The bulk of UCP3 expressed in yeast cells is incompetent for a nucleotide regulated H^+ transport

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Abstract The impact of uncoupling protein (UCP) 1, UCP3 and UCP3s expressed in yeast on oxidative phosphorylation, membrane potential and H^+ transport is determined. Intracellular ATP synthesis is inhibited by UCP3, much more than by UCP1, while similar levels of UCP3 and UCP1 exist in the mitochondrial fractions. Measurements of membrane potential and H^+ efflux in isolated mitochondria show that, different from UCP1, with UCP3 and UCP3s there is a priori a preponderant uncoupling not inhibited by GDP. The results are interpreted to show that UCP3 and UCP3s in yeast mitochondria are in a deranged state causing uncontrolled uncoupling, which does not represent their physiological function.

Key words: Uncoupling protein; Mitochondria; Saccharomyces cerevisiae; H^+-transport; Membrane potential; Oxidative phosphorylation

1. Introduction

Uncoupling proteins (UCP) are supposed to uncouple oxidative phosphorylation [1]. They are localised in the inner mitochondrial membrane and there rechannel H^+ pumped by the respiration chain. Whereas this concept is experimentally well established for UCP1 in brown adipose tissue, for the UCP variants occurring in other tissues, UCP1–4 and BCMP-1 [2], convincing experimental evidence for their function as H^+ transporters is lacking despite recent claims to the contrary, as will be discussed below. One reason is the low abundance of these new UCP variants in their respective tissues whereas UCP1 could be isolated in high amounts from brown adipose tissues. Therefore the need for heterologous overexpression for the UCP variants was even more important than for UCP1.

For the recombinant expression of the UCPs, *Saccharomyces cerevisiae* has been a preferred host. First UCP1 from brown adipose tissue was introduced into yeast cells and was quite abundantly incorporated in yeast mitochondria, without negatively influencing the growth of the yeast cells [3–6]. This expression system was extensively used as a vehicle for elucidating the structure–function relationship in UCP1 by directed mutagenesis [6–13]. Following this lead, the discoveries of UCP variant genes, UCP2, UCP3 and BCMP [14–19], also initiated their expression in *S. cerevisiae* [17–21]. The uncoupling was measured by fluorescent activating cell sorting (FACS) using a fluorescent probe for the mitochondrial membrane potential. In yeast cells expressing UCP2 and 3, a stronger decrease of membrane potential was reported than in cells expressing UCP1 [17–19]. However, it cannot be decided a priori whether the observed downshift of the fluorescence maximum in the FACS profile is caused by a decrease of the membrane potential, i.e. an uncoupling effect, or merely by a decrease of the amount of mitochondria [19]. Further the uncoupling function of UCP2 and UCP3 was investigated at the level of isolated mitochondria from yeast [20–24]. Also with UCP1 and UCP3 reconstituted from inclusion bodies in *Escherichia coli* no [25] or only a low and barely nucleotide-sensitive H^+ transport was obtained [26]. In our view, the emerging picture of UCP3 function is far from clear if one applies rigorous criteria for uncoupling protein such as a regulated H^+ transport.

Here we scrutinise the uncoupling and the H^+ transport by UCP3 expressed in yeast using experimental approaches not yet applied. As a reference we use the well studied UCP1 expression in yeast cells. At variance with other authors we conclude that, different from UCP1, UCP3 and UCP3s incorporated into yeast mitochondria exist largely in a deranged and non-physiological state which allows uncontrolled H^+ transport. Only traces of UCP3 are in the native conformation with regulated uncoupling.

2. Materials and methods

The gene for UCP1 for hamster was cloned in the pEMBL Lyex4 vector under the control of the gal 10-cy1 promoter as described [11]. Similarly the gene for human UCP3 and of UCP3s was introduced into this vector. Further full-length coding sequences of human UCP3 and UCP3s were cloned into the vector pYES2 (Invitrogen) as described previously [27]. They were also cloned into the *Bgl*II restriction enzyme site of the high expression vector pGAL110 [28].

Yeast cells were grown in selective lactate medium with 0.67% yeast nitrogen base, 2% lactic acid, 0.05% glucose, with amino acids and adenine at 40 mg/l each, pH 5.5. Galactose was added to a final concentration of 0.4% at 11 h after start of the culture. Mitochondria were isolated as described [11].

Oxidative phosphorylation in yeast cells was measured according to a method used for yeast cells carrying mutant ADP/ATP carrier [29]. In brief, cells were starved by aeration. To deplete ATP the washed cells were made anaerobic by flushing with N_2 under addition of 1% ethanol for 30 min. ATP synthesis was started by bringing the sus-

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Abbreviations: UCP, uncoupling protein; FACS, fluorescent activating cell sorting; $disC_3$, 3,3′-dipropylthiacarbocyanine; LA, lauric acid; GP, 1-glycerophosphate; CCCP, carbonylcyanide *m*-chlorophenylhydrazone

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pension in a thermostated (10°) syringe and with fast stirring under O_2; aliquots were withdrawn typically after 10, 20, 30, 60 and 120 s and extracted with $HClO_4$ for the enzymatic ATP determination.

Mitochondria were isolated from yeast cells according to the procedures specified previously [11,30]. Spheroplasts from yeast cells were generated as for the isolation of mitochondria and permeabilised by nystatin according to [31]. For the immunoblots of mitochondria, polyclonal antibodies were used, raised in rabbits against whole UCP1 and against the 14 residue long peptide of UCP3 S^{149}IHLGPSRSDRKYS^{162}.

Measurements of membrane potential in mitochondria were performed with the fluorescent cyanine dye 3,3′-dipropylthiacarbocyanine ($disC_3$) obtained from Molecular Probes. A highly sensitive fluorometer constructed in our workshop was used, with internal stirrer and a quartz fibre conducting the emitted light directly from the cuvette to the photomultiplier which had a special red-sensitive photocathode S22. Exciting light was at 620 nm, emitting light with a cutoff filter RG 645 (Schott) at >650 nm. For stabilising the signal, part of the incident light was diverted by a beam splitter and monitored by a photodiode which regulated the dynode voltage of the measuring photomultiplier for compensating the light fluctuations of the xenon arc lamp. Mitochondrial H^+ influx was measured according to the previously detailed procedure [12].

3. Results

UCP3 was introduced into yeast cells by various vectors which employ the galactose promoter. Besides the normal ('long') form of UCP3 (UCP3), the short spliced variant UCP3s was expressed in which the last 37 residues of UCP3 are omitted [28]. Fig. 1 shows that induction of human hUCP3 and hUCP3s decreases the growth rate. As a control we used cells with the 'empty' pYES2 vector. The generation time with pYES2 is only 3.1 h, with pYES2–hUCP3 5.4 h, and with pYES2–UCP3s 4.9 h. Other variations of the cell growth were tested, such as changing the carbon source to raffinose, varying the induction time and the amount of inducing galactose. Whereas the growth rate and expression levels changed, there was no principal change of those properties of the UCP3-containing cells and mitochondria, which are characterised in the following.

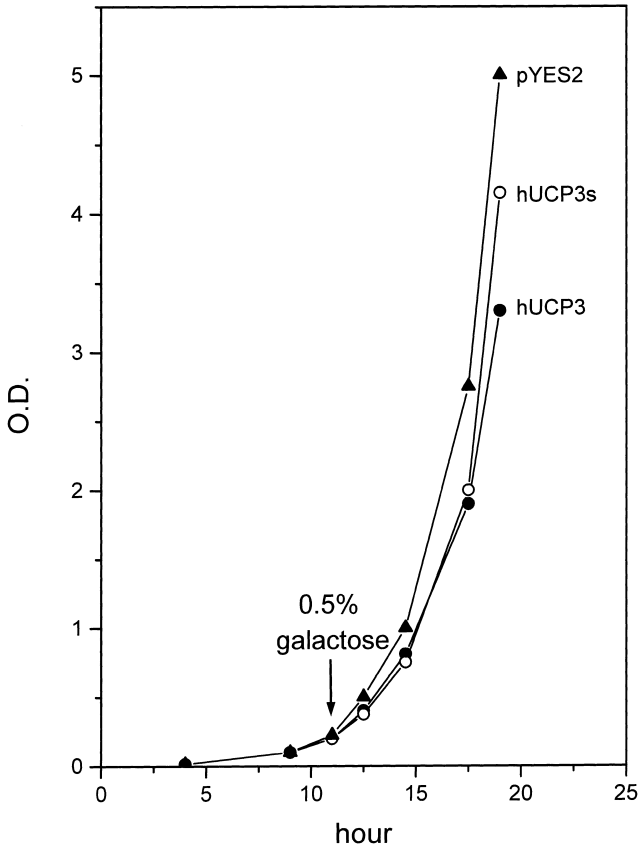

Fig. 1. The growth curves of yeast cells transfected with human UCP3 and with the short form hUCP3s. Aerobic growth in minimal nitrogen base medium with lactate as carbon source. Cells from a preculture were added to 600 ml of the standard medium (see Section 2) in culture flasks. Expression is induced by addition of 0.5% by weight final concentration of galactose at 11 h after start of the culture.

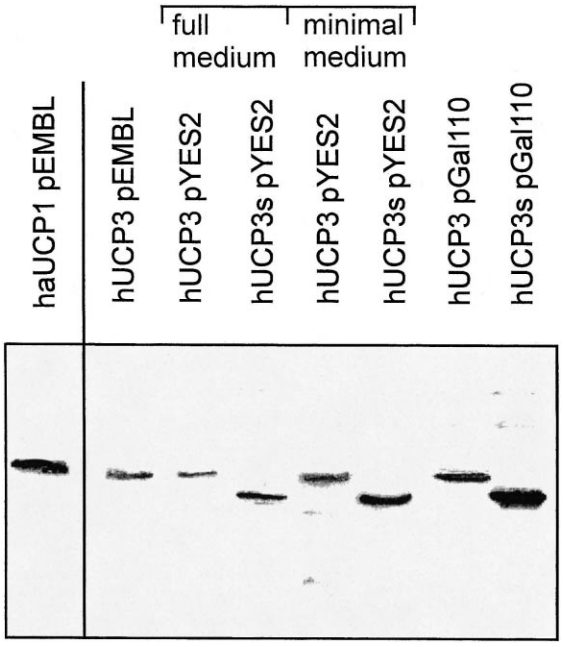

Fig. 2. Immunoblots of mitochondria isolated from UCP-transfected yeast cells. Comparison of various expression vectors (pEMBL, pYES2 medium high expression, pGal110 very high expression). For haUCP1 rabbit antiserum against whole hamster UCP1 was used, for UCP3 and UCP3s antiserum against a 14 residue peptide (see Section 2). 40 μg mitochondrial protein was added to each lane.

The content of UCP in the mitochondrial fraction was assayed by immunoblots. In Fig. 2 the levels of UCP1 and UCP3 in the expression vectors pEMBLyex4 and pYES2 are shown. The UCP1 antiserum was generated in rabbits against hamster UCP1 and the UCP3 antiserum against a peptide of 14 residues localised on the matrix side of the second domain. Different antisera had to be used for UCP1 and UCP3 since there was no cross-reaction. The blots give a semi-quantitative picture of the UCP3 content in the various preparations.

Interestingly, the content of UCP3s reaches a higher level than of UCP3. The expression can be increased by using minimal nitrogen base medium instead of 'full' medium for UCP3 and UCP3s, as noted previously for the expression of UCP1 [11]. The immunoblot also displays the higher UCP3 content with the very high expression vector pGal110. Whereas the immunostain of UCP1 can be 'calibrated' through the binding of nucleotides, this is not possible for UCP3 in yeast mitochondria, due to the lack of GDP binding and the different antibodies required for UCP1 and UCP3. The immunostain of UCP3 could be semi-quantitatively evaluated by comparison with UCP3 expressed in *E. coli* in inclusion bodies (not

shown) estimating the UCP3 protein content up to about 1–5% for UCP3 in the three vectors, and 2–10% for UCP3s in the two vectors. This compares with about 2% UCP1 in yeast mitochondria [11].

3.1. Oxidative phosphorylation in cells

The effect of the overexpressed UCPs on oxidative phosphorylation was directly assayed by measuring the ATP synthesis in yeast cells according to a previously described procedure [29]. For an accurate assessment the time progress of ATP synthesis was followed after initiating oxidative phosphorylation by addition of oxygen to the anaerobic yeast cells. The initial rates of ATP synthesis are a quantitative measure of the oxidative phosphorylation capacity (Fig. 3). In cells expressing hamster haUCP1, the phosphorylation rate is only 20% lower than in the parent strain. However, in cells expressing human hUCP3 the activity is reduced by 70%. Differently, with the expression of murine mUCP3 the oxidative phosphorylation is only inhibited by 32%.

3.2. Membrane potential measurements in mitochondria

Recording of the mitochondrial membrane potential $\Delta\Psi$ with the fluorescent probe $disC_3$ provides a highly sensitive method for assessing the uncoupling by UCP. Very small amounts of mitochondria can be used. The method can also be applied to permeabilised spheroplasts [31]. As a reference the recording of $\Delta\Psi$ with yeast mitochondria containing haUCP1 is shown (Fig. 4A). On addition of the substrate 1-glycerophosphate (GP), $\Delta\Psi$ strongly increases and on addition of lauric acid (LA) it drastically falls. GDP fully reverses the uncoupling effect of LA. To establish as a control the uncoupled state, valinomycin was added which collapses the $\Delta\Psi$ by facilitating the influx of external K^+. The uncoupler carbonylcyanide *m*-chlorophenylhydrazone (CCCP) cannot be used since its absorption interferes with the fluorescence of $disC_3$. With prior addition of GDP (not shown), LA produces only a short transitory decrease of $\Delta\Psi$ which responds to the uptake of undissociated LA and the accompanying internal pH decrease.

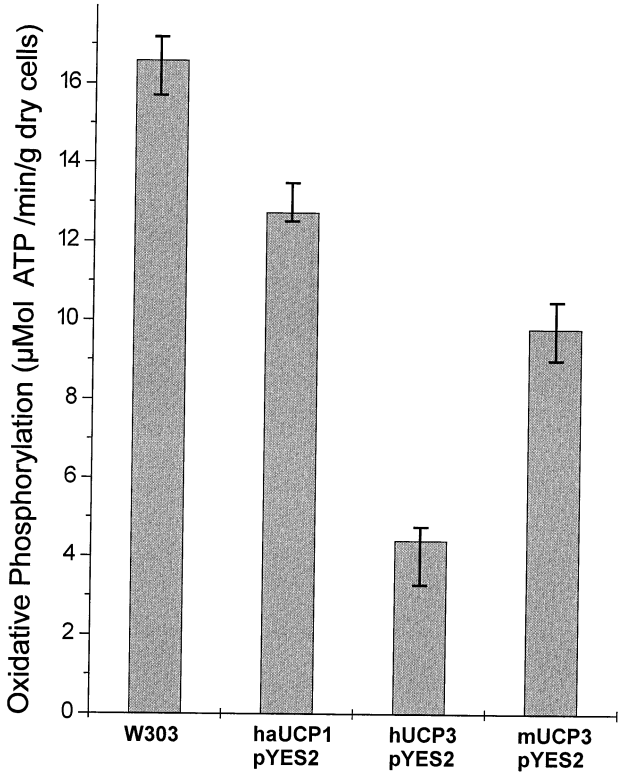

Fig. 3. Oxidative phosphorylation in yeast cells. Inhibition by the plasmid-mediated expression of UCP1 and UCP3. Yeast host cells (W303) and cells transformed with hamster haUCP1, human hUCP3 and murine mUCP3. The oxidative phosphorylation is measured as the rate of ATP formation after addition of O_2 and ethanol to anaerobic, starved cells. For details see Section 2.

In mitochondria from cells expressing human hUCP3, on addition of GP $\Delta\Psi$ develops more slowly than with haUCP1 mitochondria. LA decreases the $\Delta\Psi$ only by 20% and with GDP a minor increase is observed, followed by the slow fluorescence increase evident before the addition of LA. On prior addition of GDP, the GP-induced $\Delta\Psi$ increase becomes faster, indicating a minor GDP-sensitive recoupling. However, the small fatty acid-induced uncoupling is not prevented. In mitochondria from cells containing the 'empty' pYES2 vector a somewhat smaller decrease of $\Delta\Psi$ by LA is measured, which is GDP-insensitive. This $\Delta\Psi$ has to be subtracted from that observed in the UCP3-containing mitochondria. As a result the GDP-sensitive $\Delta\Psi$ decrease attributable to native UCP3 amounts to only about one tenth of that observed with UCP1. In mitochondria with the short form hUCP3s, the addition of 50 µM LA induces a small $\Delta\Psi$ increase which may be due to a turbidity decrease of the mitochondria. The 'non-specific' GDP-insensitive uncoupling by 100 µM LA is observed as a rule in all yeast mitochondria, independent of the transfection with UCP. In summary, due to the high a priori uncoupling, the induction of uncoupling by fatty acid in UCP3-containing mitochondria is much smaller than with UCP1 and largely unregulated, i.e. with only traces of GDP-induced recoupling.

With spheroplasts generated from the yeast cells and permeabilised by nystatin [31] or by osmotic shock, it was also possible to record the $\Delta\Psi$ with the fluorescent probe (Fig. 4B). An advantage is that the isolation of mitochondria from yeast cells, with their notoriously low yield, can be bypassed. In spheroplasts mitochondria are still in a more native environment which may modify the UCP response. As shown in Fig. 4, in spheroplasts containing haUCP1 the response of $\Delta\Psi$ to GP was as strong as in isolated mitochondria. LA and the non-metabolisable 2-Br-palmitate produced strong uncoupling, which was fully reversed by GDP. In spheroplasts containing hUCP3 a small uncoupling was produced only after addition of 100 µM LA. The response was much smaller than with UCP1, similar to that in mitochondria. This uncoupling was insensitive to GDP.

3.3. H^+ transport

Another assay for UCP function in mitochondria is the direct measurement of H^+ transport [12]. To this purpose mitochondria are incubated with 100 mM KCl and 50 µM LA. The H^+ pumping by electron transport is suppressed by antimycin. During the preincubation with KCl, first Cl^- is taken up via UCP followed by a H^+ influx and as a result mitochondria become more acidic inside, which is a prerequisite for the observed H^+ efflux in exchange for K^+ influx. On addition of valinomycin the K^+ uptake causes a $\Delta\Psi$ positive inside which drives H^+ outside through UCP. The records in Fig. 5 compare the H^+ efflux in mitochondria con-

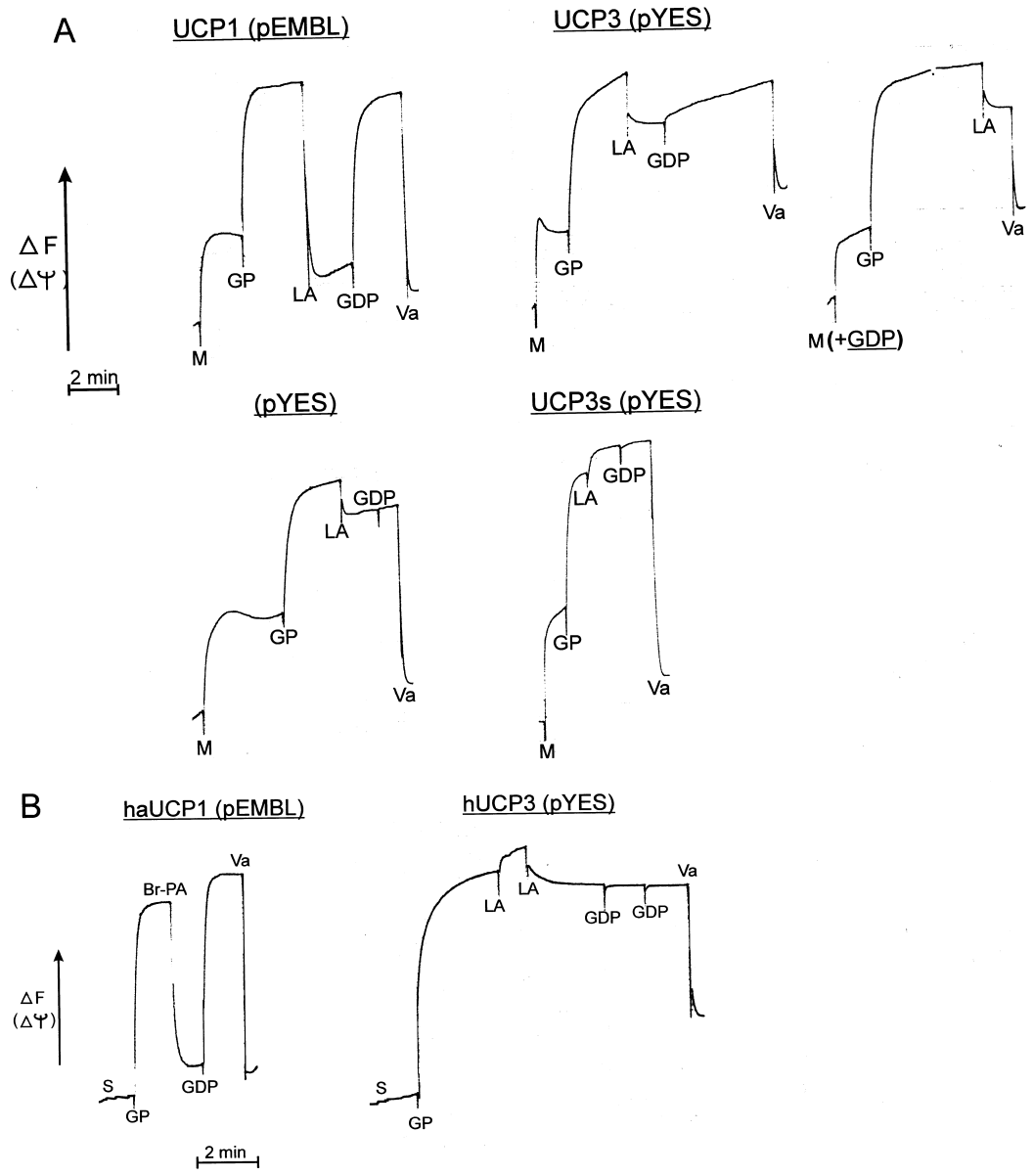

Fig. 4. A: Recording of membrane potential ($\Delta\Psi$) with the fluorescence dye $disC_3$ in mitochondria containing various UCPs, UCP1, UCP3 and UCP3s (short form). The two expression vectors used are given in brackets. 6 μg mitochondrial protein was added to a final volume of 400 μl. B: Recording of $\Delta\Psi$ with the fluorescent dye $disC_3$ in permeabilised spheroplasts (S) derived from yeast cells overexpressing UCP1 and UCP3 (see Section 2). 38–40μg spheroplasts was added to a total volume of 400 μl.

taining haUCP1 and hUCP3. A small but distinct H^+ efflux can be induced in hUCP3-containing mitochondria, which is largely suppressed on preincubation with 100 μM GTP. This effect is observed only in mitochondria from cells, when hUCP3 expression is induced by lower amounts of galactose and shorter growth time. The experiment shows that expression of hUCP3 can confer on the mitochondria a nucleotide-sensitive H^+ transport, which is, however, much weaker than with UCP1. With mitochondria containing UCP3s, no fatty acid- and nucleotide-sensitive H^+ import was detected (results not shown).

3.4. Nucleotide binding

The binding of nucleotides has been the most important assay for the intact functionality and for the quantification of UCP1 [13]. Radiolabelled and fluorescent nucleotides have been extensively used to measure the nucleotide binding to UCP1 expressed in yeast [1]. In contrast, all attempts to detect binding to mitochondria from cells expressing hUCP3, mUCP3 and hUCP3s failed, both with [^{14}C]GTP and with dansyl GTP. The limit of detection is at about 0.1 μmol/mg mitochondria.

4. Discussion

Because of the successful expression of functional UCP1 in *S. cerevisiae*, this expression system seemed to be the obvious choice for UCP3 as for the other UCP variants. UCP3 can reach the same expression levels as UCP1, depending on the expression system used. The short form UCP3s is expressed to an even higher extent. Different from UCP1, the expression of UCP3 in yeast cells significantly decreased growth and caused

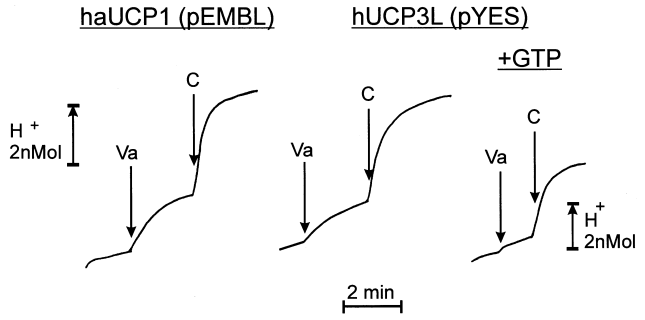

Fig. 5. Recording of H^+ efflux from mitochondria with a pH electrode. Mitochondria containing hamster haUCP1 expressed with the pEMBL Lyex4 vector and mitochondria containing human hUCP3 expressed with the pYES2 vector were used. The cells were induced with 2% galactose after 15 h and harvested after another 10 h. The calibration by addition of 1 nM H_2SO_4 is given at the left side. Mitochondria (1.5 mg/ml) were incubated in a medium containing 0.6 M mannitol, 100 mM KCl, 2 mM KH_2PO_4 and 1 mM MOPS, pH 7.2. Electron transport was inhibited by 4 μM antimycin, and the ADP/ATP carrier was blocked by 5 μM bongkrekate and 10 μM carboxyatractylate. Further 50 μM LA was added and on addition of 2 μM valinomycin (Va) H^+ release was initiated. The total H^+ release capacity was measured after addition of 2 μM CCCP. In the third experiment 100 μM GTP was added prior to the valinomycin.

some uncoupling. However, a convincing functional characterisation of UCP3 in cells and isolated mitochondria was not achieved [22–24], as we will explain in the following.

4.1. UCP3 in yeast cells

The test of uncoupling function at the cellular level by the direct determination of intracellular ATP synthesis shows only a small decrease with UCP1, but with human hUCP3 inhibition of oxidative phosphorylation up to 80% is found. The results are in agreement with the decrease of the fluorescence in the FACS scans of yeast cells on expression of UCP3 and of UCP3s [14,17]. However, the interpretation of the FACS shift is not unique since it can also be caused by fewer mitochondria in the UCP3-expressing cells [19]. Probably both partial uncoupling and a resulting decreased mitochondrial biogenesis contribute to the observed effects. The FACS scans in UCP1-expressing cells vary among the reports. Whereas Gong et al. [17] and Boss et al. [15] found a low fluorescence change in FACS by UCP1, in accordance with our oxidative phosphorylation measurements, Zhang et al. [20] reported the same strong shift as with UCP3. Also thermogenesis has been reported to be increased by about 30% in UCP3- but less in UCP1-expressing yeast [32]. The more drastic effect of UCP3 on ATP synthesis than on intracellular fluorescence and thermogenesis (80% inhibition of oxidative phosphorylation versus 30% shift of FACS) can be rationalised by the high sensitivity of oxidative phosphorylation towards the decrease of $\Delta\Psi$ in mitochondria.

4.2. UCP3 in isolated mitochondria

The recording of $\Delta\Psi$ in mitochondria by a fluorescent probe is a more sensitive method to assess the uncoupling characteristics of UCP than respiration. Yeast mitochondria containing UCP1 provide a 'master' pattern of strong $\Delta\Psi$ responses to the activators and inhibitors, to which the UCP3-containing mitochondria have to be compared. Already the initial increase of $\Delta\Psi$ by substrate addition to UCP3-containing mitochondria is more subdued than with UCP1, reflecting a fatty acid-independent uncoupling. Only a barely discernible, GDP-sensitive uncoupling induced by fatty acid can be observed occasionally. With the short form, UCP3s, the GDP-sensitive uncoupling is completely absent. Similarly, direct measurements of H^+ transport show only a small GDP-sensitive H^+ release in mitochondria containing UCP3 but none with UCP3s. The very low share of a fatty acid-inducible and nucleotide-sensitive uncoupling as probed by $\Delta\Psi$ and by H^+ transport, which are the hallmarks of UCP1 function, is remarkable in view of the high content of UCP3 in the mitochondria.

By measuring respiration of mitochondria from UCP3-expressing yeast cells, both groups [20–22,24] found an increased uncoupling which was insensitive to inhibition by nucleotides. Fatty acids stimulate respiration of mitochondria from UCP3-expressing yeast cells only very poorly, if at all. The authors conclude that the physiological regulation of uncoupling by UCP3 due to the lack of inhibition by nucleotides is different from UCP1. In contrast, we believe that the uncoupling effects reported here and by others do not reflect the normal function of UCP3 in skeletal muscle. It is our working hypothesis that the same criterion, inhibition by a low concentration of nucleotide, which has been successfully used to define H^+ transport by UCP1 also applies to the physiological function of UCP3.

With this presumption the observed results can be rationalised by assuming that UCP3 incorporated into the yeast mitochondria primarily does not exist in the native but in a deranged conformation unable to bind nucleotides. In this state it forms an unregulated pore for fatty acid-independent H^+ transport in the inner membrane. Only a minor portion of UCP3 is in a native conformation with a regulated H^+ transport. The conclusion gains support from the following facts.

(a) In the structure of UCP3 most of those residues are present which have been identified in UCP1 to participate in the nucleotide binding and in the pH regulation of binding.

(b) The short form UCP3s facilitates unregulated uncoupling as well as UCP3. The amputated protein with only five helices should be barely able to form a stable, functionally competent conformation but rather exist in a deranged folding. The uncoupling seems to be weaker as judged from the less retarded growth despite a nearly twice as high content. It seems that in UCP3s also the unregulated H^+ transport is impaired.

(c) The unregulated uncoupling is highly variable, as observed by comparing the uncoupling of human and murine UCP3 in yeast cells. Subtle differences in the amino acid composition may influence an unstable deranged conformation more than a definitive native conformation.

(d) UCP3 and UCP1 expressed in inclusion bodies of *E. coli* and reconstituted into vesicles are able to catalyse a nucleotide-sensitive Cl^- but not H^+ transport [25]. The Cl^- transport is inhibited by nucleotides with a $K_I < 1$ μM for both UCP3 and UCP1 and thus fulfils our 'classical' criterion [34] for a UCP-catalysed function. The absence of H^+ transport was attributed to the lack of a cofactor in the reconstitution from the inclusion bodies. The H^+ transport activity by UCP3 reported recently by Jaburek et al. [26] does not contradict our results, since the activity is about 40 times lower than with native UCP1 accounting for the temperature difference of measurements. With a K_I of 1 mM GTP it has a 10^4-fold lower sensitivity to nucleotides than the Cl^- transport. In

our own studies we have therefore regarded the same observation as non-specific H^+ leakage in the reconstituted vesicles. Hinz et al. [21] argue that the conformation of UCP3 in our reconstitution has an artificially increased affinity for nucleotides. Such a reasoning would be opposite to the generally accepted criterion that a more native folding of a protein creates a function such as a specific nucleotide binding site.

(e) Boss et al. [33] showed that overexpression of UCP3 in myoblasts produces a much smaller downshift in FACS than in yeast cells. Obviously on overexpression in natural mitochondria, UCP3 is in a native state where its uncoupling capacity is largely inhibited, i.e. regulated by internal nucleotides and fatty acids.

We may ask why UCP1 but not UCP3 should attain the native state on expression in yeast. The reason should exist in the different structure. There is 57% similarity between the two UCPs and the insertion into the yeast mitochondrial membrane may critically depend on a good match between the lipid environment and the protein. The phospholipid composition in yeast differs by shorter fatty acids [35] and this may be marginally tolerant to UCP1 but not any more to UCP3. The nucleotide binding site seems to be most sensitive to a disturbance of the native structure and thus loses its competence in UCP3 expressed in yeast.

The practical consequences of these findings would be to avoid the expression system in yeast for the characterisation of UCP3 and, instead, to concentrate on the *E. coli* expression system and there to explore conditions where UCP3 attains its native capacity for H^+ transport. Judged from the FACS scans of UCP2-expressing yeast cells [18,19], it seems possible that also UCP2 in yeast cells exists in a deranged conformation with similar consequences as for UCP3.

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