Ubiquinone is not required for proton conductance by uncoupling protein 1 in yeast mitochondria

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Q (coenzyme Q or ubiquinone) is reported to be a cofactor obligatory for proton transport by UCPs (uncoupling proteins) in liposomes [Echtay, Winkler and Klingenberg (2000) Nature (London) **408**, 609–613] and for increasing the binding of the activator retinoic acid to UCP1 [Tomás, Ledesma and Rial (2002) FEBS Lett. **526**, 63–65]. In the present study, yeast (*Saccharomyces cerevisiae*) mutant strains lacking Q and expressing UCP1 were used to determine whether Q was required for UCP function in mitochondria. Wild-type yeast strain and two mutant strains (CENΔCOQ3 and CENΔCOQ2), both not capable of synthesizing Q, were transformed with the mouse *UCP1* gene. UCP1 activity was measured as fatty acid-dependent, GDP-sensitive proton conductance in mitochondria isolated from the cells. The activity of UCP1 was similar in both Q-containing and -deficient yeast mitochondria. We conclude that Q is neither an obligatory cofactor nor an activator of proton transport by UCP1 when it is expressed in yeast mitochondria.

Key words: mitochondria, proton conductance, retinoic acid, ubiquinone (coenzyme Q), UCP1 (uncoupling protein 1), yeast.

INTRODUCTION

UCP1 (uncoupling protein 1), the paradigm of the UCP subfamily of mitochondrial carriers, short-circuits the proton-motive force generated by the respiratory chain in brown adipose tissue mitochondria by conducting protons across the inner membrane, partially uncoupling ATP production from respiration and leading to heat production [1,2]. An extensive literature has developed concerning the function of UCP1, and various aspects of its regulation have been given close attention. For example, activation of uncoupling by fatty acids has been widely studied, both in mitochondria and using reconstituted UCP1 (see [3] for a review). The mechanism by which fatty acids activate UCP1 is still a matter of debate, and it is still unclear whether fatty acids are the substrate transported by the protein or direct activators or cofactors for proton transport [2,3]. Studies of the binding of purine nucleoside di- and triphosphates (such as GDP) and of their regulation of UCP1 activity have also provided insights into the function of UCP1 [4] and the closely related proteins UCP2 and UCP3 [5–7].

A recent and exciting topic in the regulation of UCPs is the activation by Q (coenzyme Q or ubiquinone, any subscript indicates the number of isoprene units). Echtay et al. [8] identified Q as a regulatory cofactor for proton transport by reconstituted UCP1 in liposomes. The inability to reconstitute fatty acid-dependent proton-transport activity from UCP1 expressed as inclusion bodies in *Escherichia coli* led the authors to consider the existence of a native cofactor for UCP1 function. After testing different fractions extracted from brown adipose tissue mitochondria, UCP1-expressing yeast and UCP1-free bovine heart, they observed that a fraction containing oxidized Q gave a strong stimulation of proton transport. Authentic Q also activated proton conductance. Stimulation required fatty acids and could be inhibited by low concentrations of ATP. These observations led to the conclusion that Q was an obligatory cofactor for proton transport through UCP1 [8]. In the same way, the homologues UCP2 and UCP3 were also demonstrated to be highly active proton transporters that required Q and fatty acids for their function in liposomes [6].

More evidence of an interaction of Q with UCP1 was given independently by Tomás et al. [9], who demonstrated that Q greatly increased the affinity of UCP1 for retinoic acid, a known activator [10]. Using photo-affinity labelling, they showed that retinoic acid bound covalently to UCP1 and binding was partially inhibited by nucleotides and significantly enhanced by Q [9].

Even though Q is a cofactor required for proton transport by UCPs after reconstitution from inclusion bodies, there is no direct evidence that it has the same effect in intact mitochondria, despite the potential importance of such a function in the regulation of mitochondrial efficiency and respiration rate.

Techniques of molecular biology have allowed the relationship between the structure and function of UCP1 in mitochondria to be probed in yeast [11]. The large number of specific mutations in *Saccharomyces cerevisiae*, organized into genetic complementation groups [12], has allowed the study of several cellular mechanisms *in vitro* using yeast cells. A specific defect accounting for Q deficiency has been identified in five out of eight genetic complementation groups with enzymic defects in the Q biosynthetic pathway [13]. In particular, *coq3* mutants such as CENΔCOQ3 [14] lack 3,4-dihydroxy-5-hexaprenylbenzoate methyltransferase activity and accumulate HHB (3-hexaprenyl-4-hydroxybenzoic acid) as the predominant intermediate [15–17]. Another mutant that is also deficient in Q is *coq2* (CENΔCOQ2 in the present study [14]), which harbours a defect in 4-hydroxybenzoate:polyprenyltransferase, the enzyme that catalyses the transfer of the polyisoprenyl chain to 4-hydroxybenzoic acid [18].

To investigate whether Q is an obligatory cofactor for the proton-transport function of UCP1 in mitochondria, we induced the expression of the protein in wild-type yeast and in yeast lacking Q and then assayed proton-transport activity in isolated mitochondria. In the present study, we show that GDP-sensitive proton transport can be induced by fatty acids in mitochondria

Abbreviations used: FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; HHB, 3-hexaprenyl-4-hydroxybenzoic acid; HNE, 4-hydroxy-2-nonenal; Q, coenzyme Q or ubiquinone (any subscript indicates the number of isoprene units); SL, selective lactate; $TPMP^+$, triphenylmethylphosphonium ion; UCP, uncoupling protein.

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Table 1 Relevant genotypes and phenotypes of *S. cerevisiae* strains

Strain	Genotype	Relevant phenotype	Reference
CEN.PK2-1C	MATa *his3-Δ1, leu2-3,112, trp1-289, ura3-52 MAL2-8^c, MAL3, SUC3*	Wild-type yeast Contains Q	[19]
CENΔCOQ3	CEN.PK2-1C, *coq3::LEU2*	Lacks Coq3 (*O*-methyltransferase) activity; accumulates HHB; lacks Q	[14]
CENΔCOQ2	CEN.PK2-1C, *coq2::HIS3*	Lacks Coq2 (*p*-hydroxybenzoate:polyprenyltransferase) activity; cannot form HHB or other polyprenylhydroxybenzoates; lacks Q	[14]

from yeast that express UCP1 but lack Q. There is no measurable difference in UCP1 catalytic activity between wild-type and Q-deficient strains, showing that Q is not a required cofactor for UCP1 in yeast mitochondria.

EXPERIMENTAL

Expression of UCP1 in *S. cerevisiae*

Characteristics of the yeast strains used in the present study are shown in Table 1. Cells of the *S. cerevisiae* wild-type strain CEN.PK2-1C [19] and the derived Q-deficient strains, CENΔCOQ3 and CENΔCOQ2 [14], were transformed with either a mouse UCP1 expression construct or a plasmid containing an empty vector, using a *S.cerevisiae* EasyComp transformation kit (Invitrogen, Paisley, Scotland, U.K.). The UCP1 expression construct used in the present study (pBF307, moderate UCP1 expression) and the empty vector plasmid (pBF254) were constructed previously [20,21].

Pre-cultures of the transformed wild-type CEN.PK2-1C yeast cells were grown at 30 °C for 18–24 h in SL (selective lactate) medium {2 % (w/v) DL-lactic acid [85 % (w/w) syrup]/0.67 % (w/v) yeast nitrogen base without amino acids (Difco, Detroit, MI, U.S.A.)/0.2 % casamino acids (Difco)/0.12 % $(NH_4)_2SO_4$/0.1 % KH_2PO_4/0.1 % glucose/0.002 % L-tryptophan/0.004 % adenine}. Pre-cultures of transformed Q-deficient CENΔCOQ3 cells were grown similarly for 24–48 h in modified SL medium, supplemented with a leucine and uracil dropout amino acid cocktail instead of casamino acids. The cocktail was a 25-fold concentrated stock, consisting of (in g/l) 0.5 adenine, 0.5 histidine, 0.75 tyrosine, 0.5 arginine, 0.5 methionine, 0.75 isoleucine, 0.125 phenylalanine, 0.5 proline, 0.375 valine, 0.5 threonine, 0.875 serine, 0.25 glutamate, 0.25 aspartate, 0.5 glycine, 0.5 asparagine, 0.5 alanine, 0.75 lysine and 0.5 cysteine. Transformed Q-deficient CENΔCOQ2 cells in pre-culture were grown in SL for up to 60 h with histidine and uracil dropout amino acid cocktail, as described for CENΔCOQ3 (with 0.5 g/l leucine instead of histidine).

Pre-cultures of yeast transformed with UCP-containing (pBF307) and empty vector (pBF254) plasmids were grown to high density under the conditions described above (attenuance D_{600} 1–2) and then transferred to a culture medium [22 % DL-lactic acid (85 % syrup)/1 % yeast nitrogen base without amino acids/0.1 % glucose (AnalaR; BDH, Poole, Dorset, U.K.)/0.05 % $CaCl_2$ (Fischer Chemicals, Zürich, Switzerland)/0.05 % NaCl/0.06 % $MgCl_2$/0.1 % KH_2PO_4 (AR, Fisons Chemicals, Loughborough, Leics., U.K.)/0.1 % NH_4Cl (AnalaR)/0.8 % NaOH (AnalaR)/0.1 % casamino acids/0.04 % adenine/0.02 % L-tryptophan (pH 5.5)], for growth under adequate conditions for the induction of UCP1 expression. Since the mutant yeast had longer doubling times compared with wild-type yeast, 1 % (w/v) D-galactose (Fischer Chemicals) was added from the beginning of the cultures for wild-type CEN.PK2-1C yeast, but was added only later to the *coq* mutant cultures, so that UCP1 expression was induced by galactose for a similar time length. Wild-type CEN.PK2-1C yeast was grown with galactose for approx. 15 h, whereas Q-deficient CENΔCOQ3 and CENΔCOQ2 yeast strains were grown without galactose for 9 and 14 h respectively, followed by growth for approx. 15 h (for CENΔCOQ3) and 19 h (for CENΔCOQ2) with galactose. All cultures were incubated at 30 °C in orbital shakers at 230–250 rev./min.

Isolation of yeast mitochondria

Yeast mitochondria were isolated by the method of Stuart et al. [21] with modifications. Yeast cells from cultures with D_{600} 0.8–1.1 were harvested by centrifugation at 2900 ***g*** for 16 min at 20–23 °C, resuspended in deionized water and re-centrifuged. Pellets were resuspended in a buffer containing 100 mM Tris/HCl and 20 mM dithiothreitol (pH 9.3; Melford Laboratories, Chelsworth, Ipswich, Suffolk, U.K.), and incubated at 30 °C in an orbital shaker (220 rev./min) for 10 min. Pellets were then re-centrifuged and washed twice in a buffer containing 100 mM Tris/HCl and 500 mM KCl (pH 7.0; AnalaR). The cells were resuspended in 1 ml of iso-osmotic spheroplasting buffer [40 mM citric acid/120 mM Na_2HPO_4 (AnalaR)/1.35 M sorbitol (NBS Biologicals, Huntingdon, Cambs., U.K.)/1 mM EGTA, pH 5.8] per 1 g of cells; lyticase was added (at 3 mg/g wet weight of cells), and the suspension was incubated for 10–15 min at 30 °C in an orbital shaker (220 rev./min). All subsequent steps were performed at 4 °C. Spheroplasts were pelleted, washed twice in approx. 30 ml of a buffer containing 10 mM Tris maleate, 0.75 M sorbitol, 0.4 M mannitol, 2 mM EGTA and 0.1 % (w/v) BSA (pH 6.8) and then resuspended in 30 ml of isolation buffer [10 mM Tris maleate/0.6 M mannitol/2 mM EGTA/1 mM EDTA (Fluka, Gillingham, Dorset, U.K.)/0.5 mM Na_2HPO_4/1 % BSA, pH 6.8] with Complete protease inhibitor (Boehringer Mannheim, Mannheim, Germany; 1 tablet/40 ml of the buffer). After homogenization by 12 passes with a Wesley Coe homogenizer (clearance, 0.54 mm), homogenates were centrifuged at 800 ***g*** for 10 min and the supernatants were recovered using a pipette. The pellets were resuspended in a small volume of isolation buffer and re-centrifuged at 800 ***g*** for 10 min to collect mitochondria still retained in the pellet. The supernatants were combined and centrifuged 2–3 times at 800 ***g*** for 10 min, and the pellets were discarded. The resultant supernatants were centrifuged at 11 000 ***g*** for 10 min. The mitochondrial pellets were washed with a buffer containing 10 mM Tris maleate and 0.65 M sorbitol (pH 6.8) and centrifuged at 11 000 ***g***. The final pellet was resuspended in a small volume (150–300 μl) of the same buffer and the protein content was assayed using the Bio-Rad DC modified Lowry protein assay (Bio-Rad Laboratories, Hemel Hempstead, Herts., U.K.), with BSA as a protein standard.

Respiration assays

After isolation, mitochondria were suspended at 0.2 mg/ml protein in 2.5 ml of electrode buffer [20 mM Tris/HCl/450 mM

sorbitol/100 mM KCl/0.5 mM EGTA/5 mM $MgCl_2$/10 mM K_2HPO_4/0.1 % (w/v) defatted BSA, pH 6.8] and respiration was measured at 30 °C using a Clark-type oxygen electrode. The electrode was calibrated with air-saturated electrode buffer, assumed to contain 424.8 nmol O/ml at 30 °C [22]. We added 5 μM FCCP (carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; in ethanol, which can be oxidized by yeast mitochondria) to uncouple mitochondria at the start of measurements, and measured respiration using 0.6 mM NADH as additional substrate. Q_2 (1–2.9 μM) was added after the addition of substrate (to restore electron-transport function in Q-deficient mitochondria from CENΔCOQ2 and CENΔCOQ3 yeast strains). We used 3 μM myxothiazol (inhibitor of Complex III) to inhibit the oxygen consumption due to respiratory chain function.

Isolation and quantification of quinones

Before lipid extraction, the samples were assayed for protein concentration by the bicinchoninic acid assay (Pierce, Rockford, IL, U.S.A.). Q_{10}, the internal standard, was added to the mitochondrial samples at a concentration of 6 pmol/μl of final resuspension volume. We added 9 ml of methanol and 6 ml of light petroleum (boiling range, 37–56 °C) to each sample, and the extraction proceeded with shaking overnight in the dark at 4 °C. The samples were centrifuged at 910 ***g*** for 10 min at 4 °C. The top layers of light petroleum were removed and a second extraction was performed with 4 ml of light petroleum for an additional 1 h. The light petroleum extracts were combined and dried under nitrogen. The dried lipids were finally resuspended in 120 μl of methanol/ethanol (9:1). Quantification by HPLC linked with an electrochemical detector was performed as described previously [23]. The Q_6 detection limit is 0.22 pmol/injection, which is equivalent to 2 pmol/mg of mitochondrial protein.

Proton conductance

The kinetic response of the proton conductance pathway to its driving force can be expressed as the relationship between the oxygen consumption rate and the membrane potential when varying the potential by titration with electron-transport chain substrates. Respiration rate and membrane potential were measured simultaneously at 30 °C using electrodes that are sensitive to oxygen (Clark-type) and the potential-dependent probe $TPMP^+$ (triphenylmethylphosphonium cation). The $TPMP^+$-binding correction (method 6 in [24]) for haploid yeast mitochondria is 0.4, and the mitochondrial matrix volume is 1.8 μl/mg (E. J. Cornwall, J. L. Pakay and M. D. Brand, unpublished work).

Mitochondria (0.6 mg/ml mitochondrial protein) were incubated in electrode buffer after the addition of 3 μM myxothiazol (an inhibitor of Complex III), 10 μg/ml oligomycin (to prevent ATP synthesis), 1 μg/ml nigericin (to collapse the difference in pH value across the mitochondrial inner membrane) and 20 μM palmitic acid (hexadecanoic acid) in the electrode chamber. The chamber was closed, avoiding air bubbles, and the $TPMP^+$ electrode was calibrated with sequential additions of 1 μM $TPMP^+$ up to a final concentration of 4 μM. Respiration and membrane potential were progressively stimulated through successive steady states by the addition of D-lactate (an electron donor to cytochrome *c* in yeast mitochondria) in steps to a final concentration of 2.2 mM. FCCP (5 μM) was added at the end of each run to dissipate the membrane potential and release all $TPMP^+$ to the medium, allowing correction for any small electrode drift. A second addition of 5 μM FCCP allowed the estimation of the small correction for direct effects of the uncoupler on the $TPMP^+$ electrode signal.

Quantitative immunodetection of UCP1

Western-blot analysis was used to assess the relative levels of UCP1 expression in mitochondria from each yeast strain containing UCP1 as described previously [21]. Fresh brown adipose tissue mitochondria from warm-adapted rats, prepared by the method of Lin and Klingenberg [4] and reported in the literature to have an average of 13 μg of UCP1/mg of protein [21], were used for internal calibration. All samples were prepared with four parts of mitochondrial suspension and one part of 5 × loading buffer [250 mM Tris/HCl, pH 6.8/10 % (w/v) SDS (AnalaR)/0.5 % (w/v) Bromophenol Blue/50 % (v/v) glycerol (AnalaR)/10 % (v/v) 2-mercaptoethanol added just before use]. Samples were loaded on to SDS/12 % (w/v) polyacrylamide gel at 45 μg of protein loaded in 12 μl, except for brown adipose tissue mitochondria, for which we loaded 1–5 μg of protein in 10 μl. The gel was run at 135 V for 60 min in Tris/glycine running buffer [2.88 % (w/v) glycine (AnalaR)/0.6 % Tris/0.1 % SDS] and then placed in transfer buffer [2.9 % glycine/0.6 % Tris/20 % (v/v) methanol (AnalaR)/0.037 % SDS] for 20 min. Protein was transferred on to a nitrocellulose transfer membrane (pre-wet with deionized water and soaked in transfer buffer for 5 min) using a Bio-Rad Trans-Blot SD semi-dry electrophoretic transfer cell at 10 V for 20 min. The membrane was washed twice in 1 × PBS and blocked for 60 min in blocking buffer [PBS containing 0.1 % (v/v) Tween 20 (NBS Biologicals) and 5 % (w/v) Marvel dried skimmed milk powder] at 20–23 °C. The membrane was exposed overnight at 4 °C to a rabbit anti-mouse UCP1 primary antibody (1:4000 dilution in blocking buffer; Chemicon AB 3038; Chemicon Europe, Chandlers Ford, Hampshire, U.K.), washed twice in blocking buffer for 10 min and incubated for 45 min at 20–23 °C with an alkaline phosphatase-conjugated secondary antibody [Cell Signaling Technology (Beverly, MA, U.S.A.) anti-rabbit IgG, H&L] diluted 1:1000 in blocking buffer. Two washes with blocking buffer and two washes with a buffer containing 100 mM Tris/HCl, 100 mM NaCl and 10 mM $MgCl_2$ (pH 9.5) (each wash for 10 min) were performed, followed by the development of the membrane with CDP-Star chemiluminescent substrate solution. Membrane was exposed for approx. 15 min to a Fuji Medical X-ray film. Western-blot films were scanned with a ScanMaker X12 USL and band intensity was quantified using NIH Image, version 1.63 (http://rsb.info.nih.gov/nih-image/). UCP1 concentration in the yeast samples was linearly related to UCP1 content in brown adipose tissue mitochondria (internal standard).

Calculation of UCP1 activities

The rate of proton pumping (and therefore of proton leak at steady state) was calculated by multiplying the oxygen consumption rate by 2 (the H^+/O ratio using D-lactate as a substrate). To calculate proton conductance, proton leak rate at 92 mV (the highest common membrane potential in the present study) was divided by membrane potential, assuming ohmic kinetics of proton leak rather than the observed non-ohmic response. Strictly speaking, conductance is the tangent at a certain membrane potential to the leak curves in Figure 2, but we retain the ohmic assumption to allow comparison with published results in Table 2. Proton conductance through UCP1 was calculated in this way at 92 mV as nmol of $H^+ \cdot min^{-1} \cdot mV^{-1} \cdot (mg\ of\ protein)^{-1}$ in the presence of GDP subtracted from that in the absence of GDP. UCP1 catalytic activity [nmol of $H^+ \cdot min^{-1} \cdot mV^{-1} \cdot (\mu g\ of\ UCP1)^{-1}$] was calculated by dividing proton conductance of UCP1 at 92 mV by UCP1 concentration determined by Western-blot analysis.

Table 2 Concentration and activity of UCP1 in mitochondria from transformed yeast cells

Values for UCP1 content are the average of three determinations from Western blots (see the Experimental section). Values for proton conductance at 92 mV were taken from proton leak kinetics shown in Figure 2. GDP-sensitive proton conductance (indicating UCP1 activity) was calculated by subtracting the conductance in the presence of GDP from that in its absence for yeast mitochondria expressing mouse UCP1 from plasmid pBF307. Results are the means ± S.E.M. for three independent experiments.

Strain	UCP1 content (μg/mg of mitochondrial protein)	GDP-sensitive proton conductance*	UCP1 catalytic activity at 92 mV†
CEN.PK2-1C	2.0 ± 0.5	2.3 ± 0.6	1.2 ± 0.04
CENΔCOQ3	2.3 ± 0.7	4.2 ± 0.3	2.3 ± 0.7 ($P = 0.27$)‡
CENΔCOQ2	1.7 ± 0.1	2.7 ± 0.4	1.6 ± 0.3 ($P = 0.33$)‡
W303 (pBF307)§	0.9	1.5	1.7‖

* Assuming $H^+/O = 2$ and measured at 92 mV; units: nmol of $H^+ \cdot min^{-1} \cdot$ (mg of mitochondrial protein)$^{-1} \cdot mV^{-1}$.

† Units: nmol of $H^+ \cdot min^{-1} \cdot mV^{-1} \cdot (\mu g \text{ of UCP1})^{-1}$.

‡ Not significantly different from wild-type CEN.PK2-1C (Student's *t* test).

§ Values are from [21].

‖ Average value, for measurements at high-fatty-acid concentration and low membrane potential.

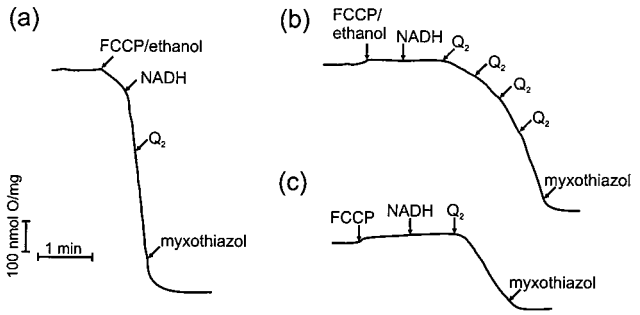

Figure 1 Respiration in mitochondria from Q-containing and -deficient yeast strains

Oxygen consumption due to respiration was measured (see the Experimental section) in mitochondria from (**a**) CEN.PK2-1C (Q-containing), (**b**) CENΔCOQ3 (Q-deficient) and (**c**) CENΔCOQ2 (Q-deficient) yeast strains expressing UCP1 from pBF307 [mitochondria from yeast containing pBF254 (empty vector) gave similar results]. Oxygen concentration was never limiting. As indicated, 5 μM FCCP (in ethanol; uncouples mitochondria), 0.6 μM NADH (respiratory substrate), 1 μM Q_2 (restores respiration in Q-deficient mitochondria) and 3 μM myxothiazol (inhibits Complex III) were added. In (**b**), Q_2 was added in successive steps up to 2.9 μM. Representative traces are shown; three different mitochondrial preparations gave similar qualitative and quantitative results.

Chemicals

All chemicals were obtained from Sigma (Poole, Dorset, U.K.) unless stated otherwise. NADH, D-lactate and GDP were dissolved in deionized water, whereas Q_2, palmitic acid, myxothiazol, nigericin, oligomycin and FCCP were dissolved in ethanol.

RESULTS

Respiration and Q levels in mitochondria from Q-containing and -deficient yeast

Unlike wild-type CEN.PK2-1C yeast, neither of the Q-deficient strains is capable of growing using ethanol as a non-fermentable carbon source (results not shown), as reported previously for other Q-deficient and respiratory mutant yeast strains [12].

A functional assay was used to confirm the absence of Q from mitochondria of the Q-deficient mutant yeast. Figure 1(a) shows that ethanol and NADH could be used as respiratory substrates by mitochondria isolated from wild-type CEN.PK2-1C (Q-containing) yeast. As expected for mitochondria with a fully competent electron-transport chain, respiration (uncoupled by FCCP) did not require addition of Q_2 and was fully inhibited by the Complex III inhibitor myxothiazol. However, mitochondria isolated from either *coq3* (Figure 1b) or *coq2* (Figure 1c) Q-deficient mutant cells did not oxidize ethanol or NADH as respiratory substrates, as a result of the absence of an electron acceptor at the level of the Q pool. Oxygen consumption was only restored when Q_2 was added to replace the missing endogenous Q_6 (Figures 1b and 1c). In both cases, myxothiazol fully inhibited oxygen consumption, showing that it was caused by electron transport through the respiratory chain (Complex III).

HPLC measurements of Q_6 content in lipid extracts from mitochondria isolated from the two mutants confirmed the Q-deficient status of mitochondria from both CENΔCOQ3 and CENΔCOQ2 yeast strains. The measured amounts of Q_6 for the *coq* mutants were lower than the estimated lower limit of detection of approx. 2 pmol/mg of mitochondrial protein, whereas levels in wild-type CEN.PK2-1C mitochondria were 2.6 nmol of Q_6/mg of protein for yeast containing empty vector and 4.4 nmol of Q_6/mg of protein for yeast expressing UCP1. These values were within the range of variation expected between individual samples, and were similar to values reported previously for this wild-type yeast strain [25].

Wild-type yeast transformed with pBF307 (moderate UCP1 expression)

As mitochondria from Q-deficient yeast did not respire using ethanol or NADH as substrate (Figures 1b and 1c), all proton conductance measurements were performed using D-lactate as a respiratory substrate, which feeds electrons to cytochrome *c* in yeast and so by-passes the respiratory defect in the mutants.

The kinetics of proton leak in mitochondria isolated from wild-type CEN.PK2-1C (Q-containing) yeast transformed with pBF307 (moderate UCP1 expression) or with empty vector is shown in Figure 2(a). As observed previously [21], proton leak in yeast mitochondria showed non-ohmic kinetics, with proton leak rate increasing steeply at higher membrane potentials. In the presence of non-esterified fatty acid (palmitic acid), mitochondria from yeast expressing moderate levels of UCP1 (filled squares) had greater proton conductance (higher proton leak rate at any potential) compared with those from empty vector controls under the same conditions (filled circles). Addition of GDP, the classic inhibitor of UCP1, returned the higher proton conductance to the basal level seen with empty vector in UCP1-containing yeast mitochondria (open squares), but had little effect in empty vector controls (open circles), showing that uncoupling was caused by UCP1 activity. Thus palmitate-activated, GDP-inhibited UCP1 activity is clearly measurable in wild-type yeast transformed with UCP1, but is absent from yeast transformed with empty vector, as reported previously [21,26,27].

Q-deficient yeast transformed with pBF307 (moderate UCP1 expression)

The UCP1-containing plasmid (pBF307) was also transformed into Q-deficient CENΔCOQ3 and CENΔCOQ2 yeast strains.

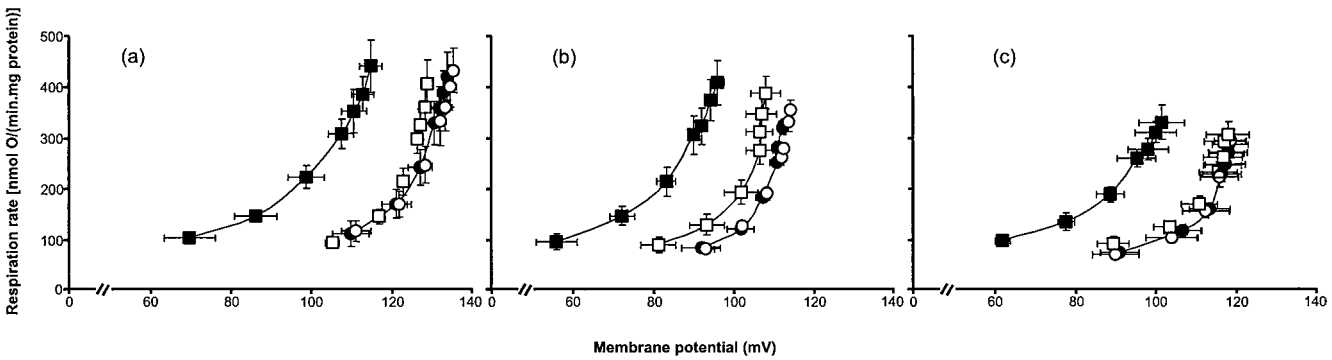

Figure 2 Kinetics of proton leak in mitochondria from CEN.PK2-1C (Q-containing) and Q-deficient yeast strains

Mitochondria were isolated from (**a**) wild-type CEN.PK2-1C, (**b**) CENΔCOQ3 and (**c**) CENΔCOQ2 yeast strains containing pBF307 (expressing mouse UCP1), as well as from paired controls (yeast with empty vector pBF254, grown under the same conditions). Oxygen consumption and mitochondrial membrane potential were measured in the presence of 0.1 % defatted BSA and 20 μM palmitic acid, and varied with D-lactate titration, as described in the Experimental section. ●, empty vector control; ○, empty vector control + 1 mM GDP; ■, mitochondria containing UCP1; □, mitochondria containing UCP1 + 1 mM GDP. Results are the means ± S.E.M. for three independent experiments.

Mitochondria were isolated from these cells and proton leak kinetics was measured to investigate whether Q was necessary for mammalian UCP1 function in yeast mitochondria. The results obtained (Figures 2b and 2c) were very similar to those obtained for the wild-type; mitochondria from Q-deficient yeast expressing UCP1 had higher proton conductance in the presence of palmitic acid (filled squares) when compared with basal proton leak levels (circles), and this effect was largely inhibited by GDP (open squares). Mitochondria from cells expressing empty vector had basal proton leak rates (filled circles), and did not show sensitivity to GDP (open circles). This result shows that UCP1 is responsible for the increase in proton conductance of mitochondria from Q-deficient yeast cells with pBF307, indicating that UCP1 can be activated even in these Q-deficient mitochondria and suggesting that UCP1 does not need Q for activity in yeast mitochondria.

The intermediate HHB accumulates in *coq3* mutants, which lack *O*-methyltransferase activity and cannot process the intermediate to Q [17], raising the possibility that HHB was capable of substituting for Q in this mutant, thereby masking the postulated Q dependence. However, this possibility is excluded since the results obtained with the CENΔCOQ2 strain (Figure 2c), which does not produce HHB, were essentially the same as those with CENΔCOQ3 (Figure 2b), which produces HHB.

UCP1 catalytic activity in wild-type and Q-deficient yeast mitochondria

The results presented above show that Q is not an obligatory cofactor for UCP1 function in yeast mitochondria. To investigate whether the presence of Q had any detectable effect on UCP1 activity in mitochondria, the catalytic activities of UCP1 were calculated in mitochondria from each yeast strain by measuring the levels of UCP1 and proton conductance at a common membrane potential (Table 2).

Levels of UCP1 expression in mitochondria from each yeast strain that contained UCP1 were determined by Western-blot analysis. As expected, UCP1 from rat brown adipose tissue mitochondria and UCP1 expressed in yeast both had molecular masses of approx. 32 kDa (results not shown), confirming previous results using similar methods [21]. The amounts of UCP1 in yeast mitochondria were calibrated with reference to mitochondria from rat brown adipose tissue used as standard.

UCP1 expression in wild-type CEN.PK2-1C yeast, which was transformed with UCP1 (pBF307), grown in lactate medium and induced with 1 % D-galactose for approx. 15 h, was approx. 2 μg of UCP1/mg of mitochondrial protein, comparable with published values [21] (Table 2). UCP1-containing (pBF307) cells from Q-deficient CENΔCOQ3 and CENΔCOQ2 were grown under the same conditions as the wild-type for approx. 15 and 19 h respectively. Mitochondria from these cells had 2.3 and 1.7 μg of UCP1/mg of mitochondrial protein (CENΔCOQ3 and CENΔCOQ2 respectively; Table 2). Thus UCP1 was expressed at similar concentrations in all three strains.

These results, together with the Q_6 measurements by HPLC, indicate that there is < 0.04 mol of Q_6/mol of UCP1 in CENΔCOQ2 and CENΔCOQ3 yeast mitochondria, whereas wild-type CEN.PK2–1C mitochondria with UCP1 contain approx. 70 mol of Q_6/mol of UCP1.

The UCP1 catalytic activity in mitochondria from each yeast strain was calculated as [proton conductance mediated by the protein at 92 mV, the highest common membrane potential]/[UCP1 content] (Table 2). There was no significant difference in UCP1 catalytic activity between mitochondria from any of the strains. Therefore, as well as being non-essential for mammalian UCP1 function in yeast mitochondria, Q is also not significantly activatory or inhibitory for UCP1 in this situation either.

DISCUSSION

The present study shows that the proton conductance catalysed by mouse UCP1 expressed in yeast mitochondria is not dependent on, or measurably influenced by, the presence or absence of Q, indicating that this molecule is neither an obligatory cofactor nor a regulator of the protein function *in situ*.

The identification of Q as a native UCP cofactor using reconstituted UCPs in liposomes [6,8] gave the first evidence that Q was involved in UCP activation. Subsequently, when the possible activation of UCPs by Q in intact mitochondria was examined, Q-induced, GDP-sensitive, fatty acid-dependent proton conductance was observed in rat kidney mitochondria. Since uncoupling activity could be prevented by superoxide dismutase, exogenous Q acts in kidney mitochondria through the production of superoxide, probably by activating UCP2 [28]. The ability of superoxide to activate UCPs was clearly demonstrated when it was shown

to increase mitochondrial proton conductance through effects on UCP1, UCP2 and UCP3 [29].

In mammalian mitochondria, GDP-sensitive superoxide activation of proton transport by UCPs can be mimicked by the addition of HNE (4-hydroxy-2-nonenal), a major product of oxidant-induced peroxidation of membrane phospholipids [30] and by 2,2′-azobis(2-methyl propionamidine) dihydrochloride, a generator of carbon-centred radicals [31]. Activation by superoxide and 2,2′-azobis(2-methyl propionamidine) dihydrochloride can be prevented by the mitochondrially targeted carbon-centred radical quencher, mitoPBN, but the activation by HNE cannot [31]. These observations support a pathway for UCP activation in which superoxide generates carbon-centred free radicals on phospholipid fatty acyl chains, and these radicals decay through lipid hydroperoxides to generate HNE and other reactive alkenals, which are the direct activators of UCPs [30–32]. Hence, HNE generated by superoxide or other oxidative stresses would induce mild uncoupling by UCPs, thus lowering the membrane potential and decreasing the production of reactive oxygen species, forming a feedback loop that protects mitochondria against overproduction of radicals [29].

Considering this model and the results of the present study, we suggest that the requirement of Q for proton transport by UCP1 in the liposome system [6,8] reflected not a direct activation of UCP1 as originally proposed, but a Q-mediated oxidative stress, leading to the production of some or all of superoxide carbon-centred radicals, lipid hydroperoxides and reactive alkenals. Therefore it is probably these products and not Q itself that induced UCP activity in the liposomes. Reports of Q-independent UCP1 activity in liposomes [5,33,34] might reflect higher background levels of oxidants in some reconstitution methods. In the yeast system studied in the present study, background oxidative processes may have produced sufficient amounts of these activators to allow full UCP1 activity when palmitate was added even in the absence of Q. A second hypothesis would be that in the reconstituted liposome system, Q is somehow required for proper folding of UCPs, thereby being essential to the protein function, whereas in the yeast system, cellular processes ensure correct folding even in the absence of Q.

After the discovery of the role of Q in the liposome system, Klingenberg [11] suggested that Q was associated with fatty acid activation of UCPs. According to two proposed models, the two species would interact by their head groups and Q would either facilitate proton transfer from the aqueous phase into the UCP or facilitate the transfer of the protonated fatty acid itself to the proton channel of UCP. The present study shows that activation of UCP1 in the presence of fatty acids is independent of Q, since the protein is similarly active in both Q-containing and -deficient yeast mitochondria. Therefore the models proposed for Q and fatty acid interaction, which were based on work done using the reconstituted system, do not apply to UCP1 *in situ*.

Tomás et al. [9] reported increased affinity of isolated UCP1 for retinoic acid in the presence of Q, and proposed a direct interaction between Q and this inducer of UCP1 activity. To examine this question, we investigated the activation of UCP1 by retinoic acid in mitochondria from Q-containing and -deficient yeast strains in the same way as described above (results not shown). We did not observe higher retinoic-acid-induced uncoupling via UCP1 in Q-containing mitochondria compared with Q-deficient mitochondria. Once again, the effect seen with the isolated protein might not be of major importance for the activation of UCP1 in mitochondria.

In conclusion, the present study shows that proton conductance mediated by mouse UCP1 is qualitatively and quantitatively the same in mitochondria isolated from transgenic Q-containing and -deficient yeast strains. Therefore Q is not an essential cofactor for UCP1 proton-transport function in yeast mitochondria, nor is it a significant activator of UCP1 under these conditions.

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