Physiological Levels of Mammalian Uncoupling Protein 2
Do Not Uncouple Yeast Mitochondria*

We assessed the ability of human uncoupling protein 2 (UCP2) to uncouple mitochondrial oxidative phosphorylation when expressed in yeast at physiological and supraphysiological levels. We used three different inducible UCP2 expression constructs to achieve mitochondrial UCP2 expression levels in yeast of 33, 283, and 4100 ng of UCP2/mg of mitochondrial protein. Yeast mitochondria expressing UCP2 at 33 or 283 ng/mg showed no increase in proton conductance, even in the presence of various putative effectors, including palmitate and all-trans-retinoic acid. Only when UCP2 expression in yeast mitochondria was increased to 4 µg/mg, more than an order of magnitude greater than the highest known physiological concentration, was proton conductance increased. This increased proton conductance was not abolished by GDP. At this high level of UCP2 expression, an inhibition of substrate oxidation was observed, which cannot be readily explained by an uncoupling activity of UCP2. Quantitatively, even the uncoupling seen at 4 µg/mg was insufficient to account for the basal proton conductance of mammalian mitochondria. These observations suggest that uncoupling of yeast mitochondria by UCP2 is an overexpression artifact leading to compromised mitochondrial integrity.

Uncoupling protein 1 (UCP1) uncouples brown adipose tissue mitochondria, causing physiologically important, hormonally regulated, thermogenic proton cycling across the inner membrane. The functions of the UCP1 homologues, UCP2 and UCP3 (1–4), are currently uncertain (5–12). They have been demonstrated to uncouple mitochondrial oxidative phosphorylation in a number of experimental models, including proteoliposomes (13), yeast heterologous expression systems (1, 2, 14–16), and transgenic mice (17). It is clear that, under some environmental or physiological condition (19–21).

We have demonstrated that expression of UCP1 in yeast mitochondria can cause a nonspecific uncoupling that is not due to protein activity per se (22, 23). This uncoupling artifact is present only at higher levels of UCP1 expression. At these levels, UCP1 expression in yeast also interferes with mitochondrial substrate oxidation. Similarly, Heidkamper et al. (24) have concluded that both UCP1 and UCP3 can be expressed in an incompetent form that interferes with ATP production. They suggest that most of the UCP3 expressed in yeast mitochondria is nonfunctional. There is considerable evidence that, under some expression regimes, a substantial proportion of the UCP1 expressed in yeast mitochondria is in fact not functional (23). In experiments with mammalian models, Cadenas et al. (18) showed that transgenic mice overexpressing UCP3 in skeletal muscle mitochondria (17) have lower state 3 rates of succinate oxidation. These observations suggest that UCP expression has compromised mitochondrial function in ways not related to uncoupling. This raises the question of whether the observed uncoupling following UCP2 or UCP3 expression might also be an artifact of expression and not represent a significant native activity of the protein. This is especially of concern because the amount of UCP2/UCP3 expressed in yeast mitochondria has not been quantified.

Recently, information has become available regarding the levels of UCP2 that are found in mammalian mitochondria. Here we use this information, and three different yeast heterologous expression systems that yield different amounts of UCP2, to assess the effects of physiological, and supraphysiological, levels of UCP2 expression in yeast mitochondria. We relate the different levels of UCP2 expression to measured proton conductance and attempt to distinguish between native UCP2 activity and expression artifact.

EXPERIMENTAL PROCEDURES

Expression of UCP2 in Escherichia coli—Human UCP2 was expressed in E. coli, where it accumulated as inclusion bodies that were subsequently harvested and used as a semipurified source of UCP2 with which to calibrate UCP2 expression levels in yeast mitochondria. A PCR product for hUCP2 was made from a human mRNA library provided by Dr. Jan Digby (Department of Clinical Biochemistry, University of Cambridge, Cambridge, United Kingdom), and its sequence was verified. It was ligated into XbaI and EcoRI restriction sites of the pET expression vector pMW172 (25). Competent C41 strain E. coli were transformed with either pET-UCP2 or the empty pET vector. Cultures were incubated in TB media with 100 µg/ml ampicillin at 37 °C at 250 rpm until the A600 reached 0.5–0.6. Expression of UCP2 was induced by addition of 1 mM isopropyl-β-D-thiogalactopyranoside. After 2 h cells were harvested by centrifugation at 3000 × g for 15 min. All centrifugation was performed at 4 °C.

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gation steps were carried out at 4 °C. Cell pellets were stored at −85 °C.

Cells were lysed in B-PER reagent (Pierce) for 10–15 min at room temperature, centrifuged at 27,200 × g for 15 min and resuspended in B-PER containing 200 μg/ml lysozyme for 5–10 min to lyse any remaining cells. Incubation bodies were harvested by centrifugation at 27,200 × g for 30 min. The resulting pellet was washed three times by resuspending the pellet into a buffer containing 150 mM potassium phosphate, 25 mM EDTA, 1 mM dithiothreitol, 1 mM ATP, pH 7.8 (13), and centrifugation at 27,200 × g. The final pellet was solubilized in 1.5% n-lauryl sarcosine for 45 min at room temperature. Insoluble material was removed by centrifugation at 27,200 × g for 15 min. The supernatant (solubilized UCP2 inclusion bodies) was harvested and stored at −85 °C.

Parity of Solubilized UCP2 Inclusion Bodies—Solubilized UCP2 inclusion bodies were electrophoresed on 19-cm 12% SDS-polyacrylamide gels for 2 h at 370 V (Fig. 1a). UCP2 content was assessed with three different stains over a range of protein loadings. For Coomassie Brilliant Blue R250 staining, protein loaded per lane was 2–20 μg (Fig. 1a). For staining with silver (Bio-Rad) and SYPRO Orange (Bio-Rad), 0.1–1.0 μg of protein was loaded. Gels were dried overnight and then scanned using a Scanner 12 USL (Microtek) scanner. Band intensities were quantified using NIH Image 1.60 (available via FTP).

The UCP2 content of inclusion bodies was quantified by comparing the UCP2 signal either to the signal obtained with bovine serum albumin (fraction V, assumed 90% pure) or to the total protein signal obtained using (27) sequence (ACCATGG) was present at the initiation ATG. Precultures of yeast transformed with pYES2 constructs were grown overnight in selective lactate (SL) media (28) (2% 600 of between 1.0 and 1.5. Yeast cells were harvested by centrifugation at 2500 × g for 5 min at room temperature, resuspended in Milli-Q grade water, recentrifuged, then subjected in buffer containing 100 mM Tris-HCl and 20 mM dithiothreitol, pH 9.3, and resuspended for 10 min at 30 °C. The cells were recentrifuged, washed twice in buffer containing 100 mM Tris-HCl and 500 mM KCl, pH 7.0, and resuspended in 5 ml of isotonic spheroplasting buffer (40 mM citric acid, 120 mM disodium hydrogen orthophosphate, 1.35 mM sorbitol, 1 mM EGTA, pH 5.8). Lyticase was added at 3 mg/ml, and the cells were incubated at 30 °C for exactly 30 min. Subsequent steps were at 4 °C. Spheroplasts were pelleted, washed twice in 40 ml of buffer containing 10 mM Tris-maleate, 0.75 mM sorbitol, 0.4 mM mannitol, 2 mM EGTA, 0.1% bovine serum albumin, pH 6.8, then resuspended in buffer containing 100 mM Tris-maleate, 0.65 mM mannitol, 2 mM EGTA, pH 6.8, then resuspended in a small volume of this buffer and assayed for protein content (31).

Respiration with NADH as Substrate—Respiration was measured at 30 °C 1.5% (v/v) ethanol in the presence of the artificial electron carrier (N,N,N′,N′-tetramethyl-p-phenyleneediamine (TMPD) was used as a well defined respiratory substrate whose oxidation could be titrated conveniently. The oxygen electrode was fitted with a methylenephosphonophosphon (TMP)-sensitive electrode to allow simultaneous measurements of membrane potential (32) and oxygen consumption (33). The potential was equal to the leak rate divided by the β’/O ratio of 4.0).

The dependence of oxygen consumption rate on membrane potential gives the kinetic response of the proton leak to its driving force. The proton conductance at each membrane potential can be read from the membrane potential were measured. Membrane potentials were calculated from TPMP concentrations outside the mitochondria, as described in Ref. 32, assuming a TPMP binding correction of 0.4 (μl/mg)−1. A different TPMP binding correction would affect all the measured values of membrane potential but would not significantly affect our conclusions.

Statistics—Means were compared using Student’s t test.
RESULTS

Expression Levels of UCP2 in Yeast Mitochondria—The levels of UCP2 expression in mitochondria isolated from transfected yeast were determined by Western blot using solubilized UCP2 inclusion bodies as calibration standards (Fig. 1). The two antibodies (C-14 and N-19) gave virtually identical results. UCP2 was expressed in yeast mitochondria at three levels that ranged from 33 ng/mg of mitochondrial protein in UCP2low yeast to 4.1 μg/mg in UCP2high yeast (Table I).

UCP2low Yeast—Induction of UCP2 expression in UCP2low yeast had no effect on yeast growth rates. The mean doubling time of UCP2low yeast in selective galactose medium was 1.83 ± 0.06 h, compared with 1.81 ± 0.03 h (S.E., n = 6) in paired controls grown under identical conditions.

Respiration rates with NADH as substrate, both coupled and uncoupled with FCCP, were not different between UCP2low mitochondria and their paired controls (Table II). Palmitate (50 μM) stimulated respiration equally in UCP2low and control mitochondria (data not shown).

The proton leak kinetics were determined in mitochondria isolated from UCP2low yeast and paired controls (Fig. 2a). Over the range of membrane potentials (driving force for proton leak), no differences in proton conductance were observed.

UCP2mid Yeast—Induction of UCP2 expression in UCP2mid yeast had no effect on growth. The doubling time of UCP2mid yeast growing in exponential phase following induction was 1.78 ± 0.06 h, compared with 1.77 ± 0.06 (S.E., n = 6) for paired controls.

Respiration rates with NADH as substrate, both coupled and uncoupled with FCCP, were not different in mitochondria isolated from UCP2mid yeast and paired controls (Table II). Palmitate (50 μM) and all-trans-retinoic acid (45 μM, buffer, pH 7.3) stimulated respiration equally in UCP2mid and control mitochondria (Fig. 3, a and b).

The proton leak kinetics of UCP2mid mitochondria were similar to control mitochondria (Fig. 2b). UCP2mid mitochondria had identical, or perhaps slightly lower, proton conductance than controls at all measured values of membrane potential.

UCP2high Yeast—Induction of UCP2 expression in UCP2high yeast significantly inhibited growth rate in the exponential phase. The doubling time of UCP2high yeast in exponential phase following induction with 1% D-galactose was 4.1 ± 0.1 h, compared with 2.6 ± 0.1 h (S.E., n = 7) for paired controls.

Mitochondria isolated from UCP2high yeast had significantly higher rates of respiration with NADH as substrate, slightly (not significantly) lowered FCCP uncoupled rates and significantly lowered respiratory control (Table II and Fig. 3c). GDP did not inhibit respiration with NADH as substrate in UCP2high mitochondria or their paired controls (Fig. 3c). 3 mM GDP slightly stimulated respiration in UCP2high mitochondria (Fig. 3c), perhaps through the nucleotide inducible proton conductance pathway (33). Our assay conditions were designed to minimize the proton leak through this pathway (33), but may not have abolished it entirely.

UCP2high yeast mitochondria had altered proton leak kinetics (Fig. 2c). At all measured membrane potentials, proton conductance was greater in UCP2high mitochondria. Membrane potentials and oxygen consumption rates can be compared for each concentration of TMPD; UCP2high mitochondria achieved a lower membrane potential, but did not respire faster than control mitochondria. Increased proton conductance normally lowers membrane potential and stimulates respiration (34). Thus, substrate (ascorbate/TMPD) oxidation was impaired in UCP2high mitochondria. Indeed, fully FCCP-uncoupled rates of NADH oxidation in UCP2high mitochondria became progressively impaired as the time between UCP2 induction and mitochondrial isolation increased (Fig. 4). This was also apparent when the oxidized substrate was ascorbate/TMPD (data not shown).

DISCUSSION

UCP2 Expression Levels in Mammalian Mitochondria—Antibodies to UCP2 are typically able to detect the presence of the protein expressed in yeast mitochondria, but the same antibodies often fail to detect UCP2 in mitochondria from mammalian
TABLE I
UCP2 expression levels in mitochondria isolated from yeast containing UCP2 expression constructs

| Yeast expression construct | Induction regime | UCP2 expression level (ng UCP2/mg mitochondrial protein) |
|---------------------------|-----------------|--------------------------------------------------------|
| UCP2low                   | Overnight 2% b-galactose | 32.5 ± 3.3                                                |
| UCP2mid                   | Overnight 2% b-galactose | 283 ± 22                                                  |
| UCP2high                  | 4-h 1% b-galactose | 4066 ± 1274                                               |

TABLE II
Respiration with NADH as substrate in mitochondria isolated from yeast containing UCP2 expression constructs

Values are in nmol of O/min/mg of mitochondrial protein, and represent means ± S.E. of five to seven separate experiments with two or three different transformants for each construct. Respiratory control ratio (RCR) = (NADH + FCCP rate)/NADH rate.

|          | NADH | NADH + FCCP | RCR |
|----------|------|-------------|-----|
| UCP2low  | 165 ± 23 | 1311 ± 245 | 7.9 ± 0.8 |
| Paired control | 161 ± 24 | 1256 ± 202 | 7.8 ± 0.4 |
| UCP2mid  | 290 ± 64 | 1849 ± 317 | 6.9 ± 0.6 |
| Paired control | 284 ± 39 | 1878 ± 151 | 6.8 ± 0.4 |
| UCP2high | 501 ± 54* | 1351 ± 81 | 2.8 ± 0.1* |
| Paired control | 260 ± 34 | 1522 ± 157 | 6.0 ± 0.4 |

* Significantly different from paired control (p < 0.01).

Fig. 2. Proton leak kinetics of mitochondria isolated from the three yeast UCP2 expression constructs. a, UCP2low and paired control; b, UCP2mid and paired control; c, UCP2high and paired control. For details, see "Experimental Procedures." Values are means ± S.E. of three to five separate experiments with two to three yeast transformants.

Thus, this amount of UCP2 expressed in yeast mitochondria did not uncouple respiration.

We increased UCP2 expression in yeast mitochondria by an order of magnitude (UCP2mid) by removing a 5'-untranslated nucleotide region that was present in the UCP2low plasmid, and inserting a Kozak sequence (27) around the initiation ATG (see "Experimental Procedures"). UCP2 expression in mitochondria isolated from these yeast was 283 ng/mg of protein, similar to levels reported for spleen mitochondria (35, 37). However, no increase in proton conductance was observed in mitochondria isolated from UCP2mid yeast. Rates of NADH oxidation in the coupled, and FCCP-uncoupled, states were unaffected by this level of UCP2 expression. Two putative effectors of UCP2 activity, palmitate (13) and all-trans-retinoic acid (at pH 7.3) (16), failed to stimulate UCP2 activity. Thus, when expressed at 283 ng/mg of protein, approximately equal to the highest level measured in mammalian mitochondria, UCP2 did not uncouple yeast mitochondria.

Is Our Assay Sensitive Enough to Detect Uncoupling by Physiological Concentrations of Active UCP2?—When UCP1 was expressed in yeast at levels (900 ng/mg of protein) similar to
UCP2mid (283 ng/mg), specific palmitate-activated and GDP-inhibitable mitochondrial uncoupling was readily measurable using our experimental methods (22, 23). If UCP2 had similar uncoupling activity, then it would have been readily observed under our experimental conditions. As it was not seen, we conclude that the native uncoupling activity of UCP2 expressed in yeast mitochondria is less than that of UCP1 (or indeed zero). The specific activity of UCP1 in yeast mitochondria is comparable to its native activity in brown adipose tissue mitochondria, indicating good insertion and folding of the protein to give a native, functional molecule (22, 23). There is no reason to suppose that, when expressed at similar levels, UCP2 is not also folded correctly in the yeast system.

Effect of Supraphysiological Concentrations of UCP2 on Proton Leak in Yeast Mitochondria—UCP2 caused increased proton conductance in yeast only when expressed at about 4 μg/mg of mitochondrial protein. This level is more than an order of magnitude higher than in UCP2mid yeast and mouse spleen mitochondria. Mitochondria isolated from UCP2high yeast were partially uncoupled. This uncoupling was apparent in the proton leak kinetics and the rates of coupled respiration of mitochondria isolated from UCP2high yeast compared with paired controls. Growth rates of UCP2high yeast were also impaired.

From Fig. 2c, the increased respiration due to UCP2 in UCP2high yeast mitochondria was 300 nmol of O/min/mg of protein at 160 mV. The proton cycling rate caused by UCP2 expression was about 1.2 μmol of H+/min/mg of yeast mitochondrial protein at 160 mV. As UCP2high yeast mitochondria have 4 μg of UCP2/mg of protein, the specific activity of UCP2 in these yeast mitochondria can be calculated as 1.2/4, or 0.3 μmol of H+/min/μg of UCP2, at 160 mV. The proton cycling due to UCP2 activity (assayed under identical conditions) in UCP2mid yeast mitochondria with 300 ng of UCP2/mg of mitochondrial protein should be 90 nmol of H+/min/mg of mitochondrial protein. Given a H+/O ratio for TMPD respiration of 4, the additional respiration attributable to UCP2 activity in these mitochondria should be 22.5 nmol of O/min/mg of protein. However, no increase in respiration was observed in
UCP2mid mitochondria, suggesting that the uncoupling seen in UCP2high mitochondria was probably not due to UCP2 activity per se, but rather to an artifactual effect of high protein expression.

When UCP1 was expressed in yeast mitochondria at levels (−11 μg/mg of protein) similar to those of UCP2 in UCP2high, an artifactual proton conductance that was insensitive to GDP was observed (22, 23). This uncoupling is not attributable to native UCP1 function, which is fully inhibitable by GDP. The artifactual GDP-insensitive proton conductance caused by 11 μg/mg UCP1 was nearly twice the proton conductance caused by 4 μg/mg UCP2 (Fig. 5), as shown by the roughly doubled respiration rate at the same membrane potential of 145 mV. Thus the uncoupling caused by high expression of UCP2 was quantitatively almost the same as the artifactual uncoupling caused by the same quantity of UCP1, strongly suggesting that the uncoupling caused by UCP2 was also an artifact of high expression.

In UCP2high mitochondria, a secondary effect of UCP2 expression was observed that could not be attributed simply to an uncoupling activity; mitochondrial substrate oxidation was inhibited. This can be seen in the proton leak kinetics (Fig. 2c); increased proton conductance should increase oxygen consumption at any concentration of substrate (34). UCP2high mitochondria were uncoupled, but at each TMPD concentration they did not respire faster. It can also be seen in fully FCCP uncoupled rates of NADH oxidation (Fig. 4), which decreased as UCP2 expression was increased by longer periods of galactose induction. A similar effect occurs at high levels of UCP1 (22, 23) (Fig. 5). Inhibition of substrate oxidation similarly accompanies UCP2 or UCP3-mediated uncoupling in a number of studies where the UCP1 homologues have been overexpressed (15, 16, 18, 24). There is no reason related to a simple uncoupling activity for this to occur, and it suggests that mitochondrial integrity was compromised at this level of UCP2 expression.

The uncoupling observed in UCP2high yeast mitochondria was insensitive to GDP. UCP1 is fully inhibited by millimolar concentrations of nucleotides (38), and it has been reported that homologues of UCP1 share this property (13, 24, 39, 40). The inability of GDP to inhibit proton conductance in UCP2high yeast mitochondria is in agreement with evidence from mammalian mitochondria. Even high concentrations of nucleotides do not lower the proton conductance of rat muscle mitochondria in which UCP2 and UCP3 are present (41, 42). In brown adipose tissue from UCP1 knockout mice, UCP2 and UCP3 mRNA rise but basal proton leak remains insensitive to nucleotides (39, 44).

If the Uncoupling Measured in UCP2high Mitochondria Was a Native Function of UCP2, Could It Make a Major Contribution to Proton Cycling in Mammalian Mitochondria?—Levels of UCP2 have been reported for spleen, lung, and stomach mitochondria. As calculated above, these are 313, 78, and 31 ng of UCP2/mg of protein, respectively. The rates of proton cycling that would be catalyzed by these amounts of UCP2 are 94, 23, and 9 nmol of H+/min/mg of protein. UCP2 protein is undetectable in heart, muscle, liver, brain, and brown adipose tissue mitochondria (35). UCP2 levels in these tissues are, therefore, lower than the lowest measurable amounts of 30 ng/mg, or indeed perhaps zero. Based on the uncoupling seen in mitochondria from UCP2high yeast, proton cycling due to UCP2 would, in mitochondria from these tissues, be less than 9 nmol of H+/min/mg of protein. In comparison, the proton cycling rates measured in mitochondria from rat liver, brain, and muscle (corrected to 30 °C assuming a Q10 of 2) are about 21, 84, and 240 nmol of H+/min/mg of protein (45). Thus, UCP2-catalyzed proton cycling might constitute less than 4% of the total rate observed in muscle, less than 11% of the rate in brain, and less than 43% of the rate in liver (where in fact UCP2 expression is believed to be restricted to Kupffer cells) mitochondria. In all cases, these are, of course, upper limits to the UCP2 contribution to proton cycling.

Only in mitochondria from spleen could the observed activity of UCP2 from mitochondria of UCP2high yeast play a significant role in proton conductance, even assuming that this activity is not artifactual. However, under standard conditions of proton leak assay, there is no evidence that spleen mitochondria have greater proton conductance than kidney, liver, or muscle mitochondria.2

Similar calculations can be made for the proton leak rate caused by UCP2 in proteoliposomes (13), with a reported Vmax of 10–30 μmol of H+/min/mg of UCP2, or about 20 nmol of H+/min/μg of UCP2. From the data reported in Ref. 13, the proteoliposome membrane potential can be estimated to be about 150 mV during the measurements. If the UCP2-dependent proton leak seen in proteoliposomes was a native function of the protein, it would account for only 20 nmol of H+/min/mg of protein, or about 6 nmol of H+/min/mg of protein, of proton cycling in spleen mitochondria. This is comparable to the leak through the phospholipid bilayer (46) and not great enough to be the major contributor to proton cycling in mammalian mitochondria.

From both sets of calculations, it is obvious that, even if the proton cycling observed in mitochondria from UCP2high yeast is a true activity, it is not great enough to account for the basal proton leak observed in mitochondria. It may, or may not, catalyze an inducible, regulated, leak, as does UCP1.

In summary, UCP2 uncouples yeast mitochondria only at supraphysiologial levels of the protein, when symptoms of mitochondrial damage appear. When expressed in yeast mitochondria at levels similar to those found in mitochondria from mouse tissues, UCP2 does not uncouple. The increased proton conductance caused by high levels of UCP2 expression is GDP-insensitive and insufficient to explain the basal proton conductance of mammalian mitochondria, although UCP2 could catalyze an inducible leak pathway. The uncoupling caused by UCP2 expression in yeast probably represents compromised mitochondrial function, and not a native UCP2 activity. This may apply also to other UCP1 homologues.

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