Uncoupling Proteins 1 and 3 Are Regulated Differently†

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ABSTRACT: Using a heterologous yeast expression system, we have previously found a marked discordance between the effects of uncoupling protein (UCP) 1 and UCP3L on basal O_2 consumption in whole yeast versus isolated mitochondria. In whole yeast, UCP3L produces a greater stimulation of basal O_2 consumption, while in isolated mitochondria, UCP1 produces a much greater effect. As shown previously and in this report, UCP3L, in contrast to UCP1, is not inhibited by purine nucleotides. In the present study, we addressed two hypothetical mechanisms that could account for the observed discordance: (i) in whole yeast, purine nucleotides inhibit UCP1 but not UCP3L and (ii) preparations of isolated mitochondria lack an activator of UCP3L that is normally present in vivo. By use of a mutant of UCP1 that lacks purine nucleotide inhibition, it is demonstrated that cytosolic concentrations of purine nucleotides present in yeast effectively inhibit UCP1 activity. This suggests that the lower activity of UCP1 compared to UCP3L in whole yeast is due to purine nucleotide inhibition of UCP1 but not UCP3L. As potential activators of UCP3L we tested free fatty acids in whole yeast and isolated mitochondria. While UCP1 was strongly activated by free fatty acids, no stimulatory effect on UCP3L was observed. In summary, this study indicates that UCP1 and UCP3L differ in their regulation by purine nucleotides and free fatty acids. This different regulation may be related to different physiological functions of the two proteins.

Uncoupling protein 1 (UCP1)[1] is a mitochondrial carrier protein expressed exclusively in brown adipose tissue. It uncouples mitochondrial respiration from ATP synthesis by mediating H^+ transport through the inner mitochondrial membrane (*1*, *2*), thus dissipating the mitochondrial transmembrane H^+ gradient and producing heat. UCP1 is believed to play an important role in thermogenesis in rodents but not in humans, in whom brown adipose tissue mass is limited.

The biochemical characteristics of UCP1, including inhibition by purine nucleotides that bind from the cytosolic side of the inner mitochondrial membrane and activation by micromolar concentrations of free fatty acids, have been studied in both isolated mitochondria and UCP1 proteoliposomes. However, it is less clear to what extent purine nucleotides and free fatty acids modulate UCP1 activity in vivo.

Two mechanisms by which free fatty acids activate H^+ transport have been suggested. According to the first model, the fatty acid carboxyl group acts as a H^+ donor in the translocation channel, compensating for the absence of resident H^+ donor/acceptor groups at critical positions in the UCP1 translocation channel (*2*, *3*). The second model, termed fatty acid protonophore model, suggests that UCP1 transports fatty acid anions rather than protons (*1*, *4*−*6*). Net H^+ influx is accomplished through fatty acid cycling, i.e., flip-flop of the protonated electroneutral fatty acid through the inner mitochondrial membrane, release of H^+ in the mitochondrial matrix, and outward transport of the fatty acid anion by UCP1. Strong arguments, which are mainly derived from studies on UCP1 proteoliposomes, have been made for and against both models. It was shown that various fatty acid derivatives, which are unable to flip-flop through lipid bilayers, can successfully activate UCP1-mediated H^+ transport in proteoliposomes (*2*, *3*). UCP1-mediated H^+ transport facilitated by these fatty acid analogues would therefore not require fatty acid cycling. Supporting the fatty acid protonophore model is the observation that alkylsulfonates, which are strong acids that cannot flip-flop, are transported by UCP1 and compete with fatty acid-activated H^+ transport (*6*, *7*).

Two other closely related members of the mitochondrial carrier family that are highly homologous to UCP1 have been identified, UCP2 (*8*, *9*), which is widely expressed, and UCP3 (*10*−*12*), which is expressed preferentially in skeletal muscle and brown adipose tissue. In human, two transcripts for UCP3 have been found, UCP3L and UCP3S (*10*, *13*). The predicted protein encoded by UCP3S is identical to UCP3L but lacks the last 37 C-terminal residues. Evidence for uncoupling activity and information on the regulation of these new homologues of UCP1 is mainly derived from studies using yeast and mammalian expression systems (*8*, *9*, *14*−*17*). These measurements include UCP2- and UCP3-induced decreases in mitochondrial membrane potential, as assessed with potential-sensitive fluorescent dyes in whole cells, and UCP-induced increases in basal O_2 consumption in whole yeast and isolated mitochondria. In addition, it was recently demonstrated that UCP2 and UCP3 mediate fatty acid activated H^+ transport in proteoliposomes, and this H^+ transport is weakly inhibited by purine nucleotides (*18*). It

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[1] Abbreviations: UCP, uncoupling protein; EDTA, ethylenediaminetetraacetic acid; BSA, bovine serum albumin; FCCP, carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone.

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was suggested that UCP2- and UCP3-mediated H^+ transport occurs according to the fatty acid protonophore model (*18*).

When heterologously expressing rat UCP1 and human UCP3L in yeast, we have previously found a discordance between the effects of UCP1 and UCP3L on respiration in whole yeast (in vivo) versus isolated mitochondria (in vitro) (*16*). When measuring the increase in basal O_2 consumption compared to control yeast carrying empty vector or expressing the mitochondrial oxoglutarate carrier (a mitochondrial carrier without uncoupling activity), UCP3L had a markedly greater effect in whole yeast compared with UCP1. In contrast, in isolated mitochondria, UCP3L was much less active compared to UCP1. Also, unlike UCP1, UCP3L appeared to be insensitive to inhibition by GDP.

In this study, we investigated two potential explanations for the observed discordance between UCP1 and UCP3L. To assess whether purine nucleotide concentrations normally present in the cytosol are sufficient to suppress UCP1 activity and therefore reduce activity of UCP1 in vivo, a UCP1 mutant, previously shown in proteoliposomes to lack purine nucleotide inhibition (*19, 20*), was expressed in yeast. The uncoupling activity of this mutant in isolated mitochondria and whole yeast was compared to that of wild-type UCP1. In addition, it was tested whether free fatty acids are activators of UCP3L in whole yeast and isolated mitochondria. The results of this study confirm that UCP1 and UCP3L are regulated differently and that this is likely accounted for, at least in part, by differential effects of purine nucleotides and free fatty acids.

EXPERIMENTAL PROCEDURES

Materials. All chemicals were of highest available purity and obtained from Sigma, except for zymolyase (ICN Biomedicals) and phenylundecanoic acid (Acros Organics). Palmitic and phenylundecanoic acid (free acids) were dissolved in dimethyl sulfoxide and added in volumes of 1−2 μL.

Expression Vectors and Mutagenesis. Constructs of rat UCP1 and human UCP3L in the pYES2 expression vector (Invitrogen, Carlsbad, CA) were prepared as previously described (*16*). The PCR primers used were designed to introduce *Hin*dIII and *Xba*I sites close to the start and stop codons, respectively. The R276L mutation in UCP1 and R282L mutation in UCP3L were generated by site-directed mutagenesis (QuikChange site-directed mutagenesis kit, Stratagene, La Jolla, CA). In UCP1, the codon CGA for Arg^{276} was changed into CTA (Leu). In UCP3L, the codon CGT for Arg^{282} was changed into CTT (Leu). All sequences were verified by DNA sequencing. Carrier gene expression is under the control of the *gal10*−*cyc1* promoter. The *Saccharomyces cerevisiae* strain INVSC1 (Invitrogen) was transformed with these vectors. Empty pYES2 vector was transformed as a negative control.

Expression of the UCPs. Yeast transformants were selected onto SC plates lacking uracil. Single colonies were inoculated into a preculture grown in synthetic complete medium without uracil (SC-ura) to an OD_{600} of approximately 3. The yeast were diluted to a final OD_{600} of 0.04 in 500 mL of SC-ura medium with 3% lactate and no glucose and grown at 30 °C with vigorous shaking. After approximately 24 h, 1% galactose was added and the cells were harvested after 8−12 h.

Analysis of Expression of UCP1 and UCP3L by Immunoblotting. The expression of UCP1 was measured with rabbit anti-mouse UCP1 IgG fraction obtained from RDI Research Diagnostics (Flanders, NJ). The expression of UCP3L was detected with rabbit anti-human UCP3 IgG fraction, prepared against a peptide representing residues 147−166 in UCP3L.

Whole Yeast O_2 Consumption. O_2 consumption of whole yeast was measured as previously described (*16*). Briefly, basal O_2 consumption and maximum O_2 consumption induced with 2.5 μM 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 3% lactate SC medium.

Isolation of Yeast Mitochondria and Polarography. Mitochondria were prepared as previously described (*16*). 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 0.1% bovine serum albumin that was omitted during the last washing step. 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 of mitochondrial protein/mL.

Statistical Analysis. Statistical analysis was performed using StatView 4.0 (Abacus Concept, Berkeley, CA). All results are presented as the mean ± SE. Student's *t* test was used to evaluate the statistical significance of differences, as indicated in the figure legends.

RESULTS

Whole yeast basal O_2 consumption in yeast expressing UCP3L was highly increased compared to the control, as illustrated in Figure 1, indicating increased H^+ leak. Expression of UCP1 resulted in a much smaller increase in whole yeast basal O_2 consumption.

In contrast, in isolated mitochondria, the increase in basal respiration of mitochondria bearing UCP3L was small compared to that of mitochondria bearing UCP1 (Figure 2). Basal mitochondrial respiration, measured in the absence of added ADP and in the presence of oligomycin, is mainly due to H^+ leakage through the inner mitochondrial membrane and is therefore a measure of uncoupling activity. Basal mitochondrial respiration was expressed as percent of the fully uncoupled rate in the presence of FCCP, corresponding to the maximum respiratory capacity, to account for day-to-day variation of mitochondrial preparations.

Figure 2 also demonstrates that increasing the pH of the incubation medium from 6.8 to 7.4 resulted in an increase in basal respiration in control yeast and yeast bearing UCP3L and UCP1. However, increasing the pH had a much greater effect on UCP3L and UCP1 than on control yeast. Basal respiration in control yeast mitochondria was increased from 27% ± 2% at pH 6.8 to 34% ± 4% at pH 7.4, basal respiration with UCP3L was increased from 40% ± 1% to 58% ± 1%, and basal respiration with UCP1 from 65% ± 4% to 92% ± 4%. This indicates that both UCP3L and UCP1 are more active in yeast mitochondria at pH 7.4 compared to pH 6.8.

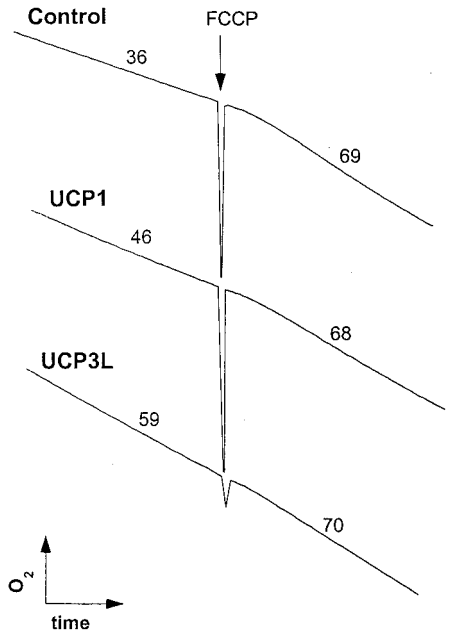

FIGURE 1: Whole yeast basal O_2 consumption in yeast expressing UCP1 and UCP3L. Whole yeast O_2 consumption was measured as described under Experimental Procedures. The addition of 2.5 μM FCCP and the respiratory rates [in nanomoles of O min^{-1} (10^8 cells)$^{-1}$] are indicated.

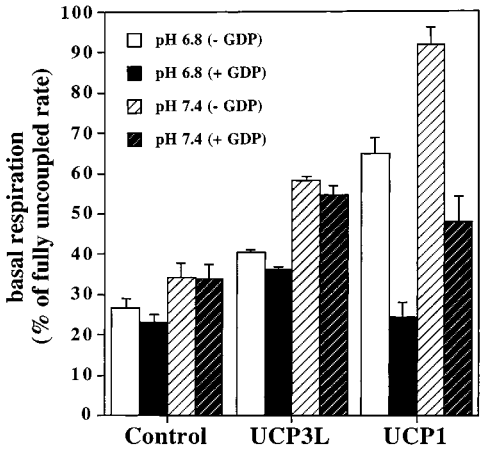

FIGURE 2: Basal respiration of isolated mitochondria at pH 6.8 and 7.4. Basal respiration was measured as descibed under Experimental Procedures in the presence of 3 mM NADH and 2.5 μg/mL oligomycin and in the absence of added ADP. The pH and the absence or presence of 1 mM GDP are indicated in the figure. The data represent the average of three independent experiments. The increases in basal respiration with UCP3L and UCP1 compared to control at pH 6.8 and at pH 7.4 are statistically significant with $p < 0.01$. The increase in basal respiration at pH 7.4 compared to pH 6.8 is not significant in the control and significant with UCP3L ($P < 0.005$) and with UCP1 ($P < 0.05$).

Figure 2 shows a lack of GDP inhibition of UCP3L. Similarly, GTP, ADP, and ATP at concentrations of 1 mM did not inhibit basal respiration of yeast mitochondria bearing UCP3L in the presence of oligomycin (data not shown).

To investigate whether the smaller effect of UCP1 on basal respiration in whole yeast compared to UCP3L is due to inhibition of UCP1, but not UCP3L, by cytosolic purine nucleotides in vivo, a mutant of UCP1 (mUCP1), carrying the R276L mutation, was generated and expressed in yeast. It has previously been shown that this mutation impairs nucleotide inhibition of UCP1 (discussed below).

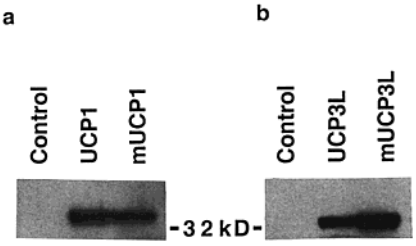

FIGURE 3: Immunoblot analysis of the expression of wild type and mutant UCP1 (a) and UCP3L (b) in isolated mitochondria. Forty micrograms of protein was used for analysis in each lane.

Expression levels of wild-type UCP1 and mutant UCP1 were compared by immunoblot analysis of isolated yeast mitochondria (Figure 3a). Expression of mUCP1 was slightly lower compared to that of wild-type protein. A 20-fold enrichment of UCP1 and mUCP1 in the mitochondrial fraction compared to whole yeast was observed (data not shown), indicating that the proteins were targeted to mitochondria. Similar expression levels of UCP1 and UCP3L were demonstrated previously (*16*).

It was shown by Modriansky et al. (*19*), when using H^+ transport measurements in UCP1 proteoliposomes, that the R276L mutant of UCP1 lacks purine nucleotide inhibition while fatty acid-activated H^+ transport was unaffected. To confirm these findings in isolated mitochondria, the basal respiratory rate of isolated yeast mitochondria in the absence of ADP and presence of oligomycin was measured (Figure 4). The respiratory rates in Figure 4a show that UCP1 and mUCP1 increased basal respiratory rate to a similar degree compared to the control. While addition of 1 mM GDP reversed the basal respiratory rate of mitochondria bearing UCP1 almost completely to the rate observed in control mitochondria, 1 mM GDP had very little effect on yeast mitochondria bearing mUCP1. The average of the results of three independent experiments are presented in Figure 4b. UCP1 and mUCP1 increased the basal respiratory rate similarly by 85% and 84%, respectively. While 1 mM GDP inhibited uncoupling activity of UCP1 completely, mUCP1 was inhibited by only 26%.

To determine whether UCP1 is inhibited in vivo by the cytosolic concentrations of purine nucleotides present in yeast, whole yeast basal O_2 consumption was measured (Figure 5). Expression of UCP1 resulted in a 12% $\pm$ 2% increase in whole yeast basal O_2 consumption compared to control yeast, while expression of mUCP1 resulted in a 33% $\pm$ 6% increase. This indicates that yeast cytosolic purine nucleotides, presumably ATP and ADP, effectively inhibit UCP1 activity. The increase in whole yeast basal O_2 consumption with mUCP1 was similar to that observed with UCP3L (35% $\pm$ 6% increase).

The residue equivalent to the Arg^{276} in UCP1 is also present in UCP3L. Given the high homology between UCP1 and UCP3L, any purine nucleotide inhibition of UCP3L, if it exists, is likely to involve the same residues. To further investigate purine nucleotide inhibition of UCP3L by purine nucleotides present in the cytosol, a mutant of UCP3L (R282L), equivalent to the R276L mutant of UCP1, was generated. Expression of mutant UCP3L (mUCP3L) did not further increase whole yeast basal O_2 consumption compared to wild-type UCP3L. The increase in whole yeast basal O_2 consumption with mUCP3L (22% $\pm$ 4% increase compared to control yeast) was actually somewhat smaller than that

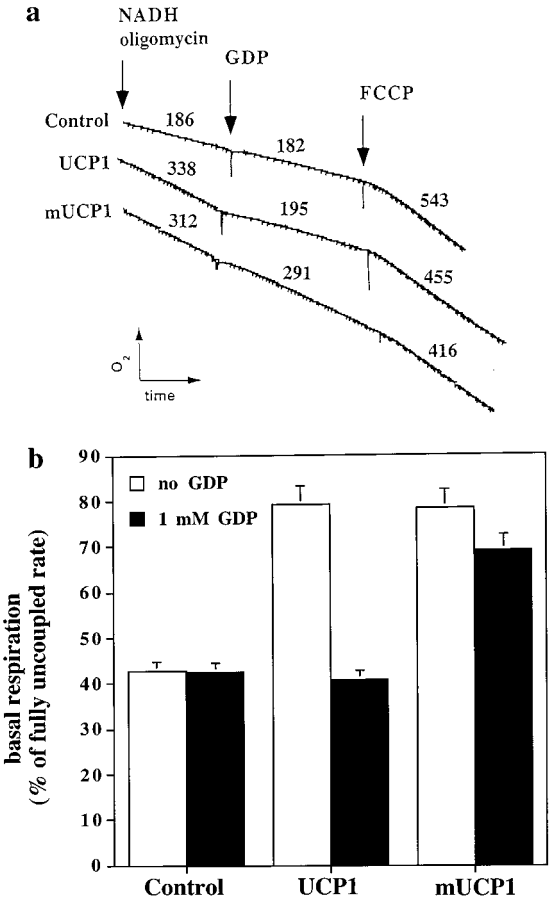

FIGURE 4: Basal respiration in mitochondria isolated from yeast expressing UCP1 and mUCP1. Mitochondrial respiration was measured as in Figure 2. (a) Respiratory tracings of one typical experiment. The addition of 1 mM GDP and 2.5 μM FCCP and the respiratory rates in nanomoles of O min^{-1} of (mg mitochondrial protein)$^{-1}$ are indicated. (b) Summary of basal respiratory rates of UCP1 and mUCP, expressed as percent of fully uncoupled respiration ($n = 3$).

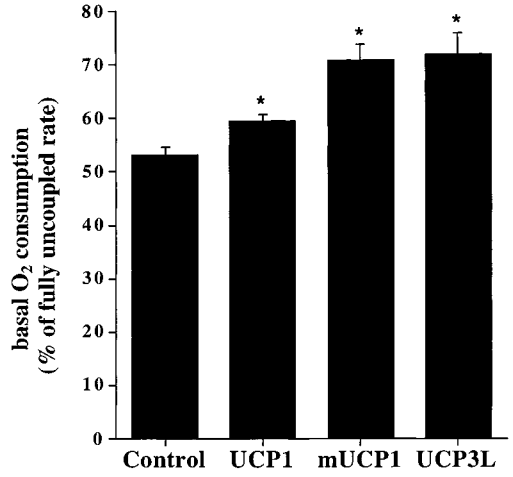

FIGURE 5: Whole yeast basal O_2 consumption of yeast expressing mUCP1. Whole yeast basal O_2 consumption was measured as described under Experimental Procedures. Fully uncoupled respiration was measured in the presence of 2.5 μM FCCP. Asterisks indicate that all differences except that between mUCP1 and UCP3L are statistically significant with $P < 0.01$ ($n = 7$).

observed with wild-type UCP3L (30% ± 6% increase) (Figure 6), despite a higher expression level of mUCP3L in yeast mitochondria (Figure 3b). This indicates that UCP3L

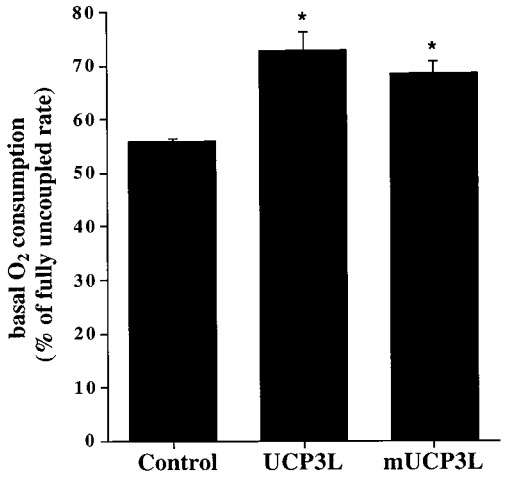

FIGURE 6: Whole yeast basal O_2 consumption of yeast expressing mUCP3L. Whole yeast basal O_2 consumption was measured as in Figure 5. Asterisks indicate that the increases in basal respiration with UCP3L and mUCP3L compared to the control are statistically significant with $P < 0.005$ ($n = 5$). The difference between UCP3L and mUCP3L is not significant.

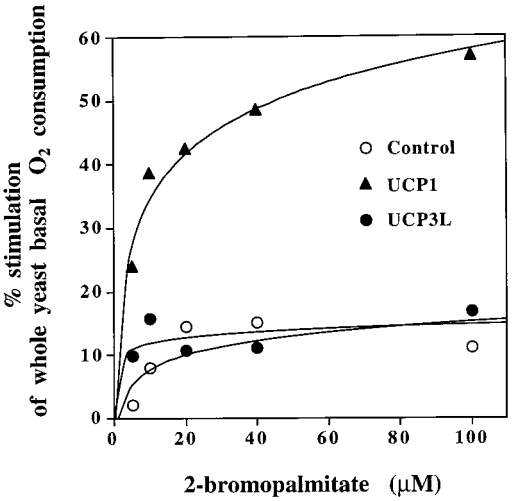

FIGURE 7: Dose−response curve for 2-bromopalmitate-induced stimulation of whole yeast basal O_2 consumption.

is not inhibited by purine nucleotides present in the yeast cytosol.

To investigate activation of UCP1 and UCP3L by free fatty acids in whole yeast, we used 2-bromopalmitate, a fatty acid derivative that is not metabolized. Thus, any observed effect on whole yeast basal O_2 consumption cannot be attributed to utilization of fatty acids as substrate. Addition of 2-bromopalmitate led to a dose-dependent increase in basal O_2 consumption in whole yeast expressing UCP1 (Figure 7). A similar bromopalmitate-induced increase in whole yeast basal O_2 consumption was observed in yeast bearing mUCP1 (data not shown). In contrast, bromopalmitate at a concentration up to 100 μM did not lead to an increase in basal respiration in yeast bearing UCP3L compared to control yeast.

To determine activation of UCP1 and UCP3L by free fatty acids in isolated mitochondria, yeast mitochondria were isolated in the presence of 2% BSA to deplete mitochondria of free fatty acids. Addition of 10 μM palmitate had very little effect on control mitochondria but increased basal respiration in mitochondria bearing UCP1 by 55% (Figure 8). A second addition of 10 μM palmitate did not lead to further stimulation (not shown), indicating that 10 μM

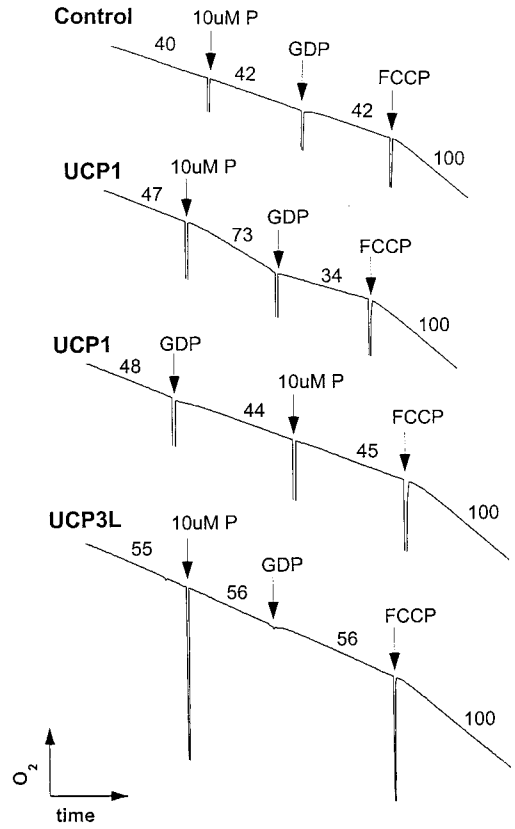

FIGURE 8: Effect of palmitate on basal respiration in isolated mitochondria. Mitochondria were isolated and resuspended in 2% BSA-containing medium to deplete mitochondria of free fatty acids. Immediately before the assay was started, mitochondria were centrifuged and resuspended in BSA-free medium. Mitochondrial basal respiration was measured as in Figure 2. The addition of 10 μM palmitate (P), 1 mM GDP, and 2.5 μM FCCP is indicated. Respiratory rates are given as percent of the fully uncoupled rate.

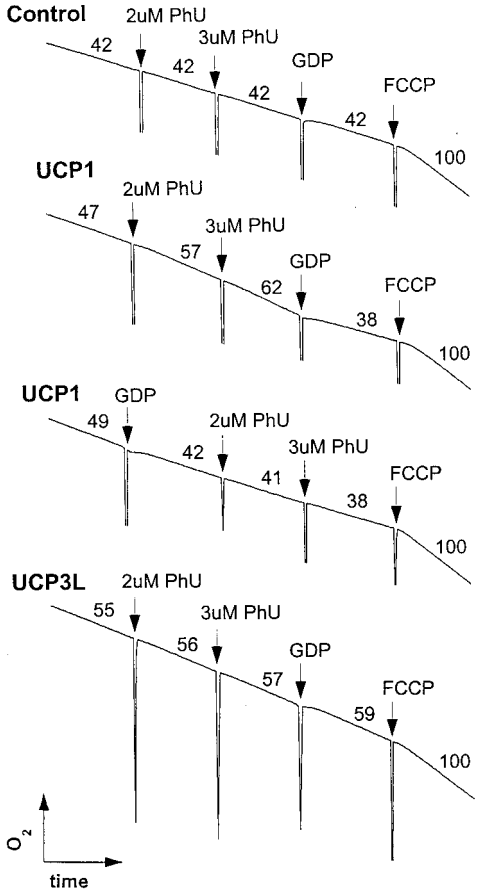

FIGURE 9: Effect of phenylundecanoate on basal respiration in isolated mitochondria. The experimental conditions are as in Figure 8. The addition of phenylundecanoate (PhU), 1 mM GDP, and 2.5 μM FCCP is indicated.

palmitate is sufficient for activation of UCP1. Addition of 1 mM GDP completely reversed the palmitate-induced stimulation (Figure 8). When GDP was added first, palmitate did not stimulate basal respiration. Palmitate was found to have no effect on mitochondria bearing UCP3L, even when the mitochondria were isolated in the presence of 2% fatty acid-free BSA.

Similarly, basal respiration of yeast mitochondria from yeast expressing UCP1 was stimulated by phenylundecanoate, a fatty acid derivative believed to be unable to flip-flop through the mitochondrial membrane (Figure 9). Phenylundecanoate at a concentration of 5 μM had no effect on the control, UCP1, if GDP was present, nor on UCP3L (Figure 9). When comparing activation of UCP1 by the two fatty acids, stimulation of basal respiration by phenylundecanoate was about half compared to stimulation by palmitate (Figure 10). Both fatty acids had only a very small effect when GDP was present.

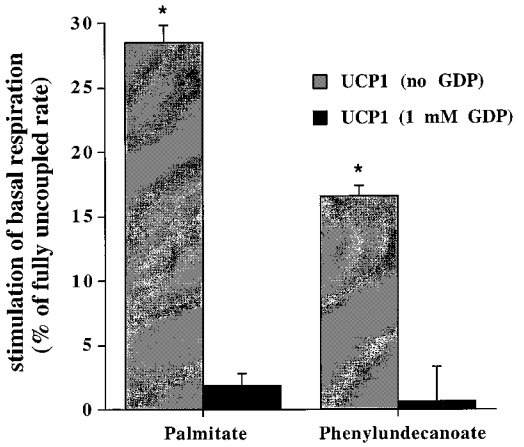

FIGURE 10: Comparison of the activation of UCP1 by palmitate and phenylundecanoate. The effect of 10 μM palmitate and 5 μM phenylundecanoate on basal respiration of yeast mitochondria bearing UCP1 in the presence or absence of GDP was measured as in Figures 8 and 9. Asterisks indicate $P < 0.005$ compared to the respiratory rates in the presence of GDP ($n = 4$).

DISCUSSION

There is increasing evidence that the recently identified uncoupling protein homologues UCP2 and UCP3 have uncoupling activity. However, relatively little is known about the biochemical regulation of UCP2 and UCP3. In a previous report, using a heterologous expression system, we found that the uncoupling activity of UCP3L in whole yeast was greater compared to that of UCP1 (*16*). In contrast, in isolated mitochondria, UCP3L was much less active than UCP1. This discordance between in vivo and in vitro effects suggests different regulation of UCP1 and UCP3L. The goal of this study was to determine which regulatory features of UCP1 and UCP3L may be responsible for the observed discordance.

For UCP1, it has been shown that fatty acid-activated H^+ transport increases with increasing pH in the range between

6.8. and 7.8 (*21*). In addition, sensitivity of UCP1 for purine nucleotide inhibition decreases with increasing pH (*22*−*25*). Both the increase in uncoupling activity and the decrease in sensitivity to purine nucleotide inhibition were also observed in this study in yeast mitochondria bearing UCP1. A significant increase in activity at pH 7.4 compared to pH 6.8 was also observed for UCP3, while the increase in the control was much smaller and not significant. These results indicate that the activity of UCP3L is modulated by the pH. Similar results have recently been reported for UCP2 (*17*).

A well-known regulatory characteristic of UCP1 is inhibition by purine nucleotides, such as GTP, GDP, ATP, and ADP. Purine nucleotides bind from the cytosolic side of the inner mitochondrial membrane with dissociation constants in the low micromolar range (*2*). The results in Figure 2 indicate that the H^+ transport activity of UCP3L is inhibited only weakly or not at all by purine nucleotides. Similarly, Jaburek et al. (*18*) have shown recently that UCP2- and UCP3-mediated H^+ transport in proteoliposomes, compared to UCP1-mediated H^+ transport, is much less sensitive to purine nucleotide inhibition. The K_i values of GTP, GDP, and ATP were 1−2 orders of magnitude higher compared to UCP1. In UCP1, several residues that are critical for purine nucleotide inhibition have been identified by use of covalently interacting nucleotide derivatives and site-directed mutagenesis, as reviewed in ref *2*. These residues include three arginines, Arg^{83}, Arg^{182}, and Arg^{276}, located in the second, fourth, and sixth transmembrane domains, respectively, which were shown to be essential for purine nucleotide inhibition (*19*). Interestingly, these residues are also present in UCP3. The low sensitivity of UCP3 for inhibition by purine nucleotides indicates that although these residues are critical, they are not sufficient for high sensitivity to purine nucleotide inhibition.

We investigated whether purine nucleotide inhibition of UCP1, in vivo, is responsible for the lower activity compared to UCP3L. For this purpose, the R276L mutant of UCP1, previously shown in proteoliposomes to lack purine nucleotide inhibition but retain full uncoupling activity (*19*), was expressed in yeast. Our study demonstrates that in isolated mitochondria, the R276L mutant has uncoupling activity similar to that of wild-type UCP1 but is poorly inhibited by purine nucleotides. Highly reduced sensitivity of mUCP1 to inhibition by cytosolic purine nucleotides led to a greater uncoupling activity in whole yeast similar to that observed with UCP3L. This suggests that UCP1, in vivo, is inhibited by purine nucleotide concentrations, presumably ATP and ADP, normally present in the cytosol and suggests strongly that purine nucelotide inhibition plays an important role in the physiological regulation of UCP1 activity.

In contrast, mutation of the equivalent arginine residue in UCP3L into leucine (R282L) actually decreased the activity of UCP3L, although this decrease was not significant. Similarly, we have previously observed reduced activity of the R282C mutant of UCP3L (*26*), suggesting that this residue may be important for uncoupling function of UCP3L. Given the high homology of UCP1 and UCP3L, any purine nucleotide inhibition of UCP3L is likely to involve a similar binding site with equivalent critical residues. Therefore, our result suggests that UCP3L is not inhibited by purine nucleotides present in the cytosol. Absent or weak purine nucleotide inhibition of UCP3L may be responsible for the much greater effect of UCP3L on whole yeast basal O_2 consumption compared to UCP1.

The discordance between the strong effect of UCP3L in vivo and weak effect in vitro led to the hypothesis of a missing activator present in vivo but absent in vitro when isolated mitochondria are studied. Given the requirement of free fatty acids for UCP1-mediated H^+ transport, free fatty acids as potential activators of UCP3L were studied in whole yeast and isolated mitochondria. It was previously shown that free fatty acids increase the basal O_2 consumption of isolated brown adipocytes, presumably by increasing mitochondrial H^+ permeability through activation of UCP1 and simultaneously by serving as fuel for mitochondrial β-oxidation (*27*). The cytosolic free fatty acid concentration was raised by addition of norepinephrine, theophylline, or dibutyryl cyclic AMP or direct addition of fatty acids such as palmitate. In the present study, 2-bromopalmitate, which has been shown to be an activator of UCP1 in proteoliposomes (*2*) and in yeast expressing UCP1 (*28*), was used to stimulate UCP1 in whole yeast and to assess whether UCP3L can also be activated by this free fatty acid analogue. Since 2-bromopalmitate is a nonmetabolizable fatty acid derivative, the observed effects on O_2 consumption cannot be due to an increase in mitochondrial β-oxidation. Micromolar concentrations of bromopalmitate stimulated whole yeast basal O_2 consumption in yeast expressing UCP1. In contrast, no activation of UCP3L by bromopalmitate was observed. In isolated mitochondria depleted of free fatty acids, UCP1 could be reactivated by addition of 10 μM palmitate. In yeast mitochondria bearing UCP3L, palmitate had no stimulatory effect on basal respiration. This result is consistent with a recent study by Rial et al. (*17*), who found no stimulation by palmitate in isolated yeast mitochondria from yeast expressing UCP2 and UCP3.

In contrast to these findings, Jaburek et al. (*18*) reported recently that UCP2- and UCP3-mediated H^+ transport, measured in UCP-proteoliposomes, requires the presence of free fatty acids, such as palmitate. The failure of bromopalmitate to activate UCP3 in vivo, as observed in our study, could possibly be due to the fact that UCP3 has already maximal activity and therefore cannot be further activated by an increase in the cytosolic concentration of free fatty acids. In isolated mitochondria, the absence of any effect of palmitate excludes the possibility that the presence of sufficient free fatty acid concentrations in vivo and their absence in vitro accounts for the observed discordance of UCP3L effects. However, it does not rule out a requirement of free fatty acids for UCP3-mediated H^+ transport. The lack of stimulation of UCP3L by palmitate in isolated mitochondria could also be due to the absence of another required activator in isolated mitochondria or the possibility that small amounts of free fatty acids, sufficient to activate UCP3, persist in vitro despite the addition of albumin. In conclusion, while our data do not rule out the possibility that small amounts of fatty acids activate UCP3L, they indicate that UCP3L is most likely not regulated by cellular free fatty acid levels. The discordant findings with respect to free fatty acid activation of UCP2 and UCP3 also emphasize that studies in proteoliposomes and in isolated mitochondria are complementary and that both are required for a thorough understanding of biochemical function and regulation of uncoupling proteins.

Similarly to palmitate, a phenyl-substituted fatty acid derivative, phenylundecanoate, also activated UCP1, but was without effect on UCP3L. Phenyl-substituted fatty acids, on the basis of their presumed inability to flip-flop through lipid bilayers, have previously been used in proteoliposomes to assess the mechanism of action of UCP1. When phenyl-substituted fatty acid derivatives have been used to activate UCP1-mediated H^+ transport in proteoliposomes, different groups have obtained different results. Phenyl-substituted fatty acids were found to activate H^+ transport to a similar degree as palmitate (*2*, *3*), while in a different study no activation was observed (*29*). Our results show for the first time that a phenyl-substituted fatty acid can activate UCP1 in isolated mitochondria. Our findings, i.e., the lack of activation of UCP3 by free fatty acids and the activation of UCP1 by phenylundecanoate, would be in contrast to the fatty acid protonophore model for uncoupling proteins, which suggests that uncoupling proteins are fatty acid anion transporters promoting fatty acid cycling through the inner mitochondrial membrane (*1*, *4*, *18*). However, it cannot be ruled out that small amounts of free fatty acids are required for UCP3 to function. Furthermore, although phenyl-substituted fatty acid derivatives have been shown to be unable to flip-flop through lipid bilayers as studied in liposomes (*30*) and isolated mitochondria (*31*), Kamp and Hamilton (*32*) found that closely related fatty acids and fatty acids with even larger attached ring groups do undergo rapid flip-flop. Of interest, in one study it was recently reported that fatty acids are not essential for UCP1 function, although they increase its uncoupling activity (*33*). It was concluded that this finding is inconsistent with UCP1 functioning as a fatty acid anion transporter.

The findings of this study indicate striking regulatory differences between UCP1 and UCP3. In contrast to UCP1, UCP3 is inhibited only weakly or not at all by purine nucleotides and is not activated by free fatty acids at concentrations where activation of UCP1 can be observed. An activator different from free fatty acids may be responsible for the greater in vivo effect of UCP3. Different biochemical regulation of UCP1 and UCP3 may also reflect the different physiological functions of these two proteins. The main physiological function of UCP1 is adaptive thermogenesis. The increase in free fatty acids upon noradrenergic stimulation of brown adipose tissue is believed to lead to activation of UCP1. Although a role of UCP3 in adaptive thermogenesis and regulation of energy expenditure has been suggested, evidence for such a function is only limited and some data are inconsistent with this idea. For instance, fasting, known to cause a decrease in resting metabolic rate, results in a paradoxical upregulation of UCP3 in skeletal muscle (*12*, *14*). UCP3-mediated uncoupling of mitochondrial respiration could also be significant for purposes other than production of heat, such as reducing the production of reactive oxygen species (*34*) or possibly modulating rate of ATP synthesis by altering the protonmotive force, the driving force for ATP synthesis. Such functions can be expected to be transcriptionally and biochemically regulated differently from UCP-mediated heat production.

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