Artifactual uncoupling by uncoupling protein 3 in yeast mitochondria at the concentrations found in mouse and rat skeletal-muscle mitochondria

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Western blots detected uncoupling protein 3 (UCP3) in skeletal-muscle mitochondria from wild-type but not UCP3 knock-out mice. Calibration with purified recombinant UCP3 showed that mouse and rat skeletal muscle contained 0.14 µg of UCP3/mg of mitochondrial protein. This very low UCP3 content is 200–700-fold less than the concentration of UCP1 in brown-adipose-tissue mitochondria from warm-adapted hamster (24–84 µg of UCP1/mg of mitochondrial protein). UCP3 was present in brown-adipose-tissue mitochondria from warm-adapted rats but was undetectable in rat heart mitochondria. We expressed human UCP3 in yeast mitochondria at levels similar to, double and 7-fold those found in rodent skeletal-muscle mitochondria. Yeast mitochondria containing UCP3 were more uncoupled than empty-vector controls, particularly at concentrations that were 7-fold physiological. However, uncoupling by UCP3 was not

stimulated by the known activators palmitate and superoxide; neither were they inhibited by GDP, suggesting that the observed uncoupling was a property of non-native protein. As a control, UCP1 was expressed in yeast mitochondria at similar concentrations to that of UCP3 and at up to 50 % of the physiological level of UCP1. Low levels of UCP1 gave palmitate-dependent and GDP-sensitive proton conductance but higher levels of UCP1 caused an additional GDP-insensitive uncoupling artifact. We conclude that the uncoupling of yeast mitochondria by high levels of UCP3 expression is entirely an artifact and provides no evidence for any native uncoupling activity of the protein.

Key words: brown adipose tissue, heart, proton leak, superoxide, UCP3.

INTRODUCTION

Brown adipose tissue (BAT) in rodents is thermogenic and is under careful hormonal regulation [1]. Heat is generated in BAT by uncoupling protein 1 (UCP1), which uncouples respiration from ATP synthesis by catalysing a rapid leak of protons across the mitochondrial inner membrane, so dissipating the proton electrochemical gradient [2]. Fatty acids stimulate uncoupling by UCP1, whereas purine nucleotides inhibit it [1,3]. The function of the close UCP1 homologues UCP2 and UCP3 is currently uncertain [3–10], although recent evidence shows that all three UCPs can catalyse a superoxide-dependent inducible proton leak [11]. UCP2 and UCP3 stimulate basal uncoupling in several experimental systems such as transgenic yeast [12–16], proteoliposomes [17] and transgenic mice [18]. However, in native systems in which the level of the UCPs has been changed by physiological alteration, such as starvation, no difference in the amount of proton conductance was found [19].

Here we report the physiological level of UCP3 in mouse and rat skeletal-muscle mitochondria. We confirm earlier reports [15,20–22] of uncoupling of yeast mitochondria by mammalian UCP3, but conclude that it is an artifact of heterologous expression usually to concentrations that are supraphysiological. This agrees with recent demonstrations that an unsuitably high expression of UCP1 [23] or UCP2 [24] in yeast causes artifactual uncoupling and that the expression of UCP3 in yeast leads to non-native uncoupling and the formation of inclusion bodies [25,26]. Taken together, these results help to explain earlier

observations that UCP3S (the truncated version of UCP3), UCP3L (the full-length UCP3) and their mutant forms cause unregulated uncoupling of yeast mitochondria [14,20,22].

EXPERIMENTAL

Expression of UCP1 and UCP3 in bacterial inclusion bodies

Mouse UCP1 and human UCP3 were expressed in inclusion bodies as described previously [23,24]. Human UCP3 expressed in *Escherichia coli* inclusion bodies was used to calibrate the UCP3 content of mouse, rat and yeast mitochondria. Human UCP3 cDNA was used to generate a PCR product with primers 5'-TAGGGATCCCATATGGTGGACTGAAGCCCTTCAG-3' and 3'-GGTAGGAATTCTCAAACGGTGATTCCG-5'. After digestion with *Nde*I and *Eco*RI it was ligated into a pET vector and UCP3 protein was expressed in *E. coli* inclusion bodies. The purity of the preparation used to calibrate UCP3 concentration was 35.3 ± 4.4 % [mean ± S.E.M. for seven values; Coomassie Brilliant Blue R250, SYPRO Orange (Bio-Rad) and silver (Bio-Rad) stains each compared against purified BSA fraction V and carbonic anhydrase and as a percentage of the total protein].

Expression of UCP1 and UCP3 in yeast

The plasmid vector pBF352 [23] was used for the expression of UCP1. By using the same strategy, a vector pBF354 was

Abbreviations used: BAT, brown adipose tissue; UCP1, uncoupling protein 1; UCP3, uncoupling protein 3.

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constructed for UCP3 expression. Human UCP3 cDNA was used as a template for PCR-amplification with the primers 5'-CAGGCTAGATCTATAATGGTTGGACTGAAGCC-3' and 3'-CAGAATAGATCTTCAAAACGGTGATTCCC-5'. The 5' primer contained the yeast consensus sequence ATAATG to ensure a high level of expression. This product was then digested with *Bgl*II and inserted into pKV49 [27]. Sequencing showed two silent point mutations that did not affect the amino acid sequence of the protein. W303 *Saccharomyces cerevisiae* diploid yeast cells were transformed with pBF352, pBF354 or pKV49 (empty-vector control) by the method of Hinnen et al. [28].

UCP1 and UCP3 were expressed as described previously [23]. A pre-culture was grown in selective 2% (w/v) lactate (SL) medium, containing 0.1% glucose to repress protein expression. At a D_{600} of 1.5 this was diluted into 500 ml of 3% (w/v) SL medium without glucose and grown overnight with good aeration. At a D_{600} of 1.5 protein expression was induced by the addition of 1% (w/v) galactose for the appropriate duration (except for 0 h of induction). The yeast cells were then harvested for mitochondrial isolation.

Yeast doubling times

Yeast pre-cultures were grown as above and diluted into 3% (w/v) SL medium containing 1% (w/v) galactose to a D_{600} of 0.2. D_{600} was measured over 12 h or more and a logarithmic growth curve was plotted. Exponential growth was measured at 1 h intervals over 5 h. Doubling time was calculated by least-squares regression.

Isolation of mitochondria

Yeast mitochondria were isolated as described previously [23,24]. Spheroplasts were prepared by enzymic digestion with lyticase followed by homogenization and differential centrifugation. Buffers for mitochondrial isolation contained 0.1% BSA except for the final washing step. Mammalian mitochondria were prepared by standard methods [29]. Mitochondria from mice in which UCP3 had been ablated (UCP3 knock-out mice) were from GlaxoSmithKline [11]. Protein concentration was measured with the biuret assay [30]. For Western blotting, yeast and BAT mitochondria were stored at -30°C ; other mitochondria were used fresh.

Quantitative immunodetection of UCP1 and UCP3

Mitochondria and solubilized inclusion bodies were separated by SDS/PAGE [12% (w/v) gel] before transfer to a PVDF membrane and subsequent Western blotting, as described previously [23]. Two antibodies against UCP1 were used: Chemicon-3038, antigenic to the C-terminal 19 residues of mouse and rat UCP1, and Chemicon-1426, antigenic to residues 145–159 of mouse and rat UCP1. Both were used at a dilution of 1 in 4000. Two antibodies against UCP3 were used: Chemicon-3044, antigenic to residues 102–118 between the second and third transmembrane domains of human UCP3, and Chemicon-3046, raised against a 14-residue peptide near the C-terminus of human UCP3. Both were both used at a dilution of 1 in 1000. UCP3 was degraded more readily than UCP1 in frozen samples, in agreement with earlier work [14,25,26], resulting in the underestimation of UCP3 in skeletal muscle in preliminary studies [31]. Only fresh skeletal muscle mitochondria were used in these experiments.

Western blot films were scanned and the band intensities were quantified with NIH Image, version 1.60 (<http://rsb.info.nih.gov/nih-image/>). The UCP content was linearly related to

UCP inclusion body protein. Mitochondrial UCP concentrations were calculated by interpolation [23].

Measurement of proton conductance in yeast

The kinetic dependence of the proton leak rate on membrane potential was measured as described previously [23,24] with ascorbate plus *N,N,N',N'*-tetramethyl-*p*-phenylenediamine (TMPD) as substrate in the presence of myxothiazol. The oxygen consumption of mitochondria (0.5 mg/ml protein) was measured in a Clark-type 3 ml oxygen electrode at 30°C . Proton leak rate was calculated by multiplying oxygen consumption by the H^{+} -to- O ratio of 4.0. Membrane potential was measured simultaneously with a triphenylmethylphosphonium-sensitive electrode [32] and was varied by increasing the concentration of TMPD and the rate of electron flow from ascorbate. Proton conductance at any membrane potential can be read from the proton leak curves.

Statistical analysis

Means were compared with Student's *t* test.

RESULTS

Concentration of UCP1 *in vivo* in hamster BAT mitochondria

Figure 1 shows the measurement of UCP1 in BAT mitochondria from room-temperature-adapted hamsters with Chemicon-1426 antibody [23] (Figure 1A) and Chemicon-3038 antibody (Figure 1B). Calibration with recombinant UCP1 allowed measurement of the UCP1 protein concentration (Table 1), giving values similar to other determinations [23]. Chemicon-3038 was raised against a peptide with one residue mismatch to hamster UCP1,

Figure 1 Immunodetection of UCP1 in mitochondria from BAT and yeast

Concentrations were calibrated with mouse UCP1 (32 kDa) expressed in *E. coli* inclusion bodies and partly purified [23]; values indicate calculated amounts of pure UCP1 loaded. (A, B) BAT mitochondria from room-temperature-adapted hamsters. (C, D) Yeast mitochondria from empty-vector controls and yeast transformed with mouse UCP1 (pBF352) induced with 1% (w/v) galactose for the durations indicated. (A, C) Detection with Chemicon-1426 antibody raised against residues 145–159 of mouse UCP1. (B, D) Detection with Chemicon-3038 antibody, raised against the 19 C-terminal residues of mouse UCP1.

Table 1 Physiological concentrations of UCP1 and UCP3 in mitochondria from animals kept at room temperature

Average values from Western blots, including those in Figures 1 and 2, are shown.

Protein	Tissue	Antibody	Concentration (μg of protein/mg of mitochondrial protein)
UCP1	Hamster BAT	1426	24 ± 5 ($n = 7$)
UCP1	Hamster BAT	3038	84 ± 43 ($n = 8$)
UCP3	Mouse skeletal muscle	3046	0.14 ± 0.05 ($n = 3$)
UCP3	Rat skeletal muscle	3046	0.12 ± 0.02 ($n = 5$)
UCP3	Rat skeletal muscle	3046	0.14 ± 0.01 ($n = 3$)
UCP3	Rat BAT	3046	0.08 ($n = 1$)
UCP3	Rat cardiac muscle	3046	undetectable ($n = 1$)

but this did not seem to decrease its affinity. Chemicon-1426 was a perfect match for hamster UCP1.

Concentration of UCP3 *in vivo* in rodent skeletal-muscle mitochondria

Chemicon-3046 antibody against UCP3 detected recombinant UCP3 and a band at 34 kDa in skeletal-muscle mitochondria from wild-type mice, but did not react with skeletal-muscle mitochondria from UCP3 knock-out mice (Figure 2A). It was therefore suitable for the measurement of UCP3 in mouse muscle mitochondria. Chemicon-3046 did not react with recombinant mouse UCP1, but did detect recombinant human UCP2 with an approx. 10-fold lower affinity than for UCP3 (Figure 2B). The lack of signal with Chemicon-3046 in skeletal-muscle mitochondria from UCP3 knock-out mice (Figure 2A) suggested that UCP2 was absent from mouse skeletal-muscle mitochondria, in agreement with previous observations [33]. We also tested the cross-reactivity of Chemicon-3044 UCP3 antibody. This antibody did not cross-react with UCP1, but had an approx. 3-fold higher affinity for UCP2 than for UCP3 (Figure 2C), so it could be used to quantify UCP3 only in tissues such as skeletal muscle that lack UCP2. Both commercial antibodies were raised against human UCP3 peptide sequences (used for calibration of the Western blots). The peptide used for Chemicon-3046 was 95% identical with the relevant mouse and rat UCP3 sequence; the peptide used for Chemicon-3044 was 71% identical with the appropriate rodent sequence. These sequence differences might have resulted in some underestimation of UCP3 in mouse and rat. However, Chemicon-3044 and Chemicon-3046 gave the same values, indicating that they had no difference in affinity. In addition, a similar sequence difference did not affect the calibration of the UCP1 Western blots (see above).

Calibration with recombinant UCP3 permitted the quantification of the UCP3 concentration in mitochondria from mouse skeletal muscle (Figure 2A) and rat skeletal muscle (Figures 2D and 2E), as shown in Table 1. Mouse and rat skeletal-muscle mitochondria contained 0.12–0.14 μg of UCP3/mg of protein. In other words, UCP3 contributed only approx. 0.014% of total protein in muscle mitochondria. This was far less than the 2–20% contribution of UCP1 in BAT mitochondria; there was between 200-fold and 700-fold more UCP1 in room-temperature-adapted hamster BAT than there was UCP3 in mouse and rat skeletal muscle.

UCP3 expression was also measured in mitochondria from rat cardiac muscle and BAT (Figure 2F and Table 1). No UCP3 was detected in cardiac muscle mitochondria; therefore if any was present it was below our detection limit (approx. 5 ng of UCP3/mg of mitochondrial protein). The UCP3 concentration

Figure 2 Immunodetection of UCP3 in mitochondria from mouse, rat and yeast

Concentrations were calibrated with human UCP3 (34 kDa) expressed in *E. coli* inclusion bodies and partly purified (see the Experimental section for details); values indicate calculated amounts of UCP3 loaded. UCP1 [23] and UCP2 [24] standards were prepared and calculated in the same way. (A) Skeletal-muscle mitochondria from UCP3 knock-out and wild-type mice. (B, C) UCP1, UCP2 and UCP3 *E. coli* inclusion-body protein. (D, E) Rat skeletal-muscle mitochondria. (F) Rat cardiac muscle and BAT mitochondria. (G, H, I) Yeast mitochondria from empty-vector controls and yeast transformed with human UCP3 (pBF354) induced with galactose for the durations indicated. (A, B, D, F, G, I) Detection with Chemicon-3046 antibody, raised against a 14-residue peptide near the C-terminus of human UCP3. (C, E, H) Detection with Chemicon-3044 raised against residues 102–118 between the second and third transmembrane domains of human UCP3.

in rat BAT mitochondria was similar to that in skeletal-muscle mitochondria (Table 1).

Effects of UCP1 and its homologues on yeast growth

Yeast expressing UCP1 and UCP3 (and also UCP2 [24]) were grown on solid and liquid media to assess the effects on growth

Figure 3 Effect of UCP expression on yeast growth

Yeast cells transformed with empty vector, or vector containing UCP1 (pBF352), UCP2 (pBF353) or UCP3 (pBF354), were grown for 48 h at 30 °C on leucine-selective yeast nitrogen-based medium containing 2% (w/v) glucose (**A**) or 2% (w/v) galactose as substrate (**B**).

rate. Figure 3 shows the results on solid medium. Yeast cells were grown either on glucose, which allows fermentative growth and represses the expression of the recombinant proteins, or on galactose, which is a substrate for oxidative growth and induces expression. Control yeast grew well on both glucose and galactose. In contrast, the growth of yeast containing UCP1 or UCP3 was almost entirely inhibited and the growth of yeast containing UCP2 was severely retarded on galactose but was normal on glucose. These results were confirmed by measuring doubling times in liquid medium, with lactate as substrate and galactose to induce expression. The doubling time of control yeast was 2.6 ± 0.0 h ($n = 7$), whereas yeast containing UCP1 (9.0 ± 0.6 ; $n = 8$, $P < 0.001$), UCP2 (4.1 ± 0.1 ; $n = 11$, $P < 0.001$) and UCP3 (9.6 ± 0.3 ; $n = 8$, $P < 0.001$) had increased doubling times. Therefore UCP1, UCP2 and UCP3 slowed the rate of growth of yeast.

Concentration of UCP1 in yeast mitochondria

Figure 1 shows that neither of the antibodies against UCP1, Chemicon-1426 [23] (Figure 1C) and Chemicon-3038 (Figure 1D), reacted with proteins in yeast containing only empty vector. Figures 1(A) and 1(B) show that both antibodies recognized a protein band at approx. 32 kDa when UCP1 expression was induced. Thus both antibodies could be used to measure UCP1 in yeast mitochondria.

Calibration with recombinant UCP1 (Figures 1A and 1B) permitted the measurement of UCP1 concentration after 0, 1, 2 and 4 h of induction. The two antibodies gave the same results, so average values were used. UCP1 is thought to act as a dimer [34,35], so results were calculated both as μg of UCP1/mg of mitochondrial protein and as pmol of UCP1 dimer/mg of mitochondrial protein (Table 2).

Even without induction by galactose (0 h), UCP1 was expressed at significant concentrations in yeast mitochondria (Figures 1A and 1B; Table 2) because of incomplete repression of the powerful promoter. At 4 h of induction, UCP1 content was $14 \mu\text{g}/\text{mg}$ of protein, approaching 50% of the concentration found in mitochondria from room-temperature-adapted hamsters (Table 1), so UCP1 expression was close to physiological levels.

Concentration of UCP3 in yeast mitochondria

Figure 2(G) shows that the UCP3 antibody Chemicon-3046 did not cross-react with proteins in yeast containing only empty vector. However, Chemicon-3044 did cross-react with yeast proteins (Figure 3H), so it was not used to measure UCP3 in yeast mitochondria. The expression of UCP3 in yeast was therefore quantified only with Chemicon-3046 (Figure 3I) at 0, 1, 2 and 4 h of induction (Table 2).

UCP3 expression in yeast increased with duration of induction. The physiological concentration of UCP3 in rodent skeletal-muscle mitochondria ($0.14 \mu\text{g}/\text{mg}$; Table 1) was reached in yeast mitochondria at 1 h of induction (Table 2). At 2 h the UCP3 concentration in yeast mitochondria was double the physiological concentration in rodents, and at 4 h it was 7-fold physiological.

Proton conductance of mitochondria from yeast expressing UCP1

Figure 4 shows the dependence of proton leak rate on membrane potential in mitochondria from control yeast and yeast expressing UCP1. Figures 4(A)–4(D) show that there was no change in proton conductance in controls during 4 h of induction by galactose. However, the kinetics of substrate oxidation did alter [for example, there was inhibition of substrate oxidation in Figure 4(B) in comparison with Figure 4(A), seen as a decrease in the maximum oxygen consumption rate]. The addition of palmitate might have increased proton conductance slightly (Table 2).

Figures 4(E)–4(H) show that the expression of UCP1 induced GDP-sensitive proton conductance, but also induced an artifactual GDP-insensitive proton conductance at longer durations. Without induction by galactose, the 'leaky' expression of UCP1 resulted in native UCP1 activity (Figure 4E), i.e. proton conductance activated by low concentrations of palmitate and fully inhibited by 1 mM GDP. The total proton conductance due to UCP1 expression increased during 4 h of induction by galactose (Figures 4E–4H). However, the native activity only doubled even though the UCP1 content increased 40-fold, so the apparent specific activity of the native component decreased with time (Table 2). A second artifactual proton conductance, defined by its insensitivity to GDP, appeared between 1 and 4 h of induction of UCP1 (Figures 4F–4H and Table 2). However, even this component did not increase linearly with UCP1 expression; by 4 h of induction by galactose a decrease in the GDP-insensitive proton conductance per UCP1 dimer was apparent (Table 2). This indicates a third phase of high UCP1 expression: UCP1 was expressed but caused no proton conductance. UCP1 was therefore expressed progressively in yeast in three forms with different properties: native GDP-sensitive proton conductance, artifactual GDP-insensitive proton conductance and a form that caused no conductance at all.

Proton conductance of mitochondria from yeast expressing UCP3

Figure 5 shows the kinetics of proton leak in mitochondria from yeast expressing UCP3. Figure 5(B) shows that there was only a small effect of UCP3 expression on proton conductance at 1 h of induction. The concentration of UCP3 at 1 h was $0.16 \mu\text{g}$ of UCP3/mg of protein (Table 2), greater than the UCP3 concentration of $0.14 \mu\text{g}/\text{mg}$ found in rodent skeletal-muscle mitochondria (Table 1). Even at 2 h (Figure 5D), there was little uncoupling; not until 4 h (Figure 5E), when UCP3 concentration was 7-fold physiological, was uncoupling obvious. Table 2 details the extent of the uncoupling, from experiments with UCP3-expressers and paired controls. As with UCP1, the proton

Table 2 Concentration and activity of mouse UCP1 and human UCP3 expressed in yeast mitochondria

UCP1 content is the average of 12 determinations with either Chemicon-1426 or Chemicon-3038 antibody. UCP3 content is the average of five determinations with Chemicon-3046, from Western blots including those shown in Figures 1 and 2. Dimer content was calculated by assuming molecular masses of 32 kDa for UCP1 and 34 kDa for UCP3. Values for proton conductance at the membrane potentials indicated were taken from the proton leak kinetics shown in Figures 4 and 5. GDP-sensitive proton conductance was measured by subtracting that in the presence of palmitate and GDP from that in the presence of palmitate; GDP-insensitive proton conductance was measured by subtracting that of the control in the presence of palmitate and GDP from that with UCP in the presence of palmitate and GDP; UCP-dependent proton conductance was measured by subtracting that of the control from that with UCP.

Conditions	UCP1 content		Proton conductance					
			GDP-sensitive, at 98 mV (native UCP1 activity)		GDP-insensitive, at 98 mV (artifactual UCP1 activity)		UCP-dependent, at 137 mV	
	(μ g of UCP/mg of mitochondrial protein)	(pmol of dimer/mg of mitochondrial protein)	(nmol of H ⁺ /min per mg of mitochondrial protein per mV)	Dimer catalytic-centre activity (min ⁻¹ · mV ⁻¹)	(nmol of H ⁺ /min per mg of mitochondrial protein per mV)	Dimer catalytic-centre activity (min ⁻¹ · mV ⁻¹)	(nmol of H ⁺ /min per mg of mitochondrial protein per mV)	Dimer catalytic-centre activity (min ⁻¹ · mV ⁻¹)
Empty-vector control								
No induction			0.5 ± 0.2					
1 h induction			0.6 ± 0.2					
2 h induction			0.5 ± 0.2					
4 h induction			0.9 ± 0.2					
UCP1								
No induction	0.32 ± 0.08	5.1 ± 1.3	3.3 ± 1.0	630 ± 270	-0.3 ± 0.2	-68 ± 39	0.5 ± 0.4	94 ± 76
1 h induction	0.70 ± 0.14	11 ± 2.1	5.4 ± 1.1	540 ± 140	0.4 ± 0.5	37 ± 47	1.6 ± 0.6	146 ± 66
2 h induction	2.2 ± 0.64	34 ± 10	3.3 ± 0.6	98 ± 34	1.0 ± 0.6	28 ± 20	3.4 ± 0.7	99 ± 35
4 h induction	14 ± 5.9	220 ± 92	7.0 ± 0.9	32 ± 15	1.9 ± 0.2	8.9 ± 3.9	> 7.6 ± 1.4	< 35 ± 15
UCP3								
No induction	0.05 ± 0.02	0.76 ± 0.34					0.6 ± 0.4	760 ± 580
1 h induction	0.16 ± 0.06	2.3 ± 0.83					0.9 ± 0.1	380 ± 150
2 h induction	0.27 ± 0.11	4.0 ± 1.6					1.5 ± 0.6	370 ± 210
4 h induction	0.91 ± 0.29	13 ± 4.3					2.0 ± 0.3	150 ± 55

Figure 4 Effect of UCP1 on the kinetics of proton leak in mitochondria isolated from yeast induced with 1% (w/v) galactose for different times

Symbols: □, △, ◇, paired empty-vector control; ■, ▲, ◆, yeast transformed with UCP1 (pBF352); ◇, ◆, no additions; □, ■, plus 50 μ M palmitate; △, ▲, plus 50 μ M palmitate and 1 mM GDP.

Figure 5 Effect of UCP3 expression on the kinetics of proton leak in mitochondria isolated from yeast induced with 1% (w/v) galactose for different times

Symbols: □, △, ◇, paired empty-vector control; ■, ▲, ◆, yeast transformed with UCP3 (pBF354); ◇, ◆, no additions; □, ■, plus 50 μ M palmitate; △, ▲, plus 50 μ M palmitate and 1 mM GDP.

conductance increased less with time than the concentration of UCP3, so the apparent specific activity decreased with time (Table 2). Thus there were at least two forms of UCP3, one that caused some uncoupling and another, at higher expression levels, that did not.

At 0.32 μ g/mg of protein, UCP1 showed native function in yeast (Figure 4E and Table 2). At 2 h of induction, the UCP3 concentration was similar (0.27 μ g/mg), so we attempted to activate any functional UCP3 that might have been present. The addition of palmitate slightly increased proton conductance, but

the effect was not inhibited by GDP (Figure 5D) and was no greater than in empty-vector controls (Figure 5C), showing that it was not due to UCP3.

DISCUSSION

Physiological concentration of UCP3

The results shown here indicate that UCP3 is present at very much lower concentrations in mammalian muscle mitochondria than is UCP1 in BAT mitochondria. This immediately suggests

that even if the uncoupling activity of UCP3 were equal to that of UCP1, it would have much less capacity to uncouple oxidative phosphorylation. Very low concentrations of UCP3 might explain the difficulties that many workers have had in detecting UCP3 in mammalian mitochondria by Western blotting, even with antibodies that seem to work adequately in yeast mitochondria with strong overexpression of UCP3.

The concentration of UCP3 in skeletal muscle (140 ng/mg of mitochondrial protein) is similar to the concentration of UCP2 in lung (78 ng/mg) and spleen (313 ng/mg) and exceeds that of UCP2 in stomach (31 ng/mg) [24,33]. Pecqueur et al. [33] have shown that UCP2 is not expressed in mouse skeletal muscle or BAT. Despite the presence of UCP2 mRNA, they did not detect UCP2 in cardiac tissue [33]. Because we detected no UCP3 in heart mitochondria, it seems that little or no uncoupling protein is expressed in the heart.

Effect of uncoupling proteins on yeast growth

Many previous studies have shown that expression of the uncoupling proteins in yeast causes retardation of growth [12,22–24,26,27,36,37]. However, yeast containing UCP1 should not be growth retarded, because pH [38] and the concentration of purine nucleotides and fatty acids [39] in yeast should be sufficient to inhibit the uncoupling by UCP1 completely. As shown previously [23], and confirmed above, UCP1 is fully inhibited by purine nucleotides when expressed at a low level in yeast. The inhibition of growth at higher expression levels is most probably due to artifactual uncoupling of the mitochondria, caused by an excessive amount of protein. Inhibition of growth therefore cannot be safely used as a demonstration of the expression of any uncoupling protein with native function.

Expression level of UCP1 and UCP3 in yeast

At all periods of induction at least 4-fold more UCP1 than UCP3 was expressed in yeast (Table 2). This variation in uncoupling-protein expression in yeast has been noted previously [14,20,25]. The only difference in the expression system was in the coding sequence of the protein itself.

Proton conductance in yeast containing UCPs

Our results indicate that there are three modes of UCP1 expression in yeast, appearing progressively as induction proceeds. At low expression levels and early induction times, UCP1 is inserted into the yeast mitochondrial membrane in a functional form that is sensitive to GDP. At higher expression levels there is GDP-insensitive, artifactual uncoupling, resulting from poor insertion, misfolding, a lack of post-translational modification or some other phenomenon. At the highest expression levels UCP1 does not affect proton conductance, probably because it does not insert into the membrane at all. Similarly, our results show that there are two modes of UCP3 expression in yeast: one that uncouples and one that does not. There is good evidence that proton conductance is completely sensitive to very low concentrations of nucleotides when catalysed by UCP3 in the presence of ubiquinone in liposomes [40,41] or mitochondria [42] and in the presence of superoxide in mitochondria [11]. However, uncoupling by UCP3 in yeast was insensitive to nucleotides (Figures 5A and 5D). We conclude that the uncoupling by UCP3 seen by us and by others in yeast corresponds to the GDP-insensitive, artifactual uncoupling that is also seen with UCP1. The expression of UCP1 in three different forms and UCP3 in two different forms is broadly in agreement with the observations

of Winkler et al. [26] and Heidkaemper et al. [25]. They suggest that little or no functional UCP3 is expressed in transgenic yeast; instead it is expressed in a form that causes artifactual uncoupling and in a form in which no uncoupling takes place. However, they also report that these artifacts are not seen when UCP1 is expressed. This difference could be attributable either to differences in absolute amounts of UCP1 in their studies or to differences in the rate at which it is expressed.

Other studies have compared uncoupling by UCP1, UCP3S, UCP3L and various mutated forms in yeast [14,20]. All proteins were concluded to have clear uncoupling characteristics. In one study, UCP3S (the truncated version of UCP3) had a higher intrinsic activity than UCP3L (the full-length UCP3) [14]. It is unlikely that UCP3S is capable of maintaining a coherent structure in the membrane; an alternative explanation for these results is that the expression of UCP3S in yeast causes a stronger artifactual uncoupling.

Effect of superoxide on the proton conductance of mitochondria from yeast expressing UCP1, UCP2 and UCP3

We analysed whether the uncoupling caused by the expression of UCP2 [24] and UCP3 was a function of native protein (as at the lowest levels of UCP1) or an artifact of heterologous expression (as at the higher levels of UCP1). We tested whether UCP2 and UCP3 in yeast displayed the properties of the native protein in mammalian mitochondria: uncoupling stimulated by superoxide and inhibited by GDP [11]. Yeast mitochondria expressing low levels of UCP1 show these properties [11], demonstrating that superoxide does stimulate uncoupling when UCP1 is functionally expressed in yeast. However, we failed to show GDP-sensitive uncoupling in the presence of superoxide in mitochondria from yeast expressing moderate levels of UCP2 and UCP3 (results not shown). This observation supports the conclusion reached above, that UCP3 (and also UCP2) is not expressed in a functional form in yeast.

Conclusions

In summary, the physiological levels of UCP3 in mammalian skeletal muscle are very low relative to UCP1 but are similar to those of other mitochondrial carrier proteins. When UCP3 is expressed in yeast there is no evidence for native function as assayed by GDP-inhibited proton conductance. Instead, it seems that virtually none of the protein is expressed in a native state and that all uncoupling caused by the expression of UCP3 in yeast by us and by others has been artifactual. Therefore the expression of UCP3 (or UCP2) in yeast does not currently provide a good experimental system for the analysis of its function. The same is true for UCP1 at high expression levels; however, at low levels it provides a good model for functional and other studies. It remains to be determined whether UCP3 uncouples mitochondria in mammalian cells *in vivo* under normal conditions or in transgenic mice overexpressing UCP3 [18,43].

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