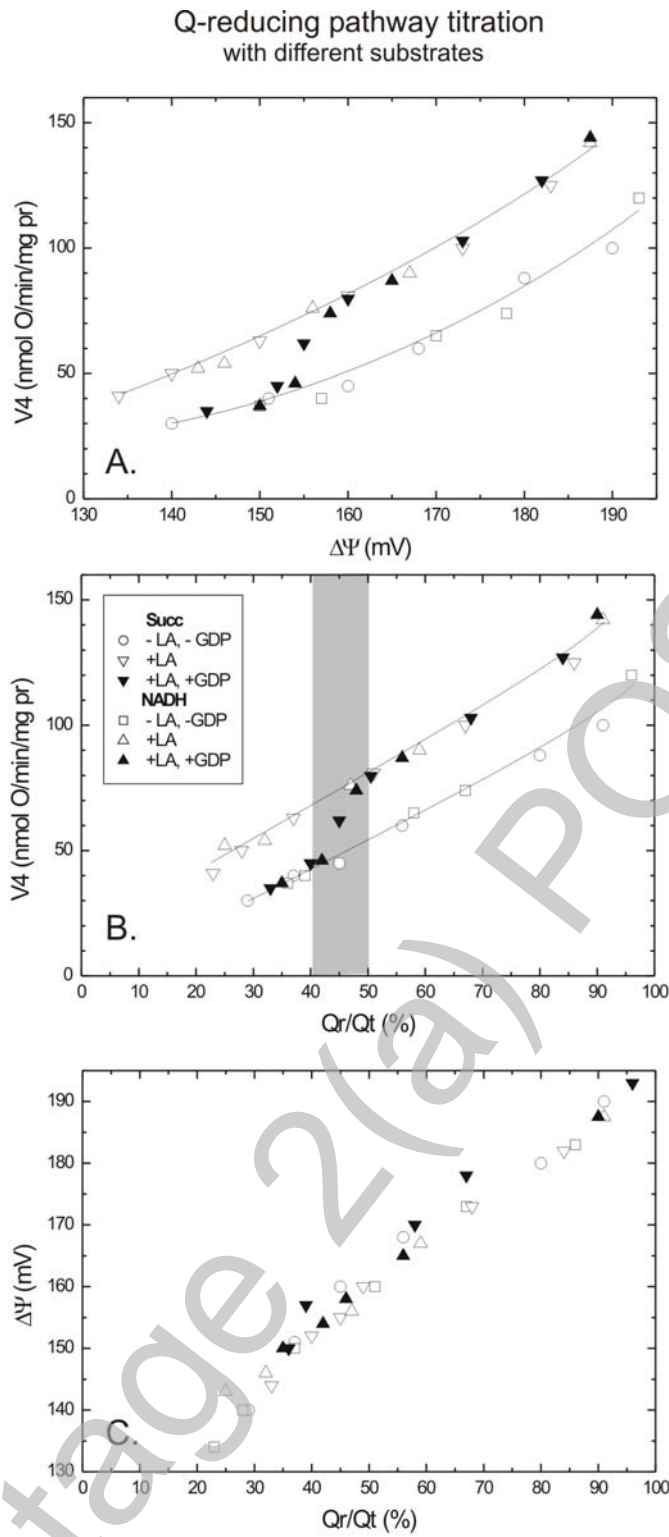


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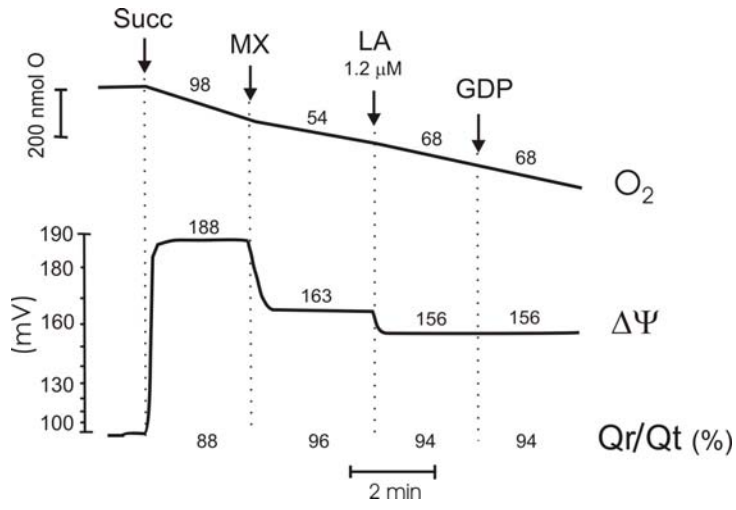
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Fig. 5



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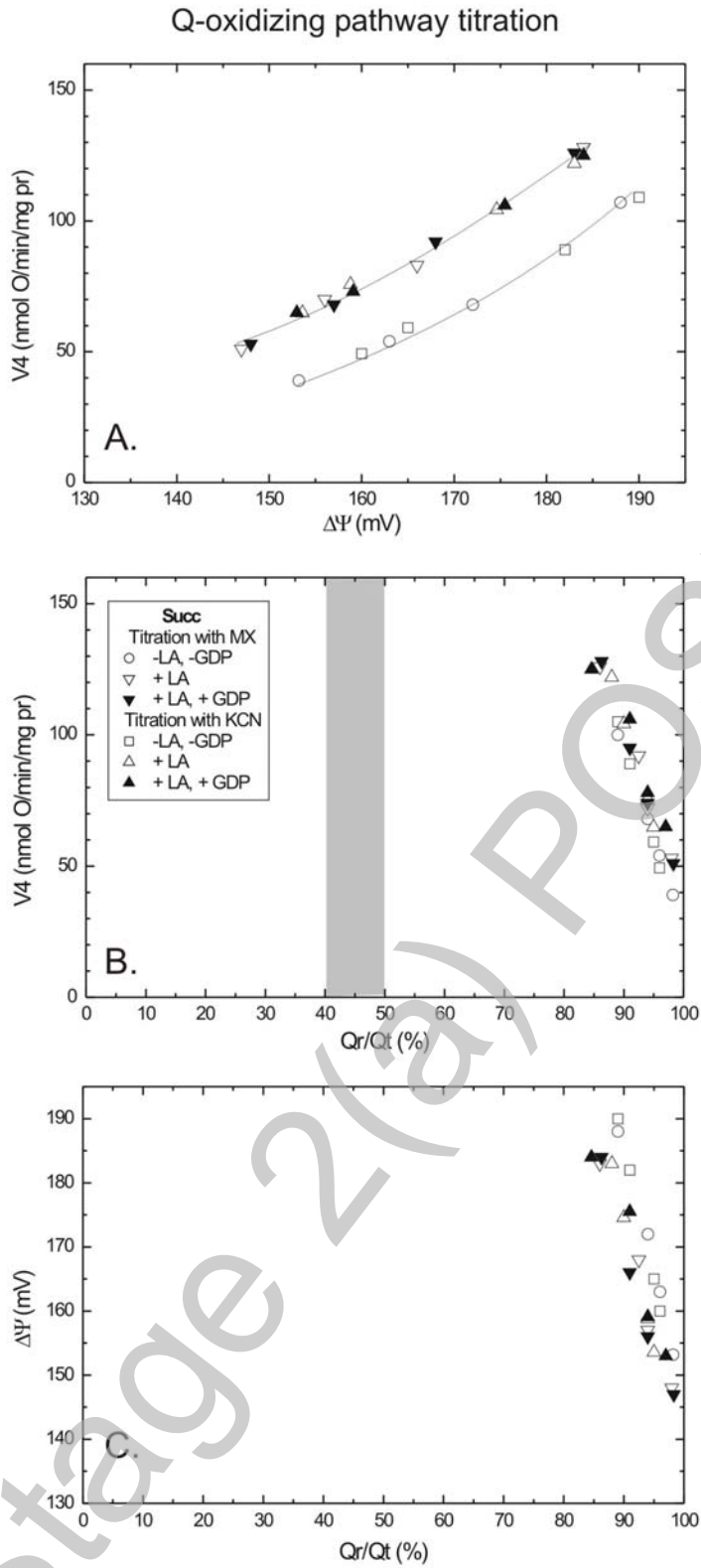
Fig. 6



Stage 2(a) POST-PRINT

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Fig. 7



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