Properties of the human long and short isoforms of the uncoupling protein-3 expressed in yeast cells

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Abstract Two splice variants of the human uncoupling protein-3 (UCP3L and UCP3S) are highly expressed in skeletal muscle. The properties of UCP3L and S have been compared to those of UCP1 in a heterologous yeast expression system under the control of the galactose promoter. Both UCP3 isoforms were found to strongly impair the coupling efficiency of respiring cells thus resulting in increased thermogenesis. The uncoupling properties of both UCP3L and S could be clearly demonstrated also in isolated yeast mitochondria both in terms of coupled respiration and in the capacity to polarize the inner membrane in conditions of limited substrate availability. Contrary to what was observed with mitochondria containing UCP1, millimolar GDP and ATP had little if any effect on the uncoupling activity of UCP3. A very marked uncoupling of whole cells and isolated mitochondria was observed at very low expression levels of UCP3S indicating that the short isoform is more active than the long one.

Key words: Uncoupling protein; Thermogenesis; Yeast; Respiration; Mitochondrion

1. Introduction

The inner membrane of mitochondria from brown fat contains an uncoupling protein (UCP1), identified more than 20 years ago [1,2], that provides a mechanism to dissipate the energy of the electrochemical proton gradient set up by the respiratory chain. The activity of UCP1 generates heat and renders the brown fat, the major organ for non-shivering thermogenesis in rodents. Recently, a few proteins have been identified that share a homology to UCP1: the ubiquitous UCP2 [3,4]; UCP3 that in human is mainly expressed in skeletal muscle [5–7]; and UCP4, a less related protein predominantly expressed in the brain [8]. UCP3 is of particular interest for the regulation of basal metabolic rate because skeletal muscle plays an important role for the regulation of thermogenesis in higher mammals [9]. The UCPs belong to the family of the anion transport proteins of the inner mitochondrial membrane. These proteins share a tripartite structure composed of three homologous segments of about 100 amino acids, each containing two transmembrane domains and a typical mitochondrial energy-transfer protein signature sequence. Two main variants of the UCP3 mRNA have been detected in human skeletal muscle where they are found in approximately equal amounts [5,10–12]. One transcript encodes the full length UCP3 (UCP3L), a protein consisting of a 312 amino acids long chain that is supposed to span 6 times the membrane with the N- and C-termini facing the inter-membrane space of mitochondria. The other UCP3 transcript, UCP3S, results from the utilization of a cleavage and polyadenylation signal located in the last intron thus terminating prematurely message elongation [13]. The encoded protein lacks the last C-terminal 37 amino acids and thus the last putative transmembrane domain. This domain is, at least in UCP1, important for purine nucleotide regulation of the activity [14]. While the potential uncoupling properties of UCP3L have been well documented [7,15–17], little is known about the functional characteristics of the truncated isoform. In this study the two isoforms have been investigated in the heterologous yeast expression system under the control of the galactose promoter and their characteristics have been compared.

2. Materials and methods

2.1. Construction of expression vectors and their introduction into yeast

The hUCP3L yeast expression vector was constructed as described previously [15]. Briefly, hUCP3L-cDNA was amplified from poly-$(A)^+$-mRNA from human skeletal muscle using two oligonucleotides (hUCP3LF-100: 5′-TCTCCTTGGACCTCCTCTCG-3′ and hUCP3-LR1124: 5′-ACGTTAGCTACCAGTGGCC-3′) derived from both the 5′ and the 3′ hUCP3L-mRNA non-coding regions. The amplified cDNA was introduced into the vector pCR2.1 (Invitrogen, Groningen, The Netherlands). In a subsequent PCR reaction with a new primer pair (hUCP3LF-*Bgl*II: 5′- GGAGATCTATGGTTGGACTG-AAGCCTTCAGACGT-3′ and hUCP3LR-*Eco*RI: 5′- GGAATTC-TTCTCAAAACGGTGATTCCCGTAACATCT-3′), the coding sequence was amplified and introduced between the *Bam*HI and the *Eco*RI recognition sites of the yeast expression vector pYeDP60. Using a different 3′ primer with homology to the 3′ end of the hUCP3S coding region (hUCP3SR-*Eco*RI: 5′-CGGAATTCACCCCTTGTA-GAAGGCTGTGGGG-3′), hUCP3S-cDNA was analogously amplified and cloned into the pYeDP60 expression vector. The coding region of hUCP1 was amplified by PCR from a pBluescriptIISK+ plasmid containing an hUCP1 insert of 945 bp (obtained from J.P. Giacobino, University of Geneva, Switzerland). The primers used for amplification contained a *Bam*HI compatible site (*Bgl*II) and a *Eco*RI site to allow insertion into the *Bam*HI/*Eco*RI linearized yeast expression vector pYeDP60 (sense hUCP1 primer: 5′-GGAAGATCTA-TAATGGGGGGCCTGACAGCCTC-3′ and antisense hUCP1 primer: 5′-GGAATTCTTATGTGGCACAGTCCATAG-3′). The PCR conditions were 94°C for 5 min and 35 cycles of 94°C for 20 s, 46°C for 1 min and 72°C for 90 s. The PCR product contained in addition to the hUCP1 coding sequence, the yeast consensus sequence ATAATG at the start codon for enhanced expression in yeast. Con-

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Abbreviations: CCCP, carbonylcyanide-*m*-chlorophenylhydrazone; UCP, uncoupling protein; TET, triethyl tin bromide; FFA, free fatty acid; BAT, brown adipose tissue; TAM, thermal activity monitor; RCI, respiratory control index; TMPD, *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylene diamine; BSA, bovine serum albumin

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structs were confirmed by double strand sequencing. These expression vectors as well as the empty pYeDP60 were transformed into the haploid yeast strain W303-B1 (*Saccharomyces cerevisiae*: Mat-a; ade2-1; his3-11, -15; leu2-3, -112; ura3-1; can^R; cyr$^+$) using the standard Li-acetate method described elsewhere [18]. Recombinant yeast was selected for uracil auxotrophy.

2.1.1. Expression of hUCP3 isoforms in yeast. Yeast cells were aerobically grown at 28°C in 0.1% casamino acids, 0.7% yeast nitrogen base, 2% lactic acid, 50 mg/l tryptophan and 40 mg/l adenine, adjusted to pH 4.5 with KOH. When the cultures reached an OD_{600} of 0.3–0.5, expression of hUCP3 isoforms was induced by the addition of 1/10 vol of 20% D-galactose, 10% yeast extract, 20% bactopeptone. The yeast cultures were analyzed and mitochondria were prepared 8 h after induction.

2.1.2. Oxygen consumption of whole cells. Respiration of yeast cells was measured at 30°C using an oxygen electrode of the Clark type (Yellow Spring Instruments, Yellow Spring, OH, USA). Washed cells were added to 3 ml of 10 mM Tris-MES (pH 6.3) supplemented with 100 mM glucose to reach a final $OD_{600} = 1.0$. After oxygen consumption reached a steady state value, triethyl tin bromide (TET) and carbonylcyanide-*m*-chlorophenylhydrazone (CCCP) were added sequentially at final concentrations of 100 μM and 10 μM, respectively.

2.1.3. Direct microcalorimetry of whole cells. Heat production from yeast cells was measured at 30°C using a TAM (Thermal Activity Monitor, Thermometric AB, Sweden) set-up to allow two measurements to be performed simultaneously (e.g. in the absence and presence of 25 μM CCCP). The buffer system was the same as for the respiration measurements but the density of the cells was lower ($OD_{600} = 0.5$). Sealed glass ampoules (volume = 3 ml) containing 100 μl of the cell suspension were introduced into the calorimeter and read against a similar volume of cell-free buffer. Heat production was recorded until steady state was reached (approximately 20 min) and ranged between 8.9 and 15.9 μW per million cells in the absence of CCCP. The effect of CCCP was expressed as % of controls.

2.1.4. Isolation of yeast mitochondria. Yeast mitochondria were prepared according to a procedure derived from Guerin et al. [19]. Briefly, spheroplasts were prepared by enzymatic digestion with 2 mg zymolyase (ICN, Luzern, Switzerland)/g wet weight yeast. After washing and homogenizing the spheroplasts with a Dounce homogenized (tight), mitochondria were fractionated by differential centrifugation. The mitochondrial pellet was washed with 600 mM mannitol, 10 mM Tris-maleate, pH 6.8, 1 mg/ml BSA and with the same medium without BSA. The final mitochondrial pellet was resuspended in reaction buffer (600 mM mannitol, 10 mM Tris-maleate, 0.2 mM EGTA and 10 mM K_2HPO_4, pH 6.8). Starting from the homogenization step, all buffers were supplemented with a protease inhibitor cocktail (*Complete* from Boehringer, Mannheim, Germany).

2.1.5. Measurements of mitochondrial respiration. Mitochondria (1 mg/ml) were incubated at 30°C in reaction buffer supplemented with 3 mM NADH and 10 μg/ml oligomycin. Oxygen consumption was monitored with a Clark type oxygen electrode. GDP (1 mM) and CCCP (1 μM) were added where indicated.

2.2. Measurements of mitochondrial membrane potential by the safranine method

Yeast mitochondria were tested at room temperature at a concentration of 1 mg protein/ml in reaction buffer supplemented with fatty acid-free BSA (1 mg/ml) and safranine-T (10 μM). Mitochondria were energized with 5 mM K-succinate in the presence of 20 μg/ml rotenone, 10 μg/ml oligomycin and 10 μM atractyloside. Absorption was measured in a 96-well microplate reader (UV max from Molecular Devices, Menlo Park, CA, USA). Based on the analysis of differential spectra of energized and deenergized mitochondria two monochromators of 530 nm and 490 nm, corresponding to a maximum and to an isosbestic point, respectively, were chosen. Aliquots of 200 μl of mitochondrial suspension were distributed into the wells containing various concentrations of malonate, GDP or vehicle and thoroughly mixed. After a 10 min incubation, the microtiter plates were read sequentially at the two wavelengths and the difference calculated. Rotenone, atractyloside, oligomycin and CCCP were prepared in DMSO. The final concentration of DMSO in all experiments was 1%, a concentration that did not affect mitochondrial membrane potential ($\Delta\Psi$).

2.2.1. Immunological detection of hUCP3. After electrophoretic separation on 13.5% SDS-polyacrylamide gels (SDS-PAGE), hUCP3 isoforms were detected using affinity purified anti-hUCP3 antibodies diluted 1000-fold (antibody AB3044 from CHEMICON International, Inc., Temecula, CA, USA) and horseradish peroxidase-linked anti-goat IgGs as secondary antibodies according to the recommendations described in the Enhanced Chemiluminescence (ECL) detection system (Amersham, Switzerland).

2.2.2. Statistical analysis. Student's unpaired *t*-test was used to test for statistical significance, which was accepted at the $P < 0.05$ level.

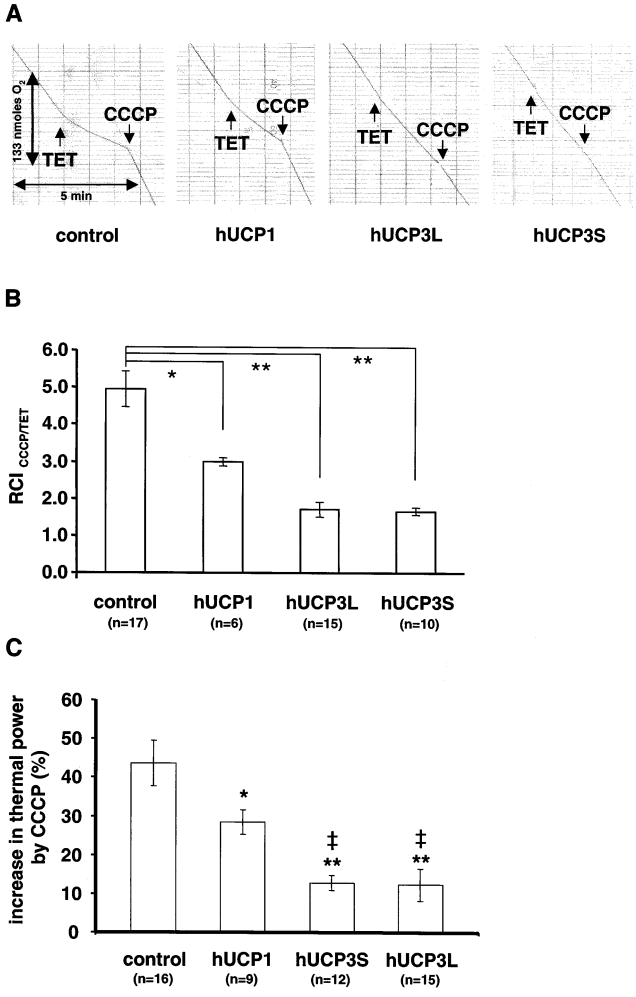

Fig. 1. Effect of the expression of UCPs on yeast cells. Respiration of intact yeast cells was analyzed using a Clark type oxygen electrode and cellular heat production was measured in a thermal activity monitor (TAM) as described in Section 2. Panel A: Original tracings illustrating the oxygen consumption of yeast cultures 8 h after galactose induction. TET (100 μM) and CCCP (10 μM) were added where indicated. Panel B: The respiratory control index (RCI), defined as the ratio between the maximal rate of respiration of cells in the presence of the uncoupler CCCP and the non-phosphorylating respiration obtained after inhibition of the ATPase by TET, was calculated from traces as those presented in panel A. Values are given as means ± S.E.M. *, $P < 0.05$; **, $P < 0.01$. Panel C: The heat production of cell cultures was measured in a TAM and the % increase obtained after challenge with the strong uncoupler CCCP (25 μM) determined. Values are given as means ± S.E.M. *, ‡, $P < 0.001$; **, $P < 0.0005$. The significance levels * and ** pertain to the comparison between the UCP expressing and the control cells; ‡ between the UCP3S or L and the UCP1 expressing cells.

3. Results

3.1. Effect of the expression of the UCP isoforms on yeast cells

Expression of UCPs in yeast was induced by the addition of galactose to the lactate containing culture. After 8 h, the respiration of whole cells was analyzed. As shown in Fig. 1A, oxygen consumption of control yeast was well coupled since it could be effectively reduced by TET (an inhibitor of oxidative phosphorylation) and strongly activated by subsequent addition of CCCP (a strong uncoupler). Respiration of UCP3S and L expressing yeast, on the other hand, was essentially uncoupled: TET had a minor inhibitory effect and the stimulation by CCCP was much less pronounced. The difference in the coupling of control and UCP3S or L yeast strains can be even better appreciated by comparing their respective respiratory control indices (RCI) as shown in Fig. 1B. The ability of a strong mitochondrial uncoupler to increase thermogenesis in yeast cells was further investigated using a microcalorimeter. Fig. 1C shows that the thermal power of control yeast could be boosted by 44% by CCCP. As already shown in a previous study [15], the level of stimulation by CCCP is indicative of the coupling state of mitochondria in the whole cell. On the other hand, only a small stimulation was observed in UCP3S and L yeast, indicating that the level of endogenous uncoupling was already very pronounced. Expression of UCP1 conferred to the yeast cells an intermediary phenotype, e.g. a modestly reduced RCI and thermogenic effect of CCCP (see Fig. 1).

Two prominent bands with apparent molecular weights of 34 kDa and 30 kDa corresponding to the expected sizes of UCP3L and UCP3S, respectively, could be visualized on immunoblots (see Fig. 2A). In spite of the identical expression strategy, which produced similar levels of mRNA (not shown), the UCP3L protein was much more abundant in whole cells (normally in the range of 20–50-fold) than UCP3S. It is possible that the shorter isoform was more susceptible to protease degradation. The antibody, however, did not detect any low molecular weight bands corresponding to degradation products of UCP3S. Both isoforms were similarly enriched (about 10–15-fold) in the mitochondrial fraction (see Fig. 2A) indicating that they were preferentially targeted to the mitochondria. A major degradation product of UCP3L of about 21 kDa was usually detected in whole cells. This product, however, did not coisolate with the mitochondria indicating that degradation occurred before insertion into the organelle. A time dependency of the expression of UCP3S and L (see Fig. 2B) was carried out and the level of uncoupling, as judged by the respiration characteristics of whole cells, was determined. Uncoupling of cell respiration was not detectable at times shorter than 1 h after induction and reached maximal levels after about 2–4 h for both UCP3S and L (not shown). Interestingly, no effect on yeast respiration was detectable at an early time point (i.e. 30 min) when the expression levels of UCP3L were comparable to those of maximal UCP3S expression, thus indicating that UCP3S has a higher specific activity in whole cells.

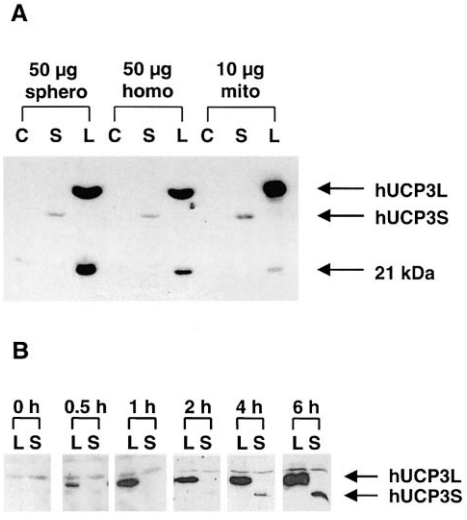

Fig. 2. Immuno detection of UCP3L and S in yeast fractions. Panel A: Yeast cells were fractionated as described in Section 2. Spheroplast (sphero), homogenate (homo) and mitochondrial (mito) proteins were separated on 13.5% SDS-polyacrylamide electrophoresis gels and the UCP3 isoforms were identified by an antibody directed against an epitope localized between the 2nd and 3rd transmembrane domains. C, S and L indicate control, UCP3S and UCP3L yeast, respectively. Panel B: The expression of UCP3L and S was investigated at different times after induction. Each lane was loaded with extract from 6 million cells.

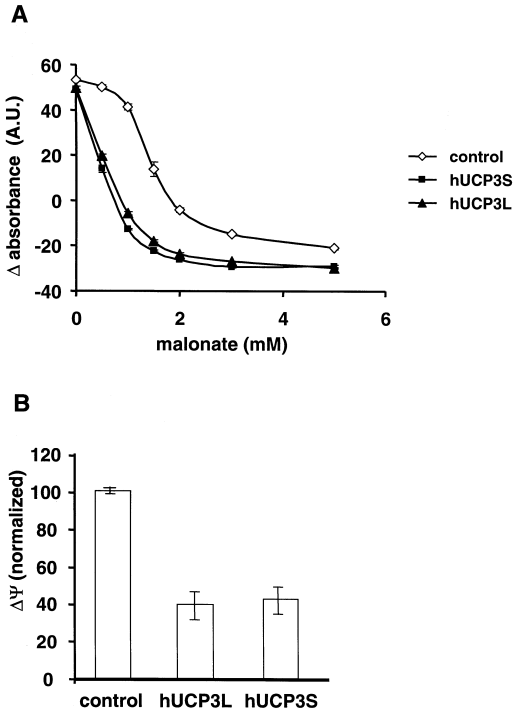

Fig. 3. Effect of UCP3L and S on the polarization of isolated mitochondria. Panel A: Mitochondria were isolated from control, UCP3L and UCP3S yeast cells and incubated at room temperature (1 mg/ml) in a reaction medium supplemented with 5 mM succinate, 1 mg/ml BSA, and in the presence of oligomycin, atractyloside, rotenone and safranine-T. Relative polarization was determined as the Δ absorbance (530–490 nm). The effect of malonate on mitochondrial polarization was determined in triplicates. Panel B: The level of polarization in the presence of 5 mM succinate and 1 mM malonate was determined in mitochondria from 10 different induction experiments. The relative polarization values are given as % of the control yeast values (means ± S.E.M., $n = 10$).

3.2. Effect of UCP expression on yeast mitochondria

The functional characteristics of mitochondria isolated from UCP3S and L expressing yeast were analyzed. The cap-

ability of mitochondria to polarize the inner membrane, when respiring on succinate as sole energy source, was evaluated by the safranine method [20]. The high level of mitochondrial membrane polarization, as judged from the difference in safranine absorbance at 530 and 490 nm, was not affected by the presence of either UCP3 isoforms when substrate supply was not limiting, indicating that mitochondria respiring under optimal substrate conditions can maintain a high level of polarization in spite of an increased proton leakage. The ability of mitochondria to maintain $\Delta\Psi$ after gradual inhibition of the succinate dehydrogenase with increasing malonate concentrations was then analyzed. This experimental approach allows detecting moderate uncoupling states of mitochondria. In fact, both UCP3 isoforms were found to induce a similar leftward shift of the malonate inhibition curves of $\Delta\Psi$ (see Fig. 3), indicating partial uncoupling.

Mitochondrial uncoupling due to overexpression of UCP1 is inhibited by purine nucleotides. Fig. 4B shows that the low respiratory control of UCP1 containing mitochondria can be significantly improved by addition of 1 mM GDP or ATP. A similar recoupling effect of GDP was observed also when analyzing the ability of the UCP1 containing mitochondria to maintain $\Delta\Psi$ in conditions of limited respiration (i.e. 5 mM succinate and 1 mM malonate; see Fig. 5B). The lowest GDP concentration tested (30 μM) had already maximal recoupling effects. Similarly to UCP1 overexpression, UCP3L also caused a clear uncoupling of mitochondrial respiration, as evidenced by the low RCI values shown in Fig. 4. Millimolar GDP or ATP, however, were completely ineffective in

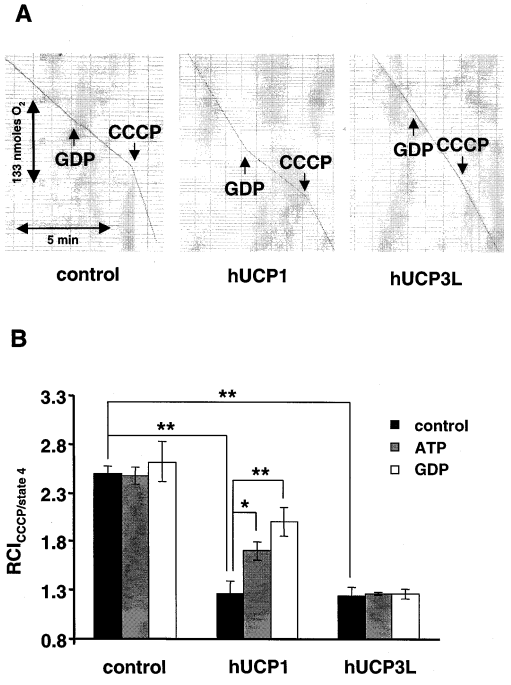

Fig. 4. Effect of purine nucleotides on the respiration of yeast mitochondria. Respiration of mitochondria (1 mg/ml) isolated from the various yeast strains was measured at 30°C in a reaction buffer containing NADH and oligomycin using a Clark electrode. Panel A: Original tracings illustrating the effect of GDP (1 mM) and CCCP (1 μM). Panel B: Evaluation of the effect of GDP (1 mM) and ATP (1 mM) on the respiratory control index (RCI) of mitochondria defined as the ratio between CCCP-stimulated respiration and state 4 respiration. Values are given as means ± S.E.M. ($n = 4$). *, $P < 0.05$; **, $P < 0.01$.

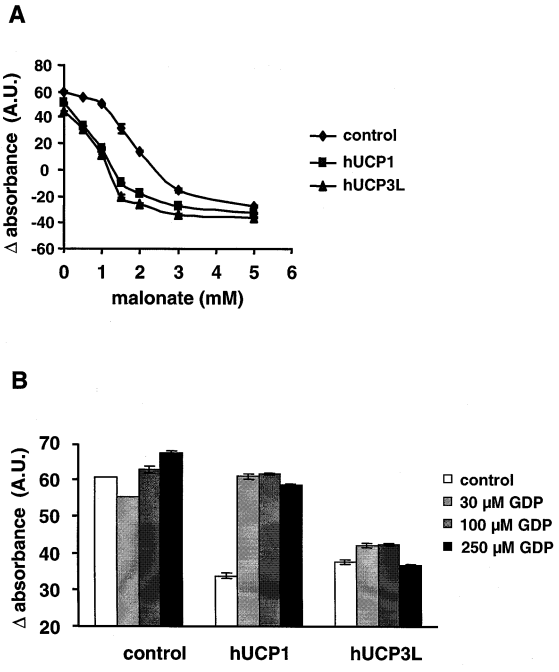

Fig. 5. Effect of GDP on the polarization of yeast mitochondria. Panel A: The polarization of mitochondria energized with succinate and the effect of malonate were measured as described in the legend to Fig. 3. Panel B: The effect of various concentrations of GDP on the polarization level observed in the presence of 1 mM malonate was determined in triplicates. Values are given as means ± S.E.M. ($n = 3$).

recoupling UCP3 expressing mitochondria (Fig. 4B shows the lack of effect on state 4 respiration and Fig. 5B on $\Delta\Psi$). The effect of GDP concentrations higher than 0.3 mM and of ATP higher than 0.05 mM could not be properly investigated in the safranine experimental approach since the polarization of control yeast mitochondria was also affected (inhibited by GDP and stimulated by ATP).

Recently, reconstitution experiments have shown that UCP1 and UCP3 need FFA for activity [21,25]. Surprisingly, the leftward shift of the malonate titration curves observed in the presence of UCP3L and S (see Fig. 3) and of UCP1 (see Fig. 5) could be observed even after addition of 1 mg/ml BSA to the reaction mixture. This observation does not necessarily imply that FFAs are not necessary for the uncoupling activity. It is possible that, in spite of the presence of BSA in the reaction medium, sufficient amounts of FFA could be retained by the mitochondria to support the uncoupling activity of UCP1 and UCP3.

4. Discussion

Several lines of evidence support the potential function of UCP3L as a mitochondrial uncoupler: Its overexpression depolarizes mitochondria in muscle-derived cell lines [16] and yeast cells [7,15,17] where it also increases mitochondrial respiration and thermogenesis [15] and the isolated recombinant protein catalyzes proton [21] transport across lipid membranes. So far, little is known about the characteristics of UCP3S, whose transcription levels in human skeletal muscle are similar to those of UCP3L [5,10–12]. Truncation of the last transmembrane domain of UCP3 could lead to major functional changes. Information necessary for targeting the

protein to the mitochondria could be lost or essential components for activity could be missing. There is some indication that the C-terminus of UCP1 could be involved in dimer formation [22], a prerequisite for activity in proteins of the mitochondrial anion transport family, or in FFA binding [23]. In addition, a short stretch of amino acids just before the 6th transmembrane domain, which is highly conserved in all known UCPs but is lacking in the truncated UCP3S, is essential for purine binding and inhibition of UCP1 activity. It is possible, therefore, that UCP3S and UCP3L could have a different susceptibility to purine nucleotide inhibition.

In this study the characteristics of UCP1, UCP3L and UCP3S were compared after expression in the heterologous yeast expression system. The uncoupling properties of all three UCPs could be demonstrated in whole yeast cells and, for the first time, a very clear effect on the membrane polarization and respiratory characteristics of isolated mitochondria could be documented. The data show that UCP3S contains sufficient information for targeting to the mitochondria. The high uncoupling activity of UCP3S implies that the C-terminus of UCP3 is not required for activity. In fact, our observations indicate that the truncated protein has a higher intrinsic activity than UCP3L, in contrast to findings in a recent publication in which both isoforms have been analyzed using a similar expression system [24]. Similar high levels of mRNA for both isoforms were found in the heterologous yeast expression system, indicating that UCP3S is more susceptible to degradation than UCP3L. Once selective and sensitive antibodies will be available, it will be possible to verify the protein expression levels of UCP3L and S in skeletal muscle.

UCP1 activity in isolated mitochondria could be inhibited, as expected, by GDP and ATP. On the other hand, we failed to see an inhibition of UCP3S (not shown) and of UCP3L (see Fig. 4B) by purine nucleotides. In contrast to our findings, a recent publication [25] has shown that translocation of chloride anions catalyzed by recombinant UCP1 and UCP3L reconstituted in proteoliposomes is inhibited by micromolar concentrations of purine nucleotides. The proteins utilized in that study, however, were only partially refolded, as no proton translocating activity of UCP1 and UCP3 could be detected, and could therefore display an abnormal purine nucleotide sensitivity. In fact, in agreement with our findings, another group [21] has very recently succeeded in demonstrating a proton translocating activity of UCP3L reconstituted in proteoliposomes that is very poorly inhibited even by millimolar purine nucleotide concentrations. The lower affinity for purine nucleotides could explain why the UCP3 isoforms were much more effective than UCP1 in uncoupling respiration in whole cells (see Fig. 1) even though no, or only small differences in the respective isolated mitochondria were observed in the absence of nucleotides.

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