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Uncoupling Protein 1 Inhibition by Purine Nucleotides Is under Control of the Endogenous Ubiquinone Redox State

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Short title: Endogenous ubiquinone redox state controls mitochondrial uncoupling protein 1 activity

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Abbreviations used: BAT, brown adipose tissue; BSA, bovine serum albumin; FFA, free fatty acids; OA, oleic acid; PN, purine nucleotides; TPP⁺, tetraphenylphosphonium; UCP, uncoupling protein; UCPI, uncoupling protein of brown adipose tissue; Q, ubiquinone; Qr or QH₂ reduced ubiquinone (ubiquinol); Qt, total endogenous pool of ubiquinone in the inner mitochondrial membrane; Qr/Qt, reduction level of ubiquinone; $\Delta\Psi$, mitochondrial membrane potential

Key words: mitochondria, proton conductance, uncoupling protein 1, ubiquinone redox state, brown adipose tissue, yeast

Synopsis

We studied free fatty acid-induced uncoupling of heterologously expressed rat UCPI in yeast mitochondria as well as UCPI in rat BAT mitochondria. The proton conductance curves and the relationship between ubiquinone reduction level and membrane potential were determined in non-phosphorylating BAT and yeast mitochondria. The ADP/O method was applied to determine the ADP phosphorylation rate and the relationship between ubiquinone reduction level and respiration rate in yeast mitochondria. Our studies of membranous ubiquinone reduction level in mitochondria demonstrate that activation of UCPI leads to a purine nucleotide-sensitive decrease in the ubiquinone redox state. Results obtained for non-phosphorylating and phosphorylating mitochondria, as the endogenous ubiquinone redox state was gradually varied by a lowering rate of the ubiquinone-reducing or ubiquinol-oxidising pathways, indicate that the endogenous ubiquinone redox state has no effect on free fatty acid-induced UCPI activity in the absence of GTP, and can only regulate this activity through sensitivity to inhibition by the purine nucleotide. At a given oleic acid concentration, inhibition by GTP diminishes when ubiquinone is reduced sufficiently. The ubiquinone redox state-dependent alleviation of UCPI inhibition by the purine nucleotide was observed at a high ubiquinone reduction level, when it exceeded 85-88%.

INTRODUCTION

UCP1 (uncoupling protein 1) is a well-characterised member of the UCP subfamily [1-5]. UCP1 short-circuits the proton electrochemical gradient generated by the respiratory chain in brown adipose tissue (BAT) mitochondria. It is responsible for adaptive thermogenesis by conducting protons across the mitochondrial inner membrane that uncouples ATP production from respiration and leads to heat production. Free fatty acid (FFA)-activated purine nucleotide (PN)-inhibited proton conductance sustained by UCP1 greatly increases the BAT respiration rate in response to hormonal stimuli. In non-thermogenic animal and plant tissues as well as in unicellular organisms, the physiological role of UCP1 homologues has not yet been well established [1-4,6,7].

Since UCPs are specialised proteins for proton electrochemical gradient dissipation, their activity must be finely regulated. Inhibition of H^+ conductance by PNs is considered diagnostic of UCP activity. In the case of mammalian UCPs (UCP1, UCP2, and UCP3), ubiquinone (coenzyme Q, Q) has been shown to be an obligatory cofactor for their action in liposomes and isolated mitochondria [8-11]. It has been postulated that ubiquinone (likely reduced form) activates proton conductance in mitochondria through the production of superoxide [10]. Products of lipid peroxidation by reactive oxygen species such as 4-hydroxy-2-nonenal are proposed to be direct activators of UCPs [12,13]. However, in a reconstituted system with heterologously expressed mammalian UCPs, no superoxide activation has been required to demonstrate the FFA-activated PN-sensitive H^+ translocation, and ubiquinone has no significant activating effect nor any effect on inhibition by PNs [14,15]. Studies with mitochondria isolated from yeast mutants expressing mouse UCP1 but lacking ubiquinone have also shown that Q is not required for proton conductance by UCP1 [16]. Taking into account the apparent affinity of reconstituted UCPs for PNs [9,15] and the concentration of nucleotides *in vivo* (at millimolar concentrations inside the cells), UCPs should be permanently inhibited under *in vivo* conditions, even in the presence of FFA, unless a regulatory factor or mechanism could alleviate the inhibition by PNs [2]. Therefore, it has been proposed that the membranous Q redox state could be a metabolic sensor that modulates PN inhibition of FFA-activated UCP1 homologues; such is the case in isolated skeletal muscle (UCP3), potato tuber (plant UCP), and *Acanthamoeba castellanii* (protozoan UCP) mitochondria respiring under phosphorylating conditions in the absence of endogenous superoxide production [17-19]. In the case of protozoan UCP, this regulatory mechanism has also been recently observed in isolated non-phosphorylating mitochondria [20]. Activation of UCP1 *in vivo* requires interactions with FFAs and their ability to overcome the inhibition of UCP1 by PNs. Therefore, the question arises as to whether UCP1 sensitivity to PNs could be regulated by the redox state of membranous Q as observed in the case of UCP1 homologues.

The aim of the present study was to examine the influence of the endogenous Q redox state on PN-inhibition of the FFA-induced activity of heterologously expressed UCP1 in yeast mitochondria as well as UCP1 in rat BAT mitochondria. We show, for the first time, that UCP1 inhibition by PN is under control of the endogenous Q redox state.

EXPERIMENTAL

Expression of UCP1 in *Saccharomyces cerevisiae*

S. cerevisiae (INVSc1 strain) cells were transformed with either a rat UCP1 low expressing construct or a plasmid containing an empty vector (pYES2.1) (Invitrogen) [21]. The preculture was grown at 28 °C under vigorous aeration in SC (*S. cerevisiae*) minimal medium containing 0.67% (w/v) yeast nitrogen base, 0.5% (w/v) $(NH_4)_2SO_4$, 0.005-0.01% (w/v) amino acids and 2% (w/v) glucose. Glucose repressed *GAL1*-promoted gene expression. Afterwards, cultures were grown in SC medium with 3% (v/v) glycerol for 48 h. Protein expression was induced in medium identical to that used for pre-culture but glucose was replaced with 2% (w/v) galactose and 5% glycerol. For both wild-type (empty vector) and UCP1-

expressing yeasts, generation time in continuously agitated cultures was ~2.5 h. Cells cultured for 20 h in 1.8 L flasks were collected in the exponential phase (with an absorbance of 1.2-1.8 at 650 nm).

UCP1 expression in mitochondria isolated from UCP1-containing cells was determined by Western-blot analysis. Concentration of ~1 μg of UCP1 per mg of mitochondrial proteins was detected, confirming previous results using similar methods [21].

Isolation of yeast mitochondria

Yeast cells (wild-type with empty vector and UCP1-expressing cells) were harvested by centrifugation at 1000 g for 10 min, re-suspended in deionised water, and centrifuged a second time. Pellets were re-suspended in a buffer containing 0.1 M Tris- H_2SO_4 (pH 9.4) and 10 mM dithiothreitol and incubated at 28 °C in an orbital shaker (160 rpm) for 15 min. Pellets were again centrifuged and washed in 1.2 M sorbitol. The cells were re-suspended in 6 ml of a buffer containing 1.2 M sorbitol and 20 mM K/K phosphate buffer (pH 7.4) per 1 g of cells. Zymolyase was added (at 1 mg/g wet weight of cells), and the suspension was incubated 28 °C under gentle agitation until about 90% of cells had converted to spheroplasts (~30-50 min). The digestion was stopped by the addition of an equal volume of ice-cold buffer containing 1.2 M sorbitol, 10 mM Tris-HCl (pH 6.8), 2 mM EGTA, and 0.2% bovine serum albumin (BSA). All subsequent steps were performed at 4 °C. Spheroplasts were pelleted, washed twice in the latter buffer, and re-suspended in an isolation buffer (0.65 mM mannitol, 10 mM Tris-HCl, pH 6.8, 0.5 mM EDTA, 0.1 mM EGTA, and 0.2% BSA) at a ratio of 9 ml buffer per 1 g of spheroplasts. After homogenisation by 10 passes with a tight Dounce homogeniser, homogenates were centrifuged at 1000 g for 10 min. The pellets were re-suspended and centrifuged again to collect mitochondria remaining in the pellet. The supernatants were combined and centrifuged at 1000 g for 10 min. The resultant supernatants were centrifuged at 10000 g for 10 min. The mitochondrial pellets were washed with a buffer containing 0.65 M mannitol and 10 mM Tris-HCl, pH 6.8 and centrifuged at 10000 g (10 min). The final pellet was re-suspended in a small volume of the same buffer.

Isolation of rat brown adipose tissue mitochondria

Brown adipose tissue mitochondria were isolated essentially as described in [22]. Interscapular and subscapular brown adipose tissues were taken from four 9- to 11-week-old male rats (250-350 g), housed at 24 °C and fed *ad libitum*. Subsequent steps were carried out at 4 °C. Tissue was placed in STE buffer (0.25 M sucrose, 1 mM EGTA, 5 mM Tris-HCl, pH 7.2) supplemented with 5% defatted BSA, minced, and homogenised in STE+BSA buffer with six strokes of an electric glass homogeniser (at middle rotation, teflon C-type piston). Then, a homogenate was filtered through two layers of gauze. The filtrate was centrifuged at 8500 g (10 min) and the pellet was re-suspended in STE buffer (supplemented with 1% BSA) and centrifuged at 700 g (10 min), and the supernatant was then centrifuged at 8500 g (10 min). The mitochondrial pellet was suspended in STE buffer, re-centrifuged and finally re-suspended in ~500 μl of STE buffer.

Mitochondrial oxygen consumption and membrane potential measurements

Oxygen uptake was measured polarographically using a Rank Bros. (Cambridge UK) oxygen electrode or a Hansatech oxygen electrode in 1.4 ml or 2.8 ml, respectively, of standard incubation media (28 °C) containing: 100 mM KCl, 10 mM Tris-HCl pH 7.0, 1 mM EGTA, 2 mM KH_2PO_4 , 1 mM MgCl_2 , and 0.1% (w/v) defatted BSA (BAT mitochondria) or 0.65 M mannitol, 10 mM Tris-HCl pH 6.8, 0.5 mM EGTA, 2 mM MgCl_2 , 10 mM with KH_2PO_4 , and 0.05% (w/v) defatted BSA (yeast mitochondria) with 0.85-1 mg of mitochondrial protein. Values of O_2 uptake are in nmol $\text{O}/\text{min}/\text{mg}$ protein. The membrane potential ($\Delta\Psi$) of mitochondria was measured simultaneously with oxygen uptake using a tetraphenylphosphonium (TPP^+)-specific electrode. After each run, 0.5 μM carbonylcyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP) was added to release TPP^+ for base-line correction. For

calculation of the $\Delta\Psi$ value, the matrix volume of mitochondria was assumed to be 2.0 μl per mg of protein. The 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. Succinate was used at 7.5 mM succinate as a respiratory substrate in the presence of 4 μM rotenone (an inhibitor of complex I). To induce UCP1 activity-mediated respiration, measurements were made in the presence of 40-43 μM oleic acid (OA). To inhibit UCP1 activity, 2 mM GTP was added.

Proton leak measurements

The proton conductance response to a driving force can be expressed as the relationship between the oxygen consumption rate and $\Delta\Psi$ (flux-force relationship) when varying the potential by titrating with respiratory chain inhibitors. Respiration rate and $\Delta\Psi$ were measured simultaneously using electrodes sensitive to oxygen and TPP^+ with isolated rat BAT mitochondria or yeast mitochondria. Proton leak rates can be calculated from respiration rates by multiplying by an H^+/O ratio of six. Measurements were performed in the absence of added ADP, i.e., in a non-phosphorylating state (resting state, State 4). To exclude the activity of an ATP/ADP antiporter, 1.8 μM carboxyatractylozide was used.

Respiratory rate, $\Delta\Psi$, and the Q redox state were varied by modulating the Q-reducing or QH_2 [reduced ubiquinone (ubiquinol)]-oxidising pathways. To decrease the rate of the Q-reducing pathway during State 4 respiration (thereby decreasing a steady-state resting respiration), titration of succinate dehydrogenase activity via increasing the concentration of malonate (a competitive inhibitor; up to 7 mM) was carried out. To decrease the rate of the QH_2 -oxidising pathway during State 4 respiration, complex IV was inhibited with cyanide (up to 20 or 50 μM in yeast or BAT mitochondria, respectively). 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 concentrations (as in Figures 3 and 7) and data from measurements with sequential inhibitor additions were afterwards combined to generate common curves (Figures 1,2,6). To assess the statistical significance of the shifts in leak curves caused by GTP, we generally compared respiration rates at the lowest common $\Delta\Psi$ (Figures 2 and 6) for pairs of curves from 3-7 independent experiments using Student's *t* test for paired data.

ADP phosphorylation rate measurements

Phosphorylating state (State 3) measurements were performed with yeast mitochondria. The ADP/O ratio was determined by an ADP pulse method with 250-450 nmoles of ADP. The measured ADP/O ratio and State 3 respiration rate (V_3) were used to calculate the rate of ADP phosphorylation ($J_p = V_3 \times \text{ADP/O}$). ATP (80 μM) was applied to activate succinate dehydrogenase.

To decrease the rate of the Q-reducing pathway during State 3 respiration (thereby decreasing steady-State 3 respiration), titration of succinate dehydrogenase activity with an increasing concentration of *n*-butylmalonate (0-5 mM), a competitive inhibitor of succinate uptake, was performed. To decrease the rate of the QH_2 -oxidising pathway during State 3 respiration, the bc_1 complex (complex III) was inhibited by antimycin A (up to 30 ng/ml).

Measurements of the ubiquinone reduction level

The redox state of Q in steady-state respiration was analysed by an extraction technique followed by HPLC detection [20]. A LiChrosorb RP-18 (10 μm) HPLC column or Polaris C18-A (5 μM) HPLC column were used for separation of Q_9 (rat BAT mitochondria) or Q_6 (yeast mitochondria), respectively. For calibration and quantification of the Q peaks commercial coenzymes (Sigma) were used. The

presented values of the redox state of Q (Q_r/Q_t) deal with the active ubiquinone pool (the difference between the total Q pool minus the inactive Q pool) in a given mitochondrial preparation [20].

RESULTS

Increasing inhibitory effect of GTP on FFA-induced and non-FFA-induced UCP1-sustained proton leak when the Q redox state is decreased in non-phosphorylating yeast mitochondria

Proton leak kinetics of yeast mitochondria containing UCP1, defined as the respiration rate needed to drive proton leak as a function of the driving force ($\Delta\Psi$), has been described previously [16,22,23-25]. Figure 1 shows the leak kinetics of yeast mitochondria with expression of modest amounts of rat UCP1 from plasmid pYES2.1. UCP1 expression did not greatly change the proton conductance, which is reflected by the relatively small increase in proton leak rate at any given $\Delta\Psi$ (no OA, no GTP, no Q-reducing pathway inhibitor) in mitochondria from yeast expressing UCP1 (Figure 1A) compared with mitochondria from yeast transformed with empty vector (Figure 1B). UCP1 was almost inactive in yeast mitochondria under conditions of no added OA (\circ), since GTP did not decrease the proton leak rate during progressive inhibition of succinate oxidation by malonate (\bullet) leading to a decrease in Q redox state from 81% to ~46% (Figure 1A). In contrast, GTP had a large inhibitory effect on the proton leak rate through OA-activated UCP1 during malonate titration (leading to decrease in the Q redox state up to ~43%) (\blacktriangle). The above experiments indicate that rat UCP1 in our yeast mitochondria was competent and required addition of FFA before large GTP-sensitive proton leak rates could be observed.

In the presence of OA, mitochondria from yeast transformed with empty vector revealed a slight proton conductance (lower proton leak at any $\Delta\Psi$) compared with those from UCP1 transformed cells (Figure 1B). Among tested free fatty acids (linoleic, myristic, oleic and palmitic acids), OA led to the smallest non-UCP1-mediated mitochondrial uncoupling (data not shown). An FFA-induced mitochondrial uncoupling effect has been previously observed in yeast mitochondria [21,23]. However, in empty vector mitochondria, OA-induced uncoupling is not sensitive to GTP during titration with malonate (decreasing the Q reduction level up to ~57%) (Figure 1B).

Decreasing inhibitory effect of GTP on FFA-induced UCP1-sustained proton leak when the Q redox state is increased in non-phosphorylating yeast mitochondria

To elucidate the role of respiratory rate and $\Delta\Psi$, we gradually decreased them with inhibitors of the QH_2 -oxidising pathway (the cytochrome pathway) leading to an increase in the Q reduction level. Figure 2 displays H^+ conductance curves (Figure 2A,C) and the relationship between Q reduction level versus $\Delta\Psi$ (Figures 2B,D) in the presence or absence of 40 μ M OA and/or 2 mM GTP, during titration with increasing concentrations of cyanide. Comparison of Figures 1A,B and 2A,C indicates that yeast mitochondria (lacking or containing UCP1) energised with succinate and titrated with inhibitors of the Q-reducing or QH_2 -oxidising pathways exhibited the same H^+ leak curves in the absence of GTP, with or without FFA. This clearly indicates that the redox state of endogenous Q does not affect the basal nor FFA-induced proton conductance in both types of yeast mitochondria.

In empty vector mitochondria during titration with cyanide, OA-induced uncoupling, which caused a slight upward replacement of the proton leak curve (Figure 2C) and a slight downward replacement of the Q redox state versus $\Delta\Psi$ (Figure 2D), was not sensitive to GTP.

In UCP1-containing yeast mitochondria, when the rate of the QH_2 -oxidising pathway was gradually decreased with cyanide, the flux-force relationship established in the presence of 40 μ M OA and in the presence or absence of 2 mM GTP indicates that inhibition of the OA-induced H^+ conductance by the nucleotide progressively diminished below $\Delta\Psi$ value of approximately 180 mV (Figure 2A). Below this value, points obtained in the presence of OA and GTP (\blacktriangledown) progressively came forward to the points

obtained in the presence of OA only (∇). For mitochondria titrated by cyanide (Figure 2A) at the lowest common $\Delta\Psi$ (168 mV), respiration rate in the presence of GTP and OA (26.2 ± 1.9 nmol O/min/mg protein, S.D.) was significantly higher ($p < 0.01$, Student's paired t test) from control (no GTP, no OA) rate (14.5 ± 2.4 nmol O/min/mg protein, S.D.) in contrast to mitochondria titrated by malonate (Figure 1A). Transition of the GTP inhibitory effect is also revealed by relationship between the Q reduction level and $\Delta\Psi$ (Figure 2B). When the Q reduction level was gradually increased, the inhibition by GTP was progressively relieved when points obtained with OA and GTP reached $\sim 88\%$ of the Q redox state (at ~ 180 mV). A further decrease in succinate oxidation with a higher cyanide concentration led to a transition of points obtained with GTP and OA (\blacktriangledown) from control relationship (no OA, no GTP) (\square) closer to an OA-induced linear relationship (∇). Although titration of succinate oxidation with cyanide (Figure 2A, 2B) comprised of the respiratory rate and $\Delta\Psi$ ranges at which GTP-sensitivity did not change during titration of the Q-reducing pathway (with malonate, Figures 1A), progressive cancellation of the inhibitory effect of GTP was revealed under these conditions. These results clearly indicate that the transition (attenuation) of the GTP inhibitory effect on OA-induced H^+ leak cannot be attributed to changes in the $\Delta\Psi$ or respiratory rate.

Figure 3A shows an example experiment in which the respiratory rate, $\Delta\Psi$, and the Q redox state were measured in UCP1-containing yeast mitochondria, concomitant with inhibition of succinate oxidation in the presence of OA by malonate or cyanide to $\sim 60\%$, leading to the same level of $\Delta\Psi$ (166-165 mV). The corresponding Q redox state was decreased from 72% to 46% by malonate or increased from 72% to 91% by cyanide. After subsequent addition of GTP, inhibition of the OA-induced H^+ leak was revealed by inhibition of the respiratory rate as well as restoration of $\Delta\Psi$ and the Q redox state. However, the inhibitory effect of GTP was much stronger when the nucleotide was added to mitochondria that had been inhibited by malonate (leading to 55% of the Q redox state) compared to those inhibited by cyanide (leading to 94% of the Q redox state). This stronger inhibition by GTP is revealed by a larger effect of the nucleotide on $\Delta\Psi$ and the Q redox state in the presence of malonate. These results indicate that efficiency of GTP to inhibit UCP1-sustained uncoupling in non-phosphorylating yeast mitochondria depends on the endogenous Q redox state. Inhibition by GTP can be diminished when Q is reduced sufficiently.

Effect of GTP in phosphorylating yeast mitochondria when the QH_2 -oxidising or Q-reducing pathways are decreased

In order to check if the dependence of UCP1 sensitivity to GTP on the endogenous Q redox state occurs also in phosphorylating yeast mitochondria containing UCP1, we measured State 3 respiratory rates as well as the ADP/O ratio in the absence or presence of 40 μM OA, with or without 2 mM GTP (Figure 4). As shown in Figure 4, State 4 respiration and $\Delta\Psi$ were perturbed (increased and decreased, respectively) by OA, while State 3 respiration and $\Delta\Psi$ remained unaffected. The respiratory control and ADP/O ratio were lowered by OA, indicating an induction of the H^+ leak. Both coupling parameters were recovered to control values when the measurement was performed in the presence of GTP. These results suggest that the OA-induced GTP-inhibited H^+ leak mediated by UCP1 expressed in yeast diverts energy from ATP synthesis during phosphorylating respiration.

The FFA-induced H^+ leak can be analysed in State 3 by applying the ADP/O method to calculate the rate of ADP phosphorylation when State 3 respiration, within the range where $\Delta\Psi$ remains constant, is titrated with various inhibitors [17-19]. In order to study the GTP sensitivity of the OA-induced H^+ leak, the rate of Q-reducing and QH_2 -oxidising pathways were gradually lessened by *n*-butylmalonate or antimycin A, respectively (Figure 5). Pair measurements of ADP/O ratios and State 3 respiration in the presence or absence of 40 μM OA, with or without 2 mM GTP, were performed for decreasing State 3 respiration. The titration range of State 3 was such that $\Delta\Psi$ remained constant (165 ± 2 mV, S.D., $n = 12$) in the absence or presence of OA (with or without GTP) for UCP1-containing yeast mitochondria. A constant ADP/O ratio (1.32 ± 0.04 , S.D., $n = 36$) was observed in all control conditions, i.e., when the Q-reducing pathway was titrated with *n*-butylmalonate either in the absence of OA (with or without GTP) or

in the presence of both OA and GTP, as well as when the QH₂-oxidising pathway was titrated with antimycin A in the absence of OA (with or without GTP) (Figure 5A). In UCP1-containing yeast mitochondria, in the presence of OA alone, the measured ADP/O ratio was lowered to 1.1 ± 0.07 (S.D., $n = 9$) and then declined with a decreasing State 3. Thus, lowering the electron flux amplified the OA-induced decrease in the ADP/O ratio.

Titration of State 3 respiration led to a linear relationship between the rate of ADP phosphorylation ($J_p = V_3 \times \text{ADP/O}$) and corresponding State 3 respiration (V_3) (Figure 5B) in all control conditions (see above) where the ADP/O constancy was found (Figure 5A). Under these conditions, the straight line intersects the abscissa axis close to the origin, indicating that no basal H⁺ leak occurs during State 3 respiration in UCP1-expressing yeast mitochondria. However, addition of OA during *n*-butylmalonate or antimycin A titration (no GTP) provoked a shift of the linear relationship between ADP phosphorylation rate and State 3 to the right. The respiration sustained by the OA-induced H⁺ leak (no GTP) was 31.5 ± 3.4 nmol O/min/mg pr. (S.D., $n = 10$).

As shown in Figure 5C, the Q redox state followed a linear relationship with decreasing State 3 respiration. Titration with *n*-butylmalonate decreased the Q reduction level (from ~65% to ~28%), while antimycin A increased the Q reduction level (from ~65% to ~95%). When the Q-reducing pathway activity was inhibited (with *n*-butylmalonate), the ADP/O ratios (Figure 5A) and the relationship between the ADP phosphorylation rate versus State 3 respiration (Figure 5B), measured in the presence of OA and GTP (when compared to plus OA, no GTP conditions) revealed an inhibitory effect of GTP on the OA-induced H⁺ leak (points are on the control lines), which thereby can be attributed to the UCP1 activity. Thus, at 65% of the Q redox state (not titrated State 3) and below this value (State 3 titrated with *n*-butylmalonate), the full inhibitory effect of GTP is observed. On the other hand, with the decreasing QH₂-oxidising pathway activity (i.e. with the increasing Q reduction level) a transition of GTP inhibitory effect was observed. Namely, with increasing concentration of antimycin A (in the presence of OA and GTP), a progressive abatement of GTP inhibitory effect was revealed by (i) a non-linear relationship between the rate of ADP phosphorylation and State 3 respiration (Figure 5B, ▼) that transitioned from a control relationship (the straight line coming from the origin) to an OA-induced linear relationship (∇), and (ii) the ADP/O ratios (Figure 5A, ▼) that progressively dropped to the ADP/O values obtained in the presence of OA only (no GTP) (∇). A weakening of the GTP inhibitory effect was observed for State 3 respiration below ~132 nmol O/min/mg pr., corresponding to a Q redox state higher than ~85%. This set of observations suggests that in phosphorylating UCP1 expressing yeast mitochondria, OA induces a UCP1-mediated H⁺ leak and inhibition of this leak by GTP is controlled by the endogenous Q redox state. Taking into account results obtained for non-phosphorylating (Figure 2) and phosphorylating (Figure 5) UCP1-expressing yeast mitochondria, it can be concluded that changes in the GTP inhibitory effect on OA-induced UCP1-mediated uncoupling are observed for the same range of Q reduction levels. For both mitochondrial energetic states, full inhibition is observed below a Q redox state of 85-88% while a progressive abatement of GTP inhibition occurs above this value.

In phosphorylating yeast mitochondria containing an empty vector, no OA-induced H⁺ leak was observed as neither the ADP/O ratio nor the linear relationship between ADP phosphorylation rate and State 3 respiration were changed in the presence of fatty acid (no GTP) (Figure 5D and 5E, respectively). It confirms that the OA-induced GTP-sensitive H⁺ leak observed in UCP1-expressing yeast mitochondria can be attributed to the UCP1 activity.

The Q redox state influences UCP1 sensitivity to GTP in mitochondria isolated from rat brown adipose tissue

To investigate whether the ability of GTP to inhibit UCP1-mediated proton conductance depends on the membranous Q redox state in its native environment, the effects of a varied Q reduction level on the endogenous (observed under applied conditions) or external OA-induced proton leaks' sensitivity to GTP were assessed in non-phosphorylating mitochondria isolated from rat BAT. It is well known that mitochondria isolated from BAT tissue are essentially uncoupled, but on addition of PNs, respiration rates

due to the UCP1-mediated proton leak are inhibited. Therefore, BAT mitochondria are not good material to study UCP1 action under phosphorylating conditions; for these studies we applied a heterologous yeast expression system (Figures 4,5) with modest amounts of rat UCP1 [21].

Figure 6 displays H^+ conductance curves (Figure 6A) and the relationship between Q reduction level versus $\Delta\Psi$ (Figure 6B) in the presence or absence of 43 μM OA and/or 2 mM GTP, when the QH_2 -oxidising or Q-reducing pathways were gradually decreased by increasing concentrations of cyanide or malonate, respectively. Rat BAT mitochondria energised with succinate and titrated with both inhibitors yielded the same H^+ leak curves (no GTP, with or without OA) (Figure 6A). Thus, the redox state of membranous Q does not affect the endogenous or OA-induced proton conductance of BAT mitochondria. In the absence of OA (no GTP), the initial Q reduction level (~65%) progressively increased up to 83% with cyanide (\square) but decreased up to 54% with malonate (\circ) (Figure 6B). In the presence of 43 μM OA (no GTP), the initial Q reduction level (~60%) progressively increased up to 80% with cyanide (∇), yet progressively decreased up to 56% with malonate (Δ) (Figure 6B).

Figure 6A demonstrates that the proton conductance of BAT mitochondria, both endogenous and external OA-induced, exhibits different sensitivity to GTP when the QH_2 -oxidising or Q-reducing pathways were inhibited. During titration with cyanide, the flux-force relationship established in the presence of GTP (with or without OA) indicated that inhibition of H^+ conductance by the nucleotide progressively diminished at $\Delta\Psi$ value below 183-184 mV (a lower slope is observed), in contrast to titration with malonate. At the lowest common $\Delta\Psi$ value in the presence of GTP (178 mV), respiration rate was significantly higher ($p < 0.05$, Student's paired t test) for mitochondria titrated by cyanide (22.5 ± 2.5 or 20.3 ± 2 nmol O/min/mg pr., S.D., without or with OA, respectively) than by malonate (9.4 ± 1.5 or 12 ± 2 nmol O/min/mg pr., S.D., without or with OA, respectively). Transition of the GTP inhibitory effect is also disclosed by the relationship between the Q reduction level and $\Delta\Psi$ (Figure 6B). In the presence of GTP (with or without OA), increasing the concentration of malonate decreased Q redox state from ~81% to ~76-74%. However, when the QH_2 -oxidising pathway was gradually inhibited with cyanide, the Q reduction level increased to a maximal value of ~86% (corresponding to a value of $\Delta\Psi$ below 183 mV). A further increase in cyanide concentration led to no further increase in the Q reduction level that remained above 82%. These results indicate that GTP's ability to inhibit UCP1-sustained uncoupling in non-phosphorylating BAT mitochondria is under control of the endogenous Q redox state.

In order to avoid possible errors due to non-steady-state conditions in the case of data from measurements with sequential inhibitor additions that are afterwards pooled together on common curves, measurements with a single inhibitor addition were performed as presented in Figure 7. This experimental approach clearly validates of the observed phenomenon. Figure 7 (A, C) displays example experiments in which the respiratory rate, $\Delta\Psi$, and the Q redox state were measured in rat BAT mitochondria under conditions where succinate oxidation in the absence (Figure 7A) or presence (Figure 7C) of 43 μM OA was inhibited by a single addition of malonate or cyanide resulting in the same value of $\Delta\Psi$ (129-130 mV). The corresponding Q redox state was decreased to 56% by malonate (both in the absence or presence of OA) or increased to 82% or 75% by cyanide (in the absence or presence of OA, respectively). After a subsequent addition of GTP, inhibition of the non-OA-induced or OA-induced H^+ leak was revealed by inhibition of the respiratory rate as well as restoration of $\Delta\Psi$ and the Q redox state. However, for both endogenous and OA-induced proton leaks, the inhibitory effect of GTP was much stronger when the nucleotide was added to mitochondria inhibited by malonate compared to those inhibited by cyanide, although the respiratory rate and $\Delta\Psi$ before GTP addition were the same. This is revealed by the greater GTP effect on $\Delta\Psi$ and the Q redox state in the presence of malonate. Thus the ability of GTP to inhibit UCP1-mediated proton conductance seems to depend on the endogenous Q redox state in BAT mitochondria just as it does in yeast mitochondria. Inhibition by GTP can be diminished when Q is reduced sufficiently.

DISCUSSION

The results presented in this study obtained with non-phosphorylating and phosphorylating UCP1-containing yeast mitochondria as well as with non-phosphorylating rat BAT mitochondria clearly indicate that UCP1 sensitivity to GTP can be regulated by the redox state of membranous Q, as has been observed previously for UCP1 homologues in isolated mitochondria of rat skeletal muscle, potato tubers, and *A. castellanii* [17-20]. It indicates a likely universal mechanism through which UCPs are regulated. It must be emphasised that in the present study, in non-phosphorylating UCP1-expressing yeast mitochondria and BAT mitochondria, at a given concentration of OA (or at no addition of external FFA), the Q redox state-dependent alleviation of PN-mediated inhibition of UCP1 is observed for a similar range of Q reduction levels as that observed for phosphorylating yeast mitochondria. Thus, for both mitochondrial energetic states and both types of mitochondria, full inhibition is observed below a Q redox state of ~85-88%. This observation, which was drawn from studies with the application of two different experimental approaches, strongly indicates that ubiquinone (likely QH₂) affects the affinity of UCP1 for GTP.

Studies with mammalian UCPs have yielded conflicting results concerning the possibility that Q may be an obligatory cofactor for their action. Namely, oxidised ubiquinone has been shown to activate PN-sensitive FFA-dependent H⁺ transport through reconstituted UCP1-3 [8,9]. Photoaffinity labelling of purified UCP1 with retinoic acid has indicated that Q increases binding of an activator [11]. On the other hand, other studies have shown that Q has no significant activating effect on FFA-dependent H⁺ translocation nor any effect on the inhibition by PN in reconstituted UCP1-3 [14]. In isolated kidney mitochondria, energised with succinate, titration with myxothiazol or cyanide (but not malonate) results in increased proton conductance only when external oxidised Q is added [10]. From these flux-force relationship studies, it has been proposed that external Q activates proton conductance in mitochondria through the production of superoxide, as superoxide dismutase inhibits Q-induced mitochondrial uncoupling. However, it has been concluded that the redox state of endogenous Q does not affect mitochondrial conductance. Therefore, the amount of endogenous ubiquinone reduced during titration with myxothiazol or cyanide is not enough to induce superoxide-stimulated proton leak until exogenous Q is added [10]. Our results suggest that the endogenous Q redox state has no effect on the basal and FFA-induced UCP1-catalysed H⁺ conductance in the absence of PNs but affects its sensitivity to inhibition by nucleotides. This conclusion can be made as determination of membranous Q reduction level was performed when H⁺ leak curves (non-phosphorylating mitochondria) or relationships between ADP phosphorylation rate and State 3 respiration rate (phosphorylating mitochondria) were established with inhibitors of the Q-reducing or QH₂-oxidising pathways.

It should be emphasised that the described regulation of UCPs by the endogenous Q redox state has so far 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 sustained by succinate oxidation [9,10,12,26-29], it is difficult to unequivocally estimate if sensitivity to PN depends on the endogenous Q redox state during titrations of the Q-reducing pathway (with malonate) or the QH₂-oxidising pathway (with cyanide or myxothiazol). However, this cannot be excluded since determinations of Q reduction level have not been performed in these experiments. Therefore, further studies are needed to explain if regulation of the sensitivity of UCP1 and its homologues to PNs through the Q redox state can also be observed under conditions when uncoupling is activated by superoxide.

The current model for the activation of UCPs by superoxide through initiation of lipid peroxidation [12,13] assumes that superoxide generated within mitochondria and a high membranous Q reduction level (as required for superoxide formation) work indirectly as UCP activators by generating carbon-centred radicals of polyunsaturated fatty acid chains of phospholipids in the mitochondrial inner membrane. However, in our opinion, these indirect effects could be a late response of UCPs as flux-force studies with isolated mitochondria do not reveal any effect of endogenously generated superoxide or the endogenous Q redox state on basal or FFA-induced activity of UCP1 or its homologues in the absence of PNs. To observe indirect activation of UCPs by superoxide (or a high Q redox state), lipid peroxidation products

or an exogenous system that generates superoxide (xanthine plus xanthine oxidase) must be applied. Our results obtained with UCP1-containing yeast and BAT mitochondria as well as those previously described for UCP1 homologues [17-20] indicate that the quick response through the endogenous Q redox state could directly regulate UCP activity. Thus, in our model for activation of UCPs, a high endogenous Q reduction level activates UCP by relieving inhibition from PNs, and this quick response does not involve superoxide formation resulting in lipid peroxidation products.

Taking into account the concentration of nucleotides in cells (in millimolar concentrations), UCPs should be permanently inhibited *in vivo*, even in the presence of FFA, unless a regulatory factor could overcome the inhibition by PN [2,17]. In the case of UCP1, it has been proposed that FFA do this in a kinetically simple competitive manner [30]. Our present results suggest that at a given FFA concentration, alleviation of PN-inhibition is dependent on the endogenous Q redox state. 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 [16]. However, there is no published data (to our knowledge) that conflicts with the observations described in this study; that is, that a highly reduced membranous Q leads to relief of PN inhibition of UCP1 activity. The present study suggests that in BAT mitochondria, the endogenous Q redox state is an additional metabolic sensor (beside FFA content) that modulates PN inhibition of UCP1 activity. Certainly, further studies are necessary to elucidate the kinetic mechanism of such regulation. However, a model describing function of reduced ubiquinone (QH₂) in modulating PN inhibition of UCP1 can be proposed (Figure 8). At a given FFA concentration, an increased amount of QH₂ could lead to decreased binding affinity of PN thereby alleviating inhibition of UCP1. QH₂ may play a role of a negative regulator of PN binding to UCP1. Conversely, at a lower QH₂ amount, PN may bound to UCP1 and proton conductance through UCP1 is inhibited.

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Figure legends:

Figure 1 Kinetics of proton leak in non-phosphorylating yeast mitochondria: effect of GTP when the Q-reducing pathway is decreased. Mitochondria isolated from yeast expressing UCP1 (A) and yeast containing an empty vector (B). Succinate oxidation was gradually decreased by increasing the concentration of malonate in the absence or presence of 40 μ M OA and in the absence or presence of 2 mM GTP. The Q redox state is indicated for initial and final points for each set of conditions. Points represents means \pm S.D. for three to six independent experiments. V4, State 4 respiration rate.

Figure 2 The effect of GTP when the QH₂-oxidising pathway is decreased in non-phosphorylating yeast mitochondria. The relationships between the respiration rate (V4) and $\Delta\Psi$ (proton leak kinetics) and the Q redox state and $\Delta\Psi$ in yeast expressing UCP1 (A, B) and yeast containing an empty vector (C, D) are presented. Titration with cyanide was performed in the absence or presence of 40 μ M OA and in the absence or presence of 2 mM GTP. Points represents means \pm S.D. for three to seven independent experiments. (A) **, respiration rates at the lowest common $\Delta\Psi$ (168 mV, vertical line) in the presence of GTP and OA are significantly different ($p < 0.01$, Student's paired *t* test) from control (no GTP, no OA).

Figure 3 The effect of GTP on the change in respiration, membrane potential, and Q redox state caused by OA-induced proton leak (UCP1 activity) when the Q reduction level is varied in UCP1-containing yeast mitochondria. (A), additions: 7.5 mM succinate (Succ), 40 μ M OA, 5 mM malonate (Mal) or 16 μ M cyanide (KCN), 2 mM GTP. The Q redox state values corresponding to given conditions are shown. After cyanide addition: solid lines and bold numbers; after malonate addition: dashed lines and numbers in italics. An example of measurements performed using mitochondria from four different preparations are shown. (B), HPLC recordings (at 290 nm) of reduced (Q_r) and oxidised (Q_{ox}) forms of membranous Q₆. Horizontal lines indicate a height of peaks obtained from samples extracted from mitochondria respiring in the presence of OA, before inhibitor additions. Calculated Q redox state values are given above (part A).

Figure 4 The effect of GTP on coupling parameters of UCP1-containing yeast mitochondria. Mitochondria were incubated in the absence (solid line) or presence of 40 μ M OA (dashed or dotted lines, plus or minus 2 mM GTP, respectively). Examples of oxygen uptake and $\Delta\Psi$ measurements are shown. Measurements of $\Delta\Psi$ allowed a fine control of the duration of State 3 respiration (vertical lines). After the

ADP pulse, respiration was uncoupled and $\Delta\Psi$ was collapsed by addition of 1 μM carbonylcyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP).

Figure 5 The effect of GTP in phosphorylating yeast mitochondria when the QH₂-oxidising or Q-reducing pathways are decreased. The relationships between ADP/O ratio (**A, D**), rate of ADP phosphorylation (**J_p**) (**B, E**) as well as Q reduction level (**C, F**) and the rate of oxygen consumption during State 3 (V3) of yeast expressing UCP1 and yeast containing an empty vector, respectively. To decrease the rate of the QH₂-oxidising pathway or the Q-reducing pathway, titration with antimycin A or *n*-butylmalonate was performed (respectively). Data were generated from nine mitochondrial preparations.

Figure 6 The effect of GTP when the QH₂-oxidising or Q-reducing pathways are decreased in non-phosphorylating rat BAT mitochondria. The relationships between the respiration rate (V4) and membrane potential (proton leak kinetics) (**A**) and Q redox state and $\Delta\Psi$ (**B**) are presented. Titration of the QH₂-oxidising pathway with cyanide and titration of the Q-reducing pathway with malonate were made in the absence or presence of 43 μM OA, and in the absence or presence of 2 mM GTP. Points represent means \pm S.D. for three to seven independent experiments. (**A**) *, respiration rates at the lowest common $\Delta\Psi$ (178 mV, vertical line) during titration with cyanide in the presence of GTP (with or without OA) are significantly different ($p < 0.05$, Student's paired *t* test) from those obtained during titration with malonate. (**B**), points of an initial State 4 (st. 4) in the absence or presence of OA or GTP before titration with malonate or cyanide are indicated.

Figure 7 The inhibition of endogenous or OA-induced UCP1 activity when the Q reduction level is varied in non-phosphorylating rat BAT mitochondria. Effect of GTP on respiration, membrane potential, and the Q redox state. Additions: 7.5 mM succinate (Succ), 43 μM OA, 2 mM GTP, 1 or 0.35 mM malonate (Mal) (**A, C**, respectively), and 50 or 32 μM cyanide (KCN) (**A, C**, respectively). The Q redox state values corresponding to given conditions are shown. After cyanide addition: solid, bold lines and bold numbers; after malonate addition: dashed lines and numbers in italics. An example of measurements performed using mitochondria from four different preparations are shown. (**B, D**), HPLC recordings (at 290 nm) of reduced (Q_r) and oxidised (Q_{ox}) forms of membranous Q₉. Horizontal lines indicate a height of peaks obtained from samples extracted from mitochondria before inhibitor additions. Calculated Q redox state values are given on left (part A and C).

Figure 8 A tentative model for the regulation of UCP1 by the membranous Q redox state. At a given fatty acid concentration, (**A**) an increased amount of QH₂ could lead to decrease in the binding affinity of PN (GTP is shown) thereby alleviating inhibition of UCP1 activity by the nucleotide. UCP1-mediated H⁺ reuptake is enhanced. (**B**) Conversely, at a lower QH₂ amount, no negative regulation of PN-binding site occurs and proton conductance through UCP1 is inhibited by PN.

Fig. 1

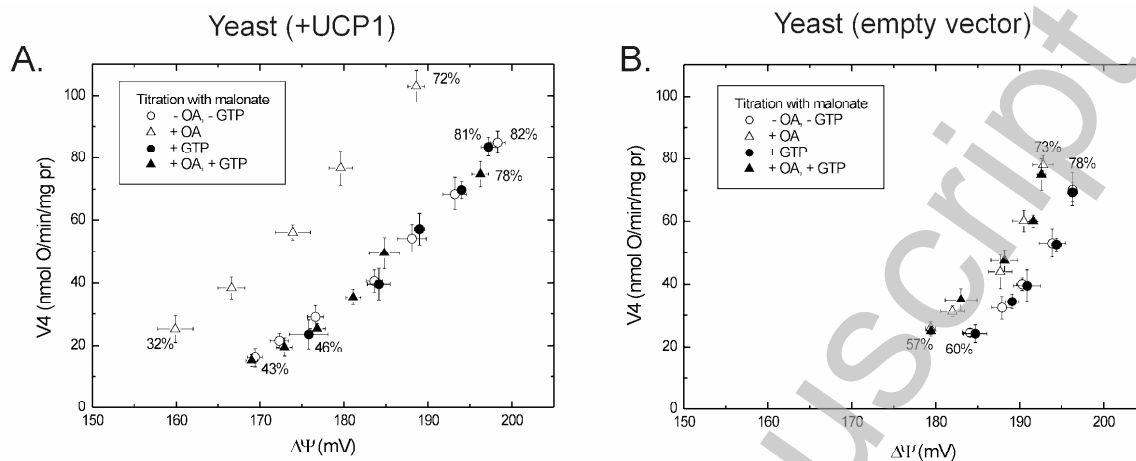
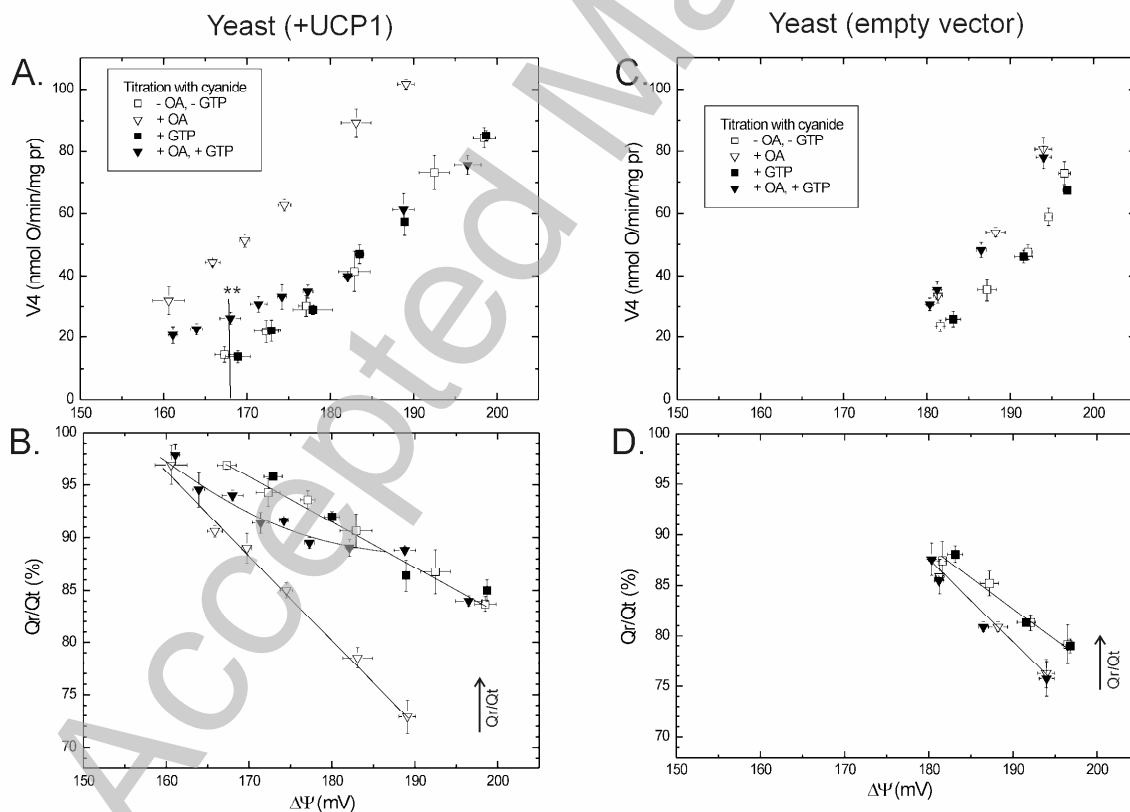
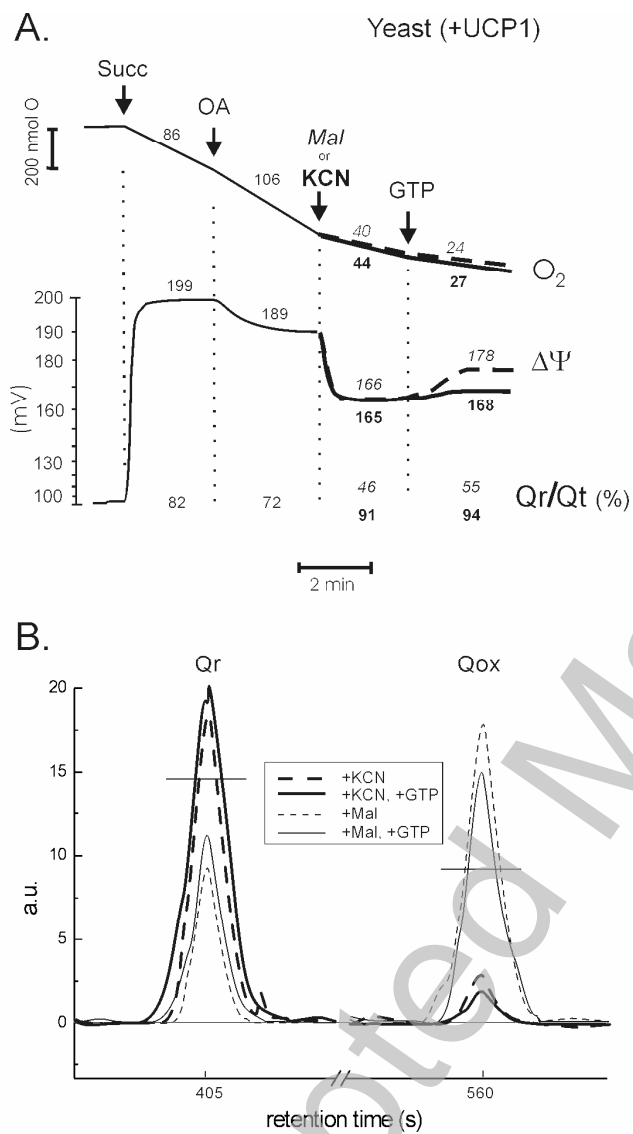


Fig. 2



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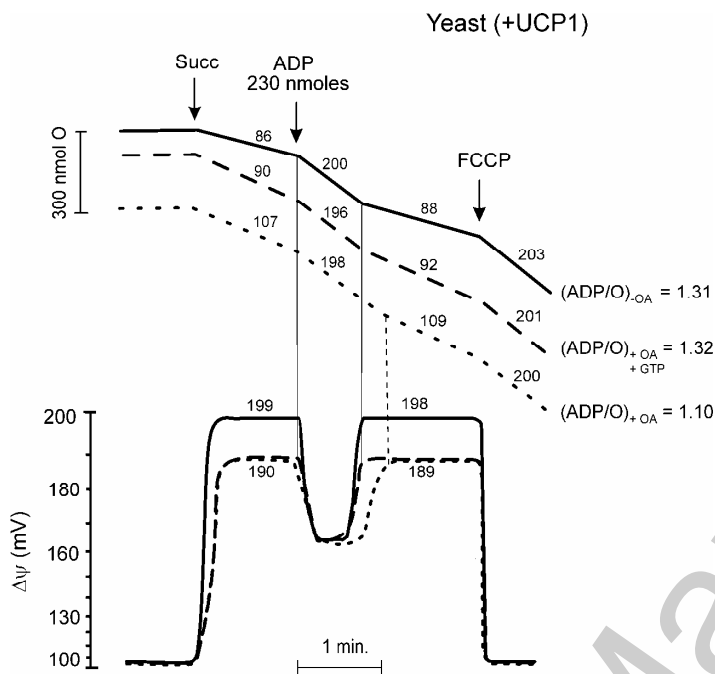
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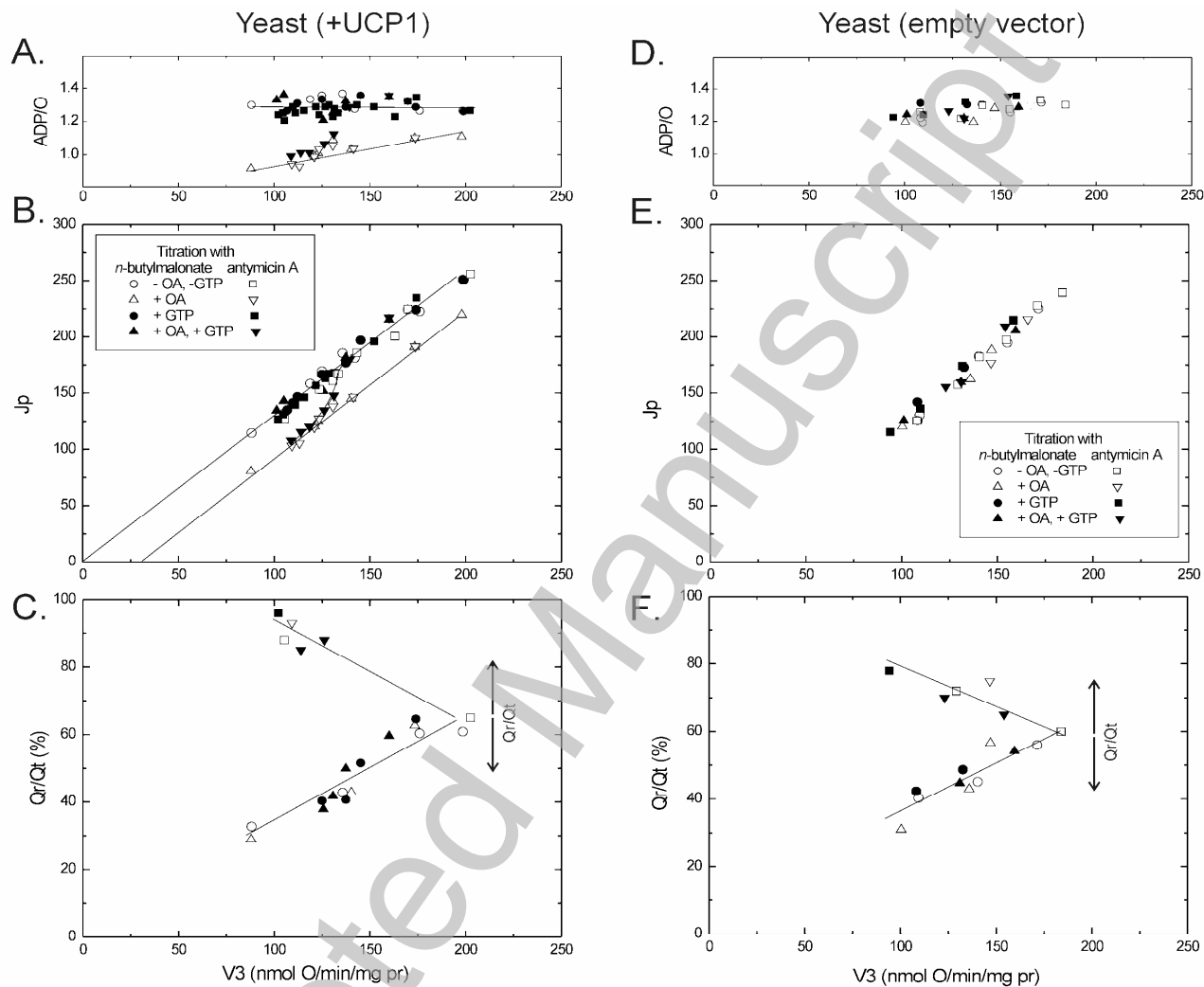
Fig. 4



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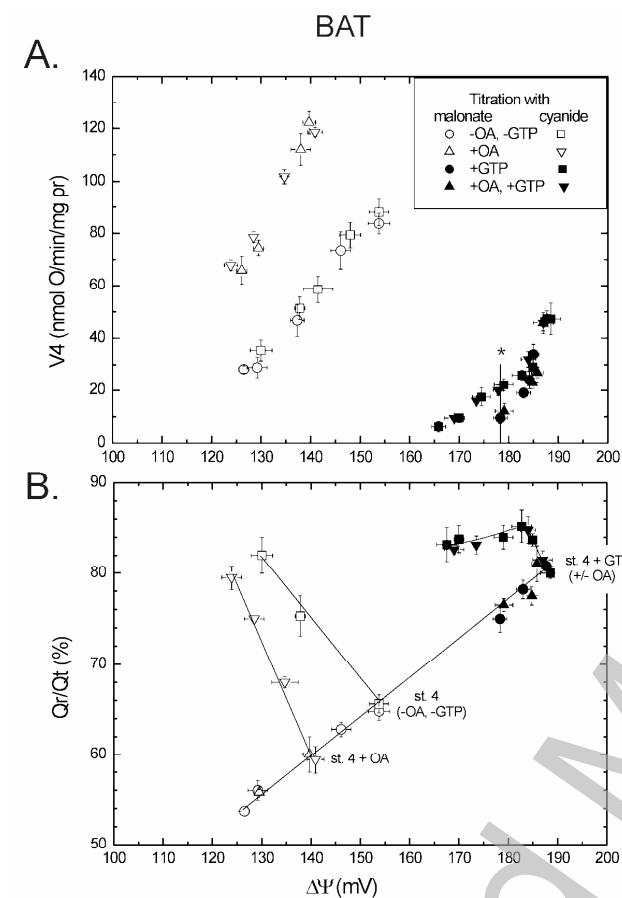
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Fig. 5



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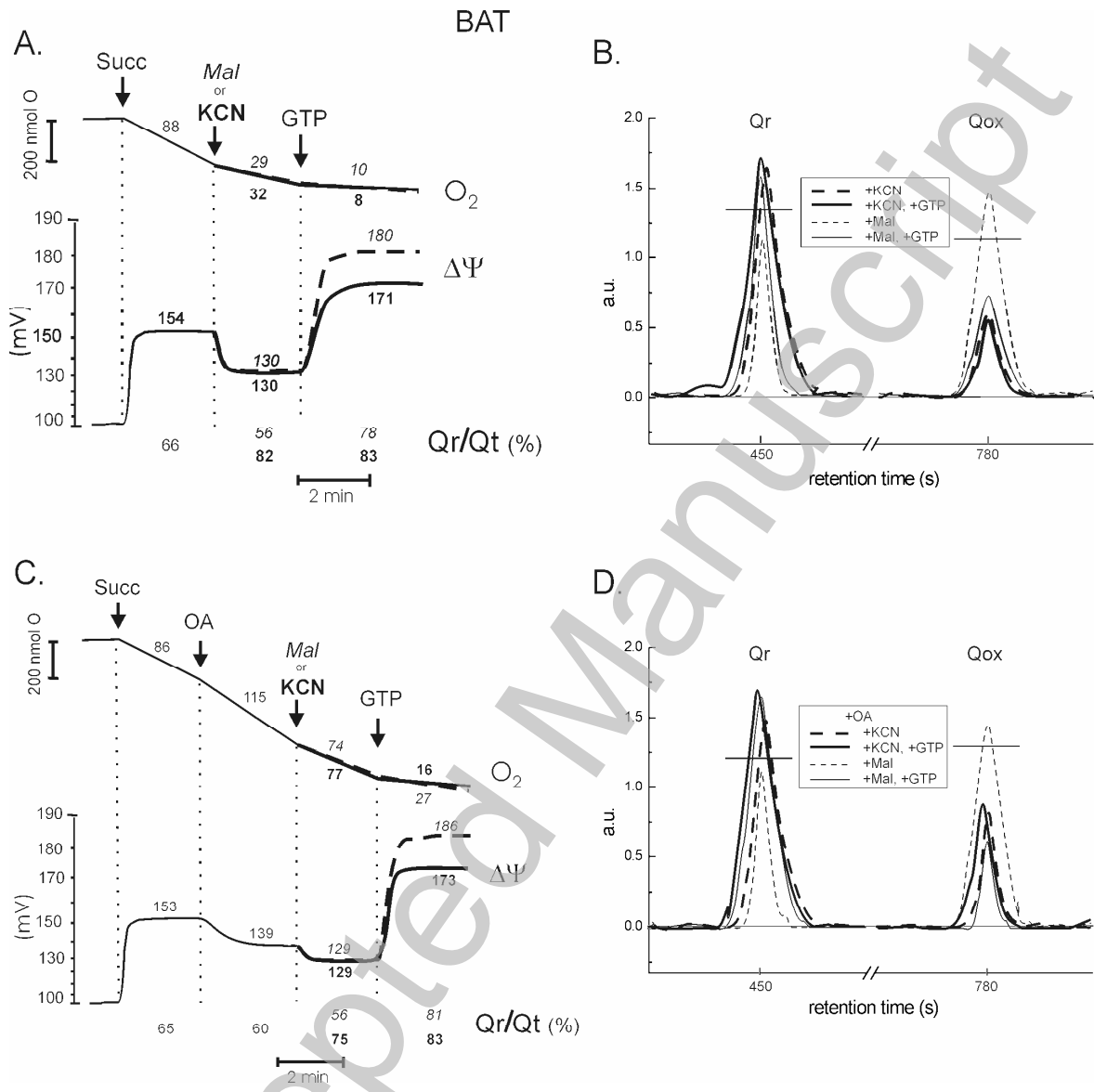
Fig. 6



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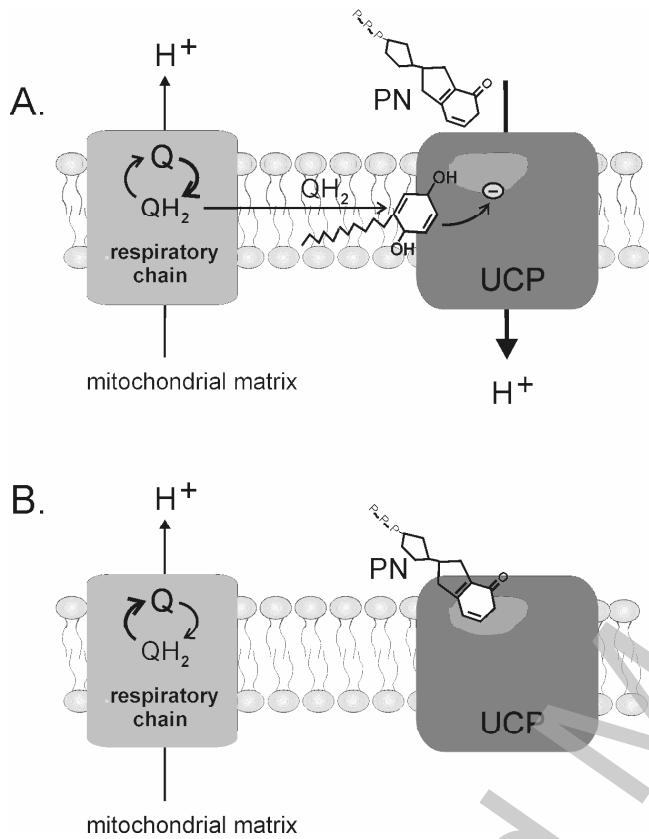
Fig. 7



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Fig. 8



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