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Assessment of uncoupling activity of uncoupling protein 3 using a yeast heterologous expression system

Chen-Yu Zhang[1,a], Thilo Hagen[1,a], Vamsi K. Mootha[a], Lawrence J. Slieker[b], Bradford B. Lowell[a,*]

[a] *Division of Endocrinology, Department of Medicine, Beth Israel Deaconess Medical Center and Harvard Medical School, 99 Brookline Ave., Boston, MA 02215, USA*

[b] *Endocrine Research Division, Lilly Research Laboratories, Eli Lilly and Company, Indianapolis, IN 46285, USA*

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Abstract Uncoupling protein 3L, uncoupling protein 1 and the mitochondrial oxoglutarate carrier were expressed in *Saccharomyces cerevisae*. Effects on different parameters related to the energy expenditure were studied. Both uncoupling protein 3L and uncoupling protein 1 reduced the growth rate by 49% and 32% and increased the whole yeast O_2 consumption by 31% and 19%, respectively. In isolated mitochondria, uncoupling protein 1 increased the state 4 respiration by 1.8-fold, while uncoupling protein 3L increased the state 4 respiration by 1.2-fold. Interestingly, mutant uncoupling protein 1 carrying the H145Q and H147N mutations, previously shown to markedly decrease the H^+ transport activity of uncoupling protein 1 when assessed using a proteoliposome system (Bienengraeber et al. (1998) Biochem. 37, 3–8), uncoupled the mitochondrial respiration to almost the same degree as wild-type uncoupling protein 1. Thus, absence of this histidine pair in uncoupling protein 2 and uncoupling protein 3 does not by itself rule out the possibility that these carriers have an uncoupling function. The oxoglutarate carrier had no effect on any of the studied parameters. In summary, a discordance exists between the magnitude of effects of uncoupling protein 3L and uncoupling protein 1 in whole yeast versus isolated mitochondria, with uncoupling protein 3L having greater effects in whole yeast and a smaller effect on the state 4 respiration in isolated mitochondria. These findings suggest that uncoupling protein 3L, like uncoupling protein 1, has an uncoupling activity. However, the mechanism of action and/or regulation of the activity of uncoupling protein 3L is likely to be different.

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Key words: Uncoupling protein 3; Yeast expression system; Energy expenditure

1. Introduction

Uncoupling proteins (UCPs)[2] are closely related mitochondrial inner membrane proteins. UCP1 was the first UCP to be identified [1–3]. Its biochemical function has been characterized extensively. UCP1 uncouples the mitochondrial respiration from ATP synthesis by dissipating the transmembrane proton gradient [4–6]. The mechanism of uncoupling is thought to be due to either direct transport of protons by UCP1 [7] or, alternatively, transport of free fatty acids anions by UCP1, allowing free fatty acids to function as cycling protonophores [8,9]. The uncoupling activity of UCP1 is inhibited by purine nucleotides, such as GDP [4]. UCP1, which is expressed exclusively in brown adipose tissue, is believed to play an important role in the thermogenesis in rodents. Its role in humans is less certain as the mass of brown adipose tissue is less abundant.

Recently, two proteins with 59% and 57% homology to UCP1 were identified, UCP2 [10,11] and UCP3 [12–14]. The UCP3 gene generates two mRNA transcripts, UCP3L, which encodes a protein similar in length to UCP1 and UCP2, and UCP3S, which encodes a protein lacking the last 37 C-terminal residues [12,15]. UCP2 is expressed in many tissues, while UCP3 in humans is found predominantly in skeletal muscle. Since skeletal muscle is believed to be a major site of adaptive thermogenesis in humans [16–20], UCP3 could be a regulator of the energy expenditure. However, to date, the biochemical function and regulation of UCP3 and UCP2 are not fully understood. Flow cytometry studies employing mitochondrial membrane potential-sensitive dyes indicate that UCP2 [10,11] and UCP3L [14,21,22] decrease the mitochondrial membrane potential. In addition, Fleury et al. [10] found that UCP2 increased the state 4 respiration in yeast mitochondria.

Recently, Bienengraeber et al. [23] found that the H^+ transport activity by UCP1, assessed by reconstitution of UCP1 into proteoliposomes, is almost completely abolished when histidine residues H145 and H147 are mutated to neutral residues and reduced to about 10% when only one of the two histidines is mutated. Of note, this histidine pair is absent in UCP2 and human UCP3 lacks the equivalent histidine, H145. It was predicted, therefore, that the H^+ transporting activity of UCP2 and UCP3 would be greatly diminished or require a different mechanism [23].

In the present study, we have utilized a yeast expression system to explore the biochemical function of UCP3L. Towards these ends, we have generated a recombinant yeast expressing UCP3L under the control of a galactose inducible promoter. For the purposes of comparison, we have also generated a yeast expressing UCP1, a carrier which has uncoupling activity, the double histidine mutant of UCP1 (H145Q and H147N) previously demonstrated to be defective in the proton transport in a proteoliposome system [23] and the oxoglutarate carrier, which mediates an electroneutral exchange of oxoglutarate for malate [24]. The oxoglutarate carrier should be informative as a negative control, particularly since it is most homologous to UCP1, UCP2 and UCP3L

*Corresponding author. Fax: (1) (617) 667 2927.
E-mail: blowell@bidmc.harvard.edu

[1] These authors share first authorship.

Abbreviations: UCP, uncoupling protein; $DiOC_6$, 3′3-dihexyloxacarbocyanine iodide; BSA, bovine serum albumin; PMSF, phenylmethylsulfonylfluoride; EGTA, ethylenebis(oxyethylenenitrilo)tetraacetic acid; EDTA, ethylenediaminetetraacetic acid; FCCP, carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone

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(31% identical), but is thought to lack uncoupling activity. These yeasts have been used to assess the effects of carrier expression on growth rates and the basal O_2 consumption in whole yeast and on the state 4 respiration in isolated mitochondria. Our data indicate that UCP3L has an uncoupling activity but is regulated differently from UCP1.

2. Materials and methods

2.1. Expression vectors and site-directed mutagenesis

Human UCP3L, rat UCP1 and human mitochondrial oxoglutarate carrier encoding sequences were obtained using PCR amplification and corresponding cDNA templates. The PCR primers used are shown in Table 1.

The oligonucleotides were designed to introduce *Hin*d III and *Xba* I sites close to the ATG (start) and TAG or TGA (stop) codons, respectively. After amplification and digestion with *Hin*d III and *Xba* I, the PCR products were introduced into the pYES2 expression vector (Invitrogen, Carlsbad, CA, USA) in which the carrier gene expression is under the control of the *gal10-cyc1* promoter. The H145Q and H147N double histidine mutant of UCP1 was generated using site-directed mutagenesis (Quickchange site-directed mutagenesis kit, Stratagene, La Jolla, CA, USA). As described in [23], the codons for H145 (CAT) and H147 (CAC) were changed to CAG (codon for glutamine) and AAC (codon for asparagine), respectively.

In addition, epitope constructs, using an influenza virus hemagglutinin epitope tag, were prepared as described in [25]. The epitope tag was added at the N-terminus of the mitochondrial carrier protein sequence.

All sequences were verified by DNA sequencing. Site-directed mutagenesis of mutant UCP1 was also confirmed by RT-PCR of isolated yeast RNA, followed by DNA sequencing.

The *Saccharomyces cerevisiae* strain INVSC1 (Invitrogen) was transformed with the pYES2 vectors containing these constructs. The empty pYES2 vector was transformed as an additional negative control.

2.2. Expression of the UCPs and the oxoglutarate carrier

Yeast transformants were selected on SC-ura plates. Single colonies were inoculated into a preculture grown in SC-ura medium (0.67% yeast nitrogen base, 0.1% KH_2PO_4, 0.12% $(NH_4)_2SO_4$, 0.1% glucose, 2% lactate, 0.1% casamino acids, 20 mg/l tryptophan, 40 mg/l adenine) to an OD_{600} of 2–4. Glucose was added in order to maintain a complete repression of the Gal-Cyc promoter during growth. The yeasts were diluted to a final OD_{600} of 0.04 in 500 ml of the same medium except that glucose was absent and lactate was present at a concentration of 3% and grown at 30°C with vigorous shaking to ensure good oxygenation. After approximately 36 h, 1% galactose was added and the cells were harvested after 8–12 h.

2.3. Analysis of expression of the UCPs and the oxoglutarate carrier by Western blotting

The expression of the UCPs was detected by Western blotting with the respective antibodies. A rabbit anti-mouse UCP1 IgG fraction was purchased from RDI Research Diagnostics (Flanders, NJ, USA). The antibody to human UCP3 was prepared against a peptide representing residues 147–166 in exon 4. The antibody was affinity-purified by the same peptide coupled to Pierce SulfoLink gel.

Western blotting of mitochondrial protein from the yeast expressing the epitope tag-coupled proteins was performed using mouse monoclonal antibody (clone 12CA5) to the peptide epitope derived from the hemagglutinin protein of human influenza virus (Boehringer Mannheim, Indianapolis, IN, USA).

2.4. Whole yeast O_2 consumption

Yeasts were harvested, washed once and resuspended in 3% lactate containing SC growth medium at approximately 2.5×10^9 cells/ml. The basal O_2 consumption and maximum O_2 consumption induced with 2 μM carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone (FCCP) were measured in a Clarke type oxygen electrode chamber at 25°C with 1 ml of cell suspension at a final concentration of 2.5×10^8 cells/ml in the 3% lactate SC medium.

2.5. Measurement of the mitochondrial membrane potential of yeast expressing UCPs and oxoglutarate carrier

The mitochondrial membrane potential was measured with the potential-sensitive dye 3′3-dihexyloxacarbocyanine iodide ($DiOC_6$) (Molecular Probes, Eugene, OR, USA) at a concentration of 25 nM. When indicated, 10 μM FCCP was added 5–10 min before the addition of $DiOC_6$. Cells were counted (10 000 events) approximately 30 min after the addition of $DiOC_6$ using a FACScan instrument (Becton Dickinson, Cockeysville, MD, USA).

2.6. Isolation of yeast mitochondria

Mitochondria were prepared as described by Arechaga et al. [26]. Spheroplasts were prepared by enzymatic digestion with zymolyase and mitochondria were isolated by differential centrifugation after homogenization of the spheroplasts. The buffer for mitochondrial isolation contained 0.6 M mannitol, 10 mM Tris/maleate pH 7.4, 0.1% bovine serum albumin (BSA), 1 mM phenylmethylsulfonylfluoride (PMSF), 1 mM ethylenebis(oxyethylenenitrilo)tetraacetic acid (EGTA) and 1 mM ethylenediaminetetraacetic acid (EDTA). BSA and PMSF were omitted during the last washing step. The mitochondria were resuspended in the isolation buffer without BSA and PMSF at a final protein concentration of approximately 10 mg/ml.

2.7. Polarography

The mitochondrial respiration was measured in a Clarke type oxygen electrode at 30°C under the following standard incubation conditions: 0.6 M mannitol, 10 mM Tris/maleate pH 6.8, 5 mM KH_2PO_4/K_2HPO_4, 0.5 mM EDTA, 3 mM NADH, 2.5 μg/ml oligomycin and approximately 0.25 mg mitochondrial protein/ml.

2.8. Statistical analysis

Statistical analysis was performed using StatView 4.0 (Abacus Concept, Berkeley, CA, USA). All results are presented as the mean ± S.E. The student's *t* test was used to evaluate the statistical significance of differences, as indicated in the figure and table legends.

Table 1
Primers for PCR amplification of mitochondrial carriers

UCP3L	
(Sense)	5′-AAGCTTGAATTC**ATG**GTTGGACTGAAGCCTCAGAC
	*Hin*d III — *Eco* RI — **start-codon**
(Antisense)	5′-TCTAGA**TCA**AAACGGTGATTGCCGTAACATCTG
	Xba I — **stop-codon**
UCP1	
(Sense)	5′-AAGCTTGAATTC**ATG**GTGAGTTCGACAACTTCCGAAGTG
	*Hin*d III — *Eco* RI — **start-codon**
(Antisense)	5′-TCTAGA**CTA**TGTGGTTGCAGTCCACTGTCTGCCG
	Xba I — **stop-codon**
Oxoglutarate carrier	
(Sense)	5′-AAGCTTGAATTC**ATG**GCGGCGACGGCGAGTGC
	*Hin*d III — *Eco* RI — **start-codon**
(Antisense)	5′-TCTAGA**TCA**GCCACTGAGGAAGAGACG
	Xba I — **stop-codon**

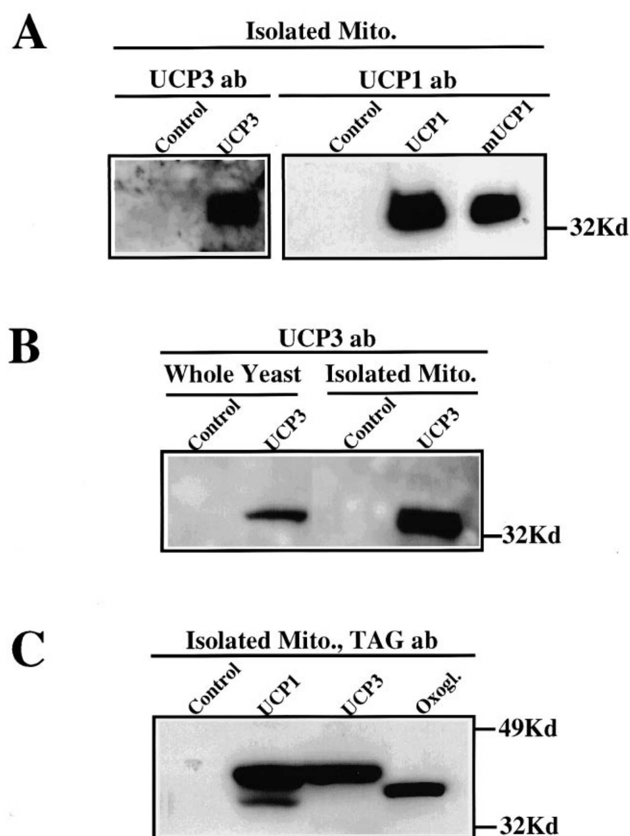

Fig. 1. (A, B) Immunoblot analysis of the expression of the UCPs. (C) Immunoblot analysis of the expression of UCP1, UCP3L and the oxoglutarate carrier in isolated mitochondria using epitope tag constructs expressed in yeast. 40 μg mitochondrial protein was used for analysis in each lane.

3. Results

3.1. Expression of the UCPs and the oxoglutarate carrier

Expression of UCP3L, UCP1 and mutant UCP1 was confirmed by Western blot analysis of isolated mitochondria, obtained 8 h after induction with 1% galactose (Fig. 1A). For UCP3L, the relative abundance of protein in whole yeast as compared to isolated mitochondria was also measured (Fig. 1B). The isolated mitochondria contained at least 10-fold more UCP3L on a per protein basis, demonstrating that UCP3L was targeted to mitochondria.

Since no suitable antibody against the oxoglutarate carrier was available, the presence of oxoglutarate carrier RNA was confirmed by Northern blot analysis (data not shown). In order to confirm that human oxoglutarate carrier protein can be expressed by yeast and to compare the expression levels of the various mitochondrial carriers, epitope tag constructs for UCP3L, UCP1 and the oxoglutarate carrier were made and expressed in yeast. Western blot analysis of isolated mitochondria in Fig. 1C shows that all three proteins were expressed at qualitatively similar levels.

3.2. The effect of UCPs on growth

Both UCP3L and UCP1 inhibited the growth of aerobically grown yeast (Fig. 2). The data in Fig. 2 represent one typical experiment. The average inhibition of growth by UCP3L, in comparison to control yeast, was 49 ± 4% ($n = 7$) after growth for 14 h. UCP1 had an intermediate effect on growth (32 ± 5% inhibition). The oxoglutarate carrier had no inhibitory effect on growth.

3.3. The effect of UCPs on whole yeast O_2 consumption

UCP3L and UCP1 increased the basal O_2 consumption of whole yeast by 31% and 19%, respectively (Table 2). The oxoglutarate carrier had no effect on the basal O_2 consumption. The uncoupling agent, FCCP, increased the O_2 consumption of all yeast strains to a similar absolute rate (approximately 100 nmol O/min/10^8 cells). The ability of UCP1 and UCP3L to increase the basal but not maximally uncoupled respiration is consistent with UCP1 and UCP3L having a partial uncoupling activity.

3.4. The effect of UCPs on the mitochondrial membrane potential

Both UCP3L and UCP1 decreased the $DiOC_6$ uptake as measured by flow cytometry (Fig. 3). Expression of the oxoglutarate carrier resulted in a slight decrease in the $DiOC_6$ fluorescence. This decrease in $DiOC_6$ uptake induced by oxoglutarate carrier expression was consistently less than that observed with UCP3L and UCP1.

3.5. The effect of UCPs in isolated mitochondria

To study the effect of UCP3L in isolated mitochondria, the capacity of UCP3L to uncouple the oxidative phosphorylation was compared with that of UCP1. The state 4 respiration was measured in the presence of oligomycin to inhibit ATP synthase. Typical tracings for the empty vector control, UCP1 and UCP3L are shown in Fig. 4a. Fig. 4b shows state 4 respiratory rates in the absence and presence of GDP and

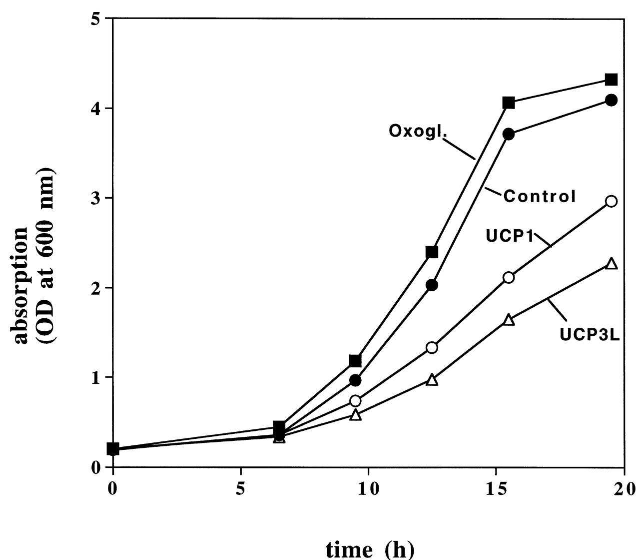

Fig. 2. The growth rate of yeast expressing UCP3L, UCP1 and the oxoglutarate carrier. Yeasts were grown at 30°C in 3% lactate and no glucose containing growth medium as described under section 2. At time zero, yeasts were diluted with growth medium to an OD_{600} of 0.20 and galactose was added (final concentration of 1%). The growth was measured by determining the absorption at 600 nm. (●) control (empty vector); (Δ) UCP3L; (○) UCP1; (■) oxoglutarate carrier. The experiment was repeated seven times with similar results. Means and standard errors are given in the text. One typical experiment is presented.

Table 2
Whole yeast O_2 consumption

	Basal O_2 consumption (nmol O/min/10^8 cells)	FCCP-induced O_2 consumption (nmol O/min/10^8 cells)	Basal O_2 consumption (% of rate with FCCP)
Control ($n=11$)	46.2 ± 1.3	104.0 ± 3.2	44.6 ± 1.2
UCP1 ($n=11$)	54.8 ± 3.7*	96.3 ± 6.2	56.9 ± 1.5**
UCP3L ($n=11$)	60.7 ± 3.7*	99.5 ± 5.7	61.3 ± 3.4**
Oxogl. ($n=4$)	47.7 ± 3.9	104.9 ± 11.9	45.5 ± 2.6

The whole yeast O_2 consumption was measured in the 3% lactate containing medium. 2 μM FCCP was added to determine the maximum respiratory capacity. $*P<0.01$ versus the empty vector (Control) and oxoglutarate carrier (Oxogl.) controls.

maximally uncoupled respiratory rates after the addition of FCCP. As expected, UCP1 increased the state 4 respiration by approximately 2-fold. The small increase of the state 4 respiration observed with UCP3L was not significant. The oxoglutarate carrier had no effect on the state 4 respiration. Of note, both UCP1 and UCP3L, but not the oxoglutarate carrier, decreased the maximally uncoupled respiration in the presence of FCCP. To correct for the different maximum respiratory activities and the day to day variation of mitochondrial preparations, the results in Fig. 4c were expressed as percentage of the FCCP-stimulated respiration. The increase in state 4 induced by UCP3L, when expressed as percentage of the FCCP-stimulated respiration, is statistically significant compared to the empty vector and oxoglutarate carrier controls. 1 mM GDP blocked the increase of the state 4 respiration with UCP1 completely, but had no effect on the state 4 respiration in mitochondria bearing UCP3L.

We also studied the effect of a modified version of UCP1 carrying the H145Q and H147N mutations, previously shown by Bienengraeber et al. [23] to have a markedly decreased H^+ transport activity in proteoliposomes. Unexpectedly, mutant UCP1 uncoupled the mitochondrial respiration to almost the same degree as UCP1. This uncoupling activity is specific, since it was completely blocked by GDP (Fig. 4d).

4. Discussion

UCP1 plays an important role in the adaptive thermogenesis by uncoupling the mitochondrial respiration from ATP synthesis and increasing the energy expenditure [5,6]. Based on the high sequence homology between UCP1 and UCP3L, it was predicted that UCP3L would have a similar function [12,13,14]. However, except for a UCP3L-induced decrease in the mitochondrial membrane potential [14,21,22], the biochemical function of UCP3L has not yet been established.

In the present study, UCP3L expression reduced the growth and increased the whole yeast basal O_2 consumption. These effects of UCP3L in whole yeast were even stronger than those observed with UCP1. Although these parameters are not specific for the uncoupling activity, the fact that the well-characterized UCP1 had similar effects, while the oxoglutarate carrier had no effect, suggests that the two UCPs have similar biochemical activities.

Expression of UCP3L and UCP1 decreased the $DiOC_6$ uptake by whole yeast, as assessed using flow cytometry. However, expression of the oxoglutarate carrier also decreased the $DiOC_6$ uptake albeit to a much smaller degree. Given that the $DiOC_6$ fluorescence depends on both the mitochondrial membrane potential and the amount of mitochondria present in yeast, our data raise the possibility that some portion of this measurement may be prone to non-specific effects. Alterna-

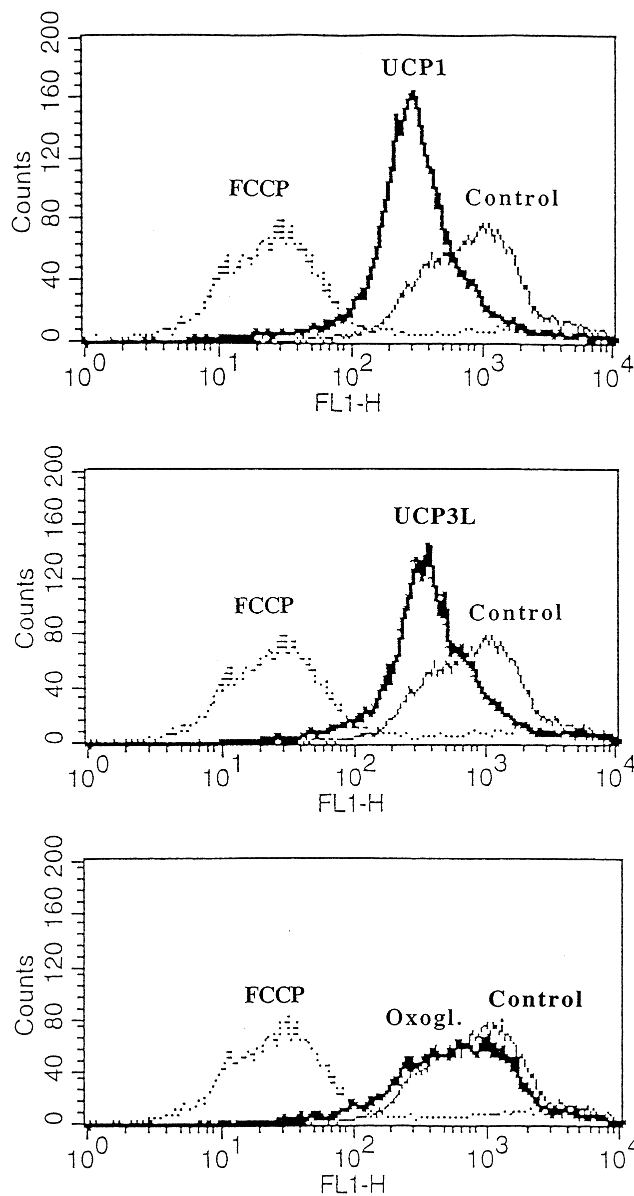

Fig. 3. The mitochondrial membrane potential of yeast expressing UCPs and oxoglutarate carrier. Fluorescence histograms of cells expressing UCP1, UCP3L, oxoglutarate carrier and empty vector are presented. In each panel the left most histogram (light line) shows the effects of the uncoupler FCCP and the right most histogram (light line) shows the cells with empty vector treated with $DiOC_6$. The dark lines show the cells with UCPs or oxoglutarate carrier treated with $DiOC_6$.

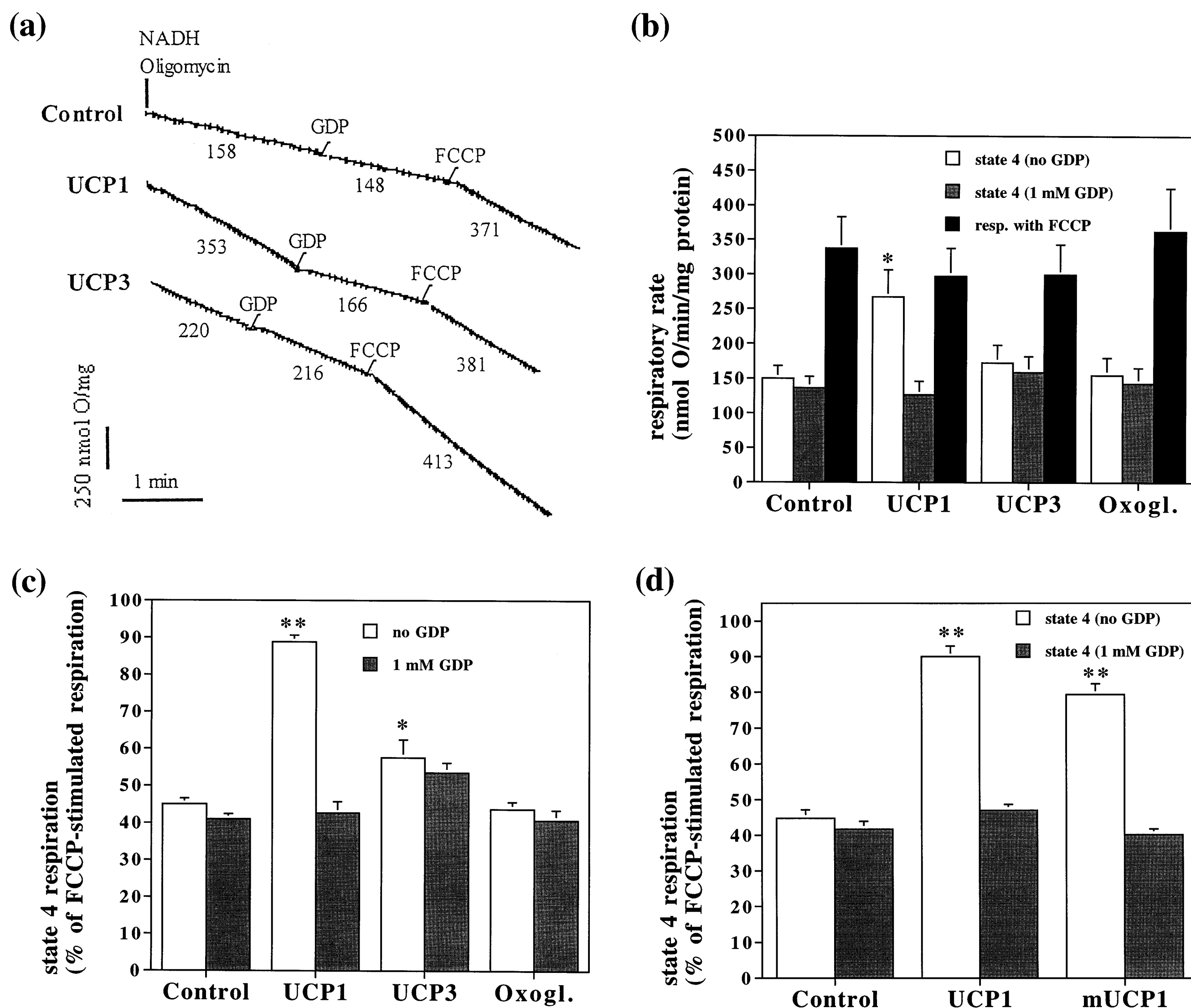

Fig. 4. Respiration in isolated mitochondria. (a) Mitochondrial respiration was measured as described under section 2. Representative tracings for the empty vector control (Control), UCP1 and UCP3L are shown. The addition of 3 mM NADH, 2.5 μg/ml oligomycin, 1 mM GDP and 2.5 μM FCCP as well as the O_2 consumption in nmol O/min/mg mitochondrial protein are indicated. (b) State 4 respiratory rates in the absence and presence of 1 mM GDP and respiratory rates in the presence of 2.5 μM FCCP are expressed in nmol O/min/mg mitochondrial protein. (c) State 4 respiratory rates are expressed as percentage of the maximally uncoupled respiratory rate in the presence of FCCP. The data in b and c represent the average of five experiments. $^*P<0.05$; $^{**}P<0.0001$ versus the empty vector (Control) and oxoglutarate carrier (Oxogl.) controls. (d) State 4 respiration with mutant UCP1 (mUCP1). State 4 respiratory rates are expressed as percentage of the maximally uncoupled respiratory rate in the presence of FCCP. The data represent the average of three experiments. $^{**}P<0.0001$ versus the empty vector control.

tively, the oxoglutarate carrier may have an effect on the membrane potential unrelated to uncoupling of the mitochondrial respiration.

To directly assess the uncoupling activity of UCP3L, the state 4 respiration (i.e. respiration in the absence of exogenous ADP) was measured in isolated mitochondria. Oligomycin was added to ensure that endogenous ADP would not stimulate ATP synthase. UCP1 expression produced a large increase in the state 4 respiratory rate, expressed as nmol O/min/mg mitochondrial protein (Fig. 4b). A small, not significant increase was observed with UCP3. Of note, the maximum respiratory rate in the presence of FCCP was decreased in mitochondria containing UCP1 and UCP3L, although this decrease was not significant. When results were expressed as percentage of the FCCP-stimulated respiration (Fig. 4c), UCP3L increased the state 4 respiration significantly in comparison to that observed in empty vector and oxoglutarate carrier controls. This finding is consistent with UCP3L having uncoupling activity.

While GDP inhibited uncoupling induced by UCP1 completely, GDP at a concentration of 1 mM did not reverse the uncoupling activity of UCP3L. Since the increase in the state 4 respiration with UCP3L was small, a partial GDP inhibition cannot be ruled out. However, our data indicate that GDP does not inhibit UCP3L with an affinity similar to that for GDP inhibition of UCP1, suggesting a different regulation of UCP1 and UCP3L.

In contrast to the stronger effect of UCP3L compared to UCP1 in whole yeast cells (inhibition of the growth rate, 49% versus 32%; stimulation of O_2 consumption, 31% versus 19%),

UCP3L uncoupled the respiration in isolated mitochondria to a much lesser degree than UCP1. This discrepancy could be due to the fact that UCP1 in whole cells is inhibited by the presence of endogenous purine nucleotides, that were absent when isolated mitochondria were studied. On the other hand, it is possible that one or more unidentified endogenous activators of UCP3L are present in whole yeast but are missing under the incubation conditions of isolated mitochondria. Our contrasting findings of the effects of UCP1 and UCP3L in whole yeast cells and in isolated mitochondria, as well as the difference in GDP inhibition of the two proteins, suggest that the biochemical regulation of UCP3L is distinct from UCP1.

We also studied the double histidine mutant of UCP1 in isolated mitochondria. This mutant was shown previously by Bienengraeber et al. [23] to have a greatly reduced H^+ transport activity after reconstitution into proteoliposomes. In contrast, our measurements of the state 4 respiration in isolated mitochondria indicate a similar, only slightly smaller effect of mutant UCP1 compared to wild-type UCP1. This suggests that although the histidine pair is required for the maximum H^+ transport activity in proteoliposomes [23], it is not essential for stimulation of state 4 respiration in isolated mitochondria. Thus, a lack of this histidine pair in UCP2 and UCP3 does not by itself rule out the possibility that these carriers are uncoupling proteins.

In summary, our data indicate that UCP3L has an uncoupling activity when expressed in yeast. The mechanism and regulation of UCP3L-induced uncoupling of the mitochondrial respiration appears to be different from UCP1 since the effects of UCP1 and UCP3L in whole yeast versus isolated mitochondria are discordant. The fact that both UCP1 and UCP3L, but not the oxoglutarate carrier, have an uncoupling activity demonstrates biochemically that they belong to a subfamily of the much larger mitochondrial carrier superfamily.

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