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UCP3 Expressed in Yeast Is Primarily Localized in Extramitochondrial Particles

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Previously it was concluded (1) that, differently from UCP1, on expression in *Saccharomyces cerevisiae,* UCP3, and UCP3 short (UCP3s) are in a deranged state, allowing for unregulated uncoupling. Here we show that the bulk of UCP3 and UCP3s is in extramitochondrial aggregates whether expressed with high or medium expression vectors. The evidence is based on the insolubility of most UCP3 and UCP3s in nonionic detergents such as Triton X100, in contrast to UCP1. Using very high expression vector, macroscopic evidence for extramitochondrial UCP3 containing particles is a viscous white sediment surrounding the mitochondrial fraction which contains UCP3 as inclusion body type aggregate. Together with the previous data it is concluded that uncoupling due to small amounts of incorporated, deranged, and nucleotide insensitive UCP3 prevents incorporation of the bulk of UCP3 into mitochondria. This finding also provides a simple and stringent assay for the state of heterologously expressed in mitochondrial membrane proteins.

Key Words: **uncoupling protein; mitochondria; *S. cerevisiae;* inclusion bodies; solubility; detergents.**

The heterologous expression in yeast was widely used in an attempt to characterise the uncoupling by uncoupling proteins (UCP) (2–8). As assayed by FACS in yeast cells, most UCPs seem to uncouple mitochondria more than the "classical" UCP1. They inhibit growth and downshift the signal of a mitochondrial membrane potential probe. Mitochondria isolated from yeast cells expressing UCP3 were reported to exhibit uncoupled respiration which, differently from UCP1, is not regulated by fatty acids sand nucleotide (9–12). This lead to the conclusion that the uncoupling by UCP3 is not controlled by nucleotides.

Abbreviations used: UCP, uncoupling protein; UCP3s, short form of UCP3; GP, glycerol-1-phosphate; LA, lauric acid; DTE, dithioerythritol; PMSF, phenyl-methane-sulfonyl-fluoride; BSA, bovine serum albumin; FACS, fluorescence activated cell sorting.

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In a previous paper we extended these observations by showing that UCP3 expression inhibits oxidative phosphorylation in yeast cells in contrast to UCP1 (1). Further, in isolated mitochondria containing UCP3, with a membrane potential probe primarily an unregulated uncoupling was observed, different from UCP1 containing mitochondria. It was concluded that UCP3 and the short form UCP3s are incorporated in yeast mitochondria in a state of imperfect folding, which allows for an unspecific H^+ leaking pore (1). Recently, we reported that UCP1, 2, and 3 require coenzyme Q (CoQ) as a cofactor which has to be added to obtain H^+ transport with UCPs reconstituted from CoQ free sources, such as inclusion bodies from *Escherichia coli* (13, 14). Under these conditions UCP3 was shown also to facilitate a fatty acid dependent H^+ transport which can be inhibited by low concentration of nucleotide.

Here we report the finding that UCP3 expressed in yeast, different from UCP1, is in an aggregated state, apparently in extramitochondrial particles, both when expressed with very high to medium expression vectors. In cells with very high expression vector growth is strongly impaired and the mitochondrial fraction has macroscopic aggregates in a viscous layer containing UCP3. These results resolve the paradox of high UCP3 expression and lack a regulated uncoupling response in yeast mitochondria. They support the previous conclusion (1) that UCP3 in contrast to UCP1 expressed in yeast is in a deranged state and that functional studies of UCP3 and possibly also of other UCPs expressed in yeast have to be reinterpreted and do not reflect their physiological function. The results also provide a simple test as to whether in yeast the heterologously expressed UCPs or other mitochondrial proteins are incorporated into the mitochondria.

MATERIALS AND METHODS

The genes for the human long (hUCP3) and short form (hUCP3s), for the murine UCP3 (mUCP3) and for the antisense hUCP3 were cloned into the Bg/II restriction enzyme site of the high expression vector pGAL110 (15) and transfected into *S. cerevisiae.* Yeast cells were grown under strong aeration in a selection medium with 0.67%

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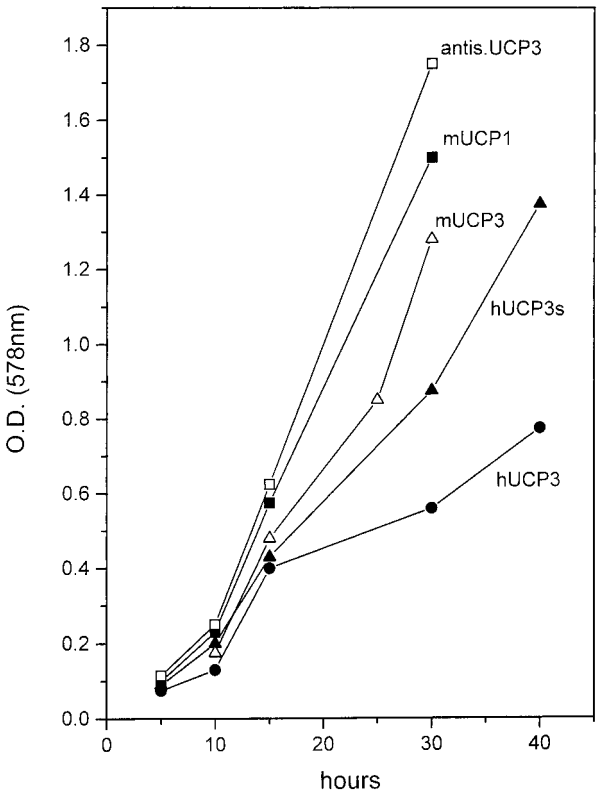

FIG. 1. Growth curves of yeast transfected with the very high expression vector pGAL110 containing the cDNA of human (hUCP3), murine UCP3 (mUCP3), the short form hUCP3s and as controls antisense UCP3 and murine UCP1. Aerobic growth in minimal nitrogen base medium with lactate as carbon source. Cells from a preculture were added to 600 ml of standard medium (see Materials and Methods). Expression is induced by addition of 0.5% by weight of galactose at 10 h after start of the cultures.

yeast nitrogen base, and with amino acids and adenine at 40 mg/l. As carbon source served 2% lactic acid and 0.05% glucose. The pH was adjusted to 5.5. For the induction 0.4% galactose was added at 10 h after start of the culture. From these cells, after enzymatic lysis, spheroplasts were generated using a mixture of "Novozym" and zymolyaseand therefrom, mitochondria and mitochondrial equivalent fractions were prepared as described in (16). For the membrane potential measurements, small batches of spheroplasts were prepared starting with 100 mg wet weight cells which were incubated in 650 μl 1 M sorbitol, 20 mM K phosphate, pH 7.5 under addition of 20 μl zymolyase (10mg/ml) and under gentle slaking at 30° until O.D. was reduced to half. After centrifugation at 1000 rpm the pellet is resuspended in 600 μl and centrifuged again and finally resuspended in 0.6 M mannitol, 20 mM Tris pH 7.2, 2 mM PMSF, 0.5 mM EDTA, 0.1 mM EGTA under further addition of 20 μg nystatin according to (17).

Oxidative phosphorylation in yeast cells was measured according to a method described in (17). It is based on the generation of ATP after addition of O^2 and substrates to an anaerobic yeast cell suspension. The time progress of ATP synthesis was followed in aliquots withdrawn at typically 10, 20, 60, 120, and 300 s or at longer intervals with the UCP3 expressing strains.

For the immunoblots polyclonal antibodies were used, raised in rabbits against whole UCP1 isolated from hamster brown adipose tissues and against the 14 residues long peptide in human UCP3 S^{149}IHLGPSRSDRKYS^{162}.

The membrane potential was recorded using the fluorescent cyanine dye 3,3′ dipropyl-thiacarbocyanine ($disC_3$). A sensitive fluorometer was constructed with a quartz fibre collecting the emitted light to the photomultiplier which had a red sensitive S22 photocathode. Exciting light was at 620 nm and emitted light was measured with a cut off filter >650 nm. In a beam splitter a portion of the exciting light was monitored by a photodiode for stabilizing the exciting light by regulating the photomultiplier voltage. For these measurements 6 μg protein mitochondria or 70 μg spheroplasts were incubated in 375 μl of a medium containing 20 mM K-phosphate, 0.25 mg/ml BSA, 0.1 μM $disC_3$. Additions during the recording were: 250 μm GDP or GTP, 1 μM valinomycin, pH 6.8.

RESULTS

In previous studies we used the medium to high expression vectors pEMBL and pYES2 for the heterologous expression in *S. cerevisiae* (1). To further increase the yield of UCP3 we applied a very high expression vector pGAL110 (15), which also uses a galactose dependent promoter. In addition to the human UCP3, the murine mUCP1 and mUCP3 were introduced with this vector into yeast. Cells containing antisense DNA of in pGAL 110 served as a control. Whereas the very high expression of mUCP1 affects the growth rate only little, it is strongly decreased by the very high expression of murine mUCP3 and nearly stalled with (human) hUCP3 (Fig. 1). Even after 40 h the culture reaches an O.D. of only 0.7 whereas with UCP3 in the pYES vector

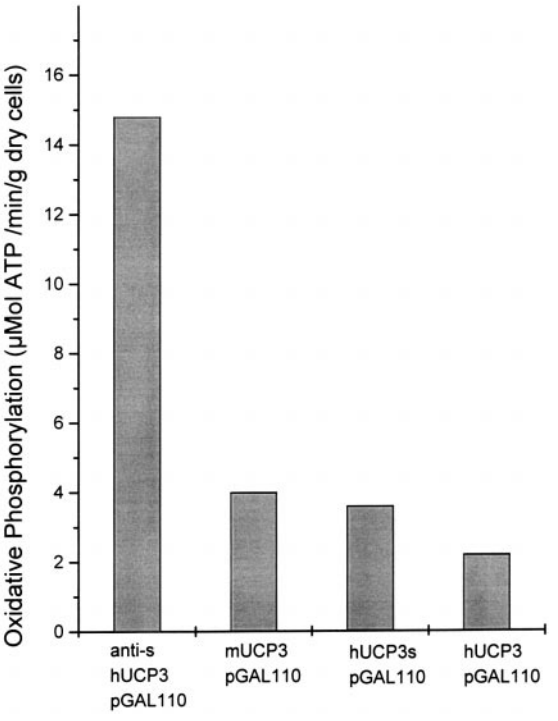

FIG. 2. Oxidative phosphorylation in yeast cells. Measurement of the rate of ATP formation under the influence of transfected UCP3 and UCP3s. In the control plasmid the antisense cDNA of hUCP3 was incorporated. The increase of the intracellular ATP levels with time was determined after addition of O_2 and ethanol to anaerobic starved yeast cells. For details see Ref. 12.

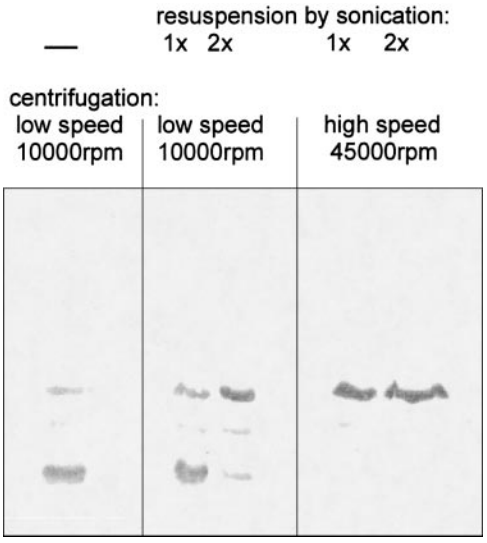

FIG. 3. Identification of UCP3 and its degradation products by immunostain in the "mitochondrial" equivalent fraction. hUCP3 was expressed in yeast with the pGAL 110 vector. In the left box, UCP3 is identified in the fraction sedimented by the usual centrifugation for isolating mitochondria. In the middle box, the sediment was washed by additional sonication and centrifugation. In the third box, washing by sonication and centrifugation at high speed.

after 20 h an O.D. of 3.0 was attained (1). The expression of the short form, hUCP3s, also strongly affects growth but less than UCP3.

In these yeast cells, oxidative phosphorylation was measured according to the procedure described in the preceding paper. As shown in Fig. 2, the rate of ATP synthesis is suppressed by the expression of hUCP3 in the pGAL 110 vector to 80% as compared to the control strain with the antisense DNA. Also the expression of the short form hUCP3s caused a 75% inhibition of oxidative phosphorylation. The expression of mUCP3 in pGAL 110 ensued 70% inhibition, as compared to the only 35% inhibition when expressed in pYES2 (1). The isolated mitochondrial fraction formed a viscous sediment, in which the small mitochondrial pellet was surround by a large whitish layer. On resuspension and prolonged sonication the whole fraction formed a homogenous, more translucent slightly brown colored pellet.

In the preceding paper the immunoblot (Fig. 2 in (1)) included the mitochondrial fractions containing UCP3 and UCP3s in the pGAL 110. The expression level of both UCP3 and UCP3s in the pGAL 110 vector was clearly higher than in pYES2. As previously noted, in the mitochondrial fraction UCP3 is more sensitive to proteolytic degradation than UCP1. This effect is much more pronounced with the high expression vector pGAL 110. As shown in Fig. 3, proteolytic degradation is extensive in the sediment after one day at 4°, although high amounts of PMSF are routinely added as early as for the breakage of spheroplasts. Further addition of the protease inhibitors leupeptin, pepstatin and proteinin, could not markedly decrease the proteolysis of UCP3. However, repeated washing by resuspension with sonication and high speed centrifugation at 45,000*g* diminished markedly proteolytic activity (Fig. 3). Obviously the adhering proteolytic enzymes are soluble and can thus be removed from the insoluble material containing UCP3. No proteolytic degradation of mUCP1 expressed in pGAL 110 was noted.

The uncoupling was scrutinized in these fractions with the sensitive fluorescent membrane potential probe $disC_3$ (Fig. 4). When UCP3 is expressed with the very high expression vector pGAL 110, only a very small $\Delta\psi$ can be generated by addition of the substrate glycerol-1- phosphate (GP), indicating a strong a priori uncoupling. Addition of lauric acid causes only a small GDP insensitive $\Delta\psi$ decrease. A similar response of $\Delta\psi$ was observed with spheroplasts generated from the UCP3 containing cells showing that the lack of a regulated uncoupling in the mitochondrial fractions was not caused by proteolytic damage during the isolation from cells. In the corresponding fraction from control cells with the antisense DNA of UCP3, the strong $\Delta\psi$ generated by substrate is only little reduced by 50 μM LA and this effect is insensitive to GDP (not shown). As shown above (Fig. 1), expression of the murine mUCP3

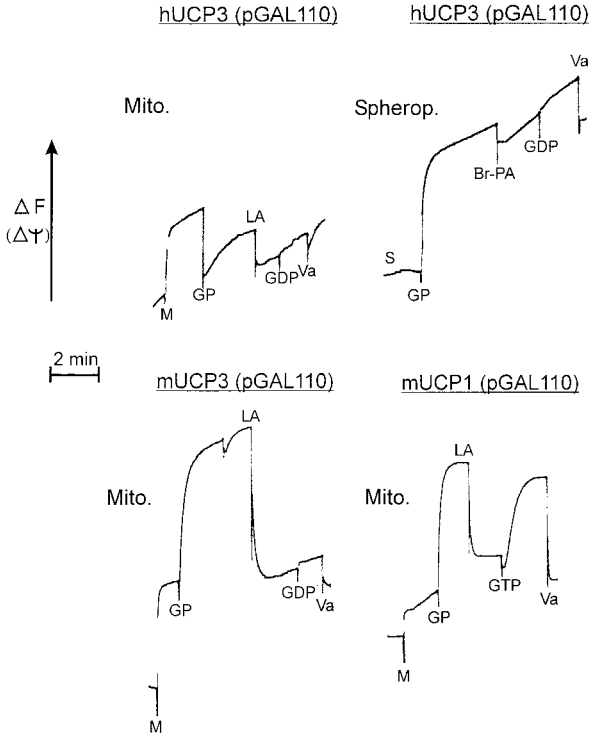

FIG. 4. Recording of membrane potential ($\Delta\psi$) with the fluorescence dye $disC_3$ in mitochondria (mito.) and spheroplasts (spherop.). Six micrograms mitochondrial protein or 70 μg spheroplasts are added to 400 μl total volume medium. M, mitochondria; S, spheroplasts; GP, 1-glycerolphosphate; LA, lauric acid; Va, valinomycin.

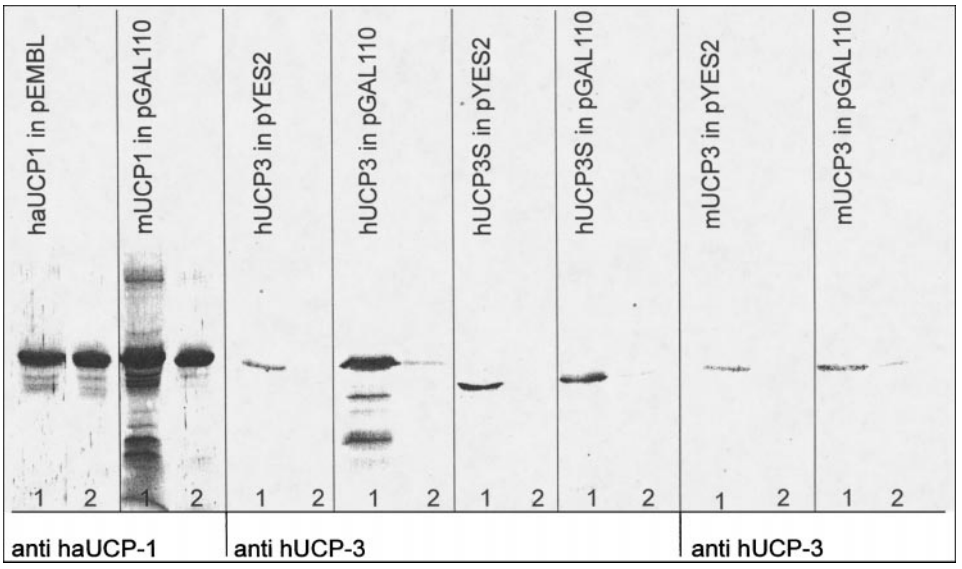

FIG. 5. The insolubility of UCP3 in Triton X100, contrasting to the solubility of UCP1, expressed in yeast mitochondria. Composite immunoblot of SDS gels. The mitochondrial fraction is isolated from yeast transformed by various vectors for the expression of UCP1, UCP3, and UCP3s. (ha, hamster; h, human; m, murine). The total protein (lane 1) versus the Triton soluble protein (lane 2) of each mitochondrial fraction is analysed by immunostaining. Polyclonal antibodies against whole haUCP1 or against the synthesized peptide S146 to S159 of UCP3 are used. For the solubilisation assay, the mitochondrial sediments (10,000 rpm fractions, see Materials and Methods) are treated with a solution containing 3.6% Triton X100, 1.2 M NH_4 acetate, 1 mM EDTA, 5 mM PMSF, 1 mM DTE, at a detergent/protein ratio of 3.65. After homogenising and incubation for 20 min at 0°, the extract is centrifuged at 45,000 rpm for 20 min. From the supernatant the protein is precipitated by TCA and redissolved with SDS.

prevented cell growth less than hUCP3. The yield of mitochondria was also higher and glycerol-1-phosphate induced a somewhat stronger $\Delta\psi$ increase (Fig. 4). The uncoupling by 100 μM LA was GDP insensitive. Cells in which the murine UCP1 is highly expressed with pGAL 110, produced ample mitochondria with a high content of mUCP1 (see Fig. 5) Here the response of $\Delta\psi$ (Fig. 4) to substrate, fatty acid, and to GTP resembled that with the pYES2-haUCP1 mitochondria and mitochondria from brown adipose tissue: a high $\Delta\psi$ produced by the substrate followed by a "classical" regulated uncoupling with fatty acid and GDP was evident. Thus, the previously reported difference between the function of UCP1 and UCP3 in mitochondria was most dramatic with the high expression by pGAL 110.

To further understand the function of UCP3 we attempted to isolate and reconstitute UCP3 from the mitochondrial fractions. For this purpose UCP was extracted with non-ionic detergents such as $C_{10}E_5$ or Triton X100. With these detergents UCP1 is totally solubilised from the yeast mitochondria (*16*). To our great surprise, however, UCP3 could not be solubilised from the mitochondrial fractions as monitored in immunoblots. Variation of salt additions, such as of 0.15 M Na_2SO_4 or 1 M NH_4 acetate which enhance the extraction of mitochondrial carriers from mitochondria, failed to solubilise hUCP3. Extraction failed also with other detergents which were known to be more aggressive than Triton X100, such as the dipol detergent lauryl-dimethylaminoxide which easily dissolves the paradigm ATP/ADP carrier. Only sarcosyl, usually reserved for the solubilisation of inclusion bodies from *E. coli* (19, 20),completely dissolved hUCP3. The same behaviour towards detergents was observed with the short form hUCP3s and with mUCP3 expressed in yeast.

To document this solubilisation pattern, immunoblots were made in parallel of mitochondrial fractions extracted with SDS and with Triton X100 (Fig. 5). The SDS extract represents the total content of UCP and the Triton extract should represent the fraction of UCP which according to our long standing experience is incorporated into the mitochondria. A drastic contrast between UCP1 and UCP3 is observed. Whereas both haUCP1 and mUCP1, even with very high expression vector pGal 110, are completely solubilised with Triton X100, only traces of UCP3 are extracted with Triton X100. Both human UCP3 and murine UCP3 are largely insoluble. This insolubility of UCP3 in Triton X100 is found whether expressed in yeast with the medium or very high expression vectors. It also extends to the short form UCP3s.

DISCUSSION

The successful expression of UCP1 from rat and hamster in *S. cerevisiae* with functional characteristics similar to those described for UCP1 in mitochondria from brown adipose tissue, triggered the use of this expression system also for the characterisation of the

new UCP variants UCP2, 3, 4, and BMCP (2–4, 6, 21). However, as concluded in the preceding paper, from studies of cells and isolated mitochondria (1), UCP3 expressed in yeast is not in a functionally competent state, since UCP3 causes unregulated uncoupling, i.e., an uncoupling which is not activated by fatty acids and not inhibited by nucleotides. Only traces of a regulated uncoupling and H^+ transport could be observed. The unregulated uncoupling was suggested not to represent the physiological function of UCP3 and to result from an incompletely folded state of UCP3 in the yeast mitochondria. This conclusion was at variance with the view of other authors (9–12) who, using different experimental approaches, reported also an unregulated uncoupling but regarded it as a physiological function.

Here we show that UCP3 and the short form UCP3s expressed in yeast is in a different physical state as UCP1 as judged by the solubility towards detergents. Various nonionic detergents which are used for extraction of native mitochondrial carriers are unable to solubilise the bulk of UCP3. This behaviour is emphasized by the contrast to UCP1, which generated even under the very high expression pGAL110 vector, is fully Triton soluble. The insolubility of UCP3 is independent of the three vectors used, pEMBL, pYES2, and pGAL 110, i.e., independent of the level of UCP3 synthesis. Also the short form UCP3s is mostly insoluble in nonionic detergents. Only ionic detergents such as sarkosyl solubilise UCP3, similar to UCP3 aggregated in inclusion bodies on expression in *E. coli.* These findings provide evidence that in yeast cells UCP3 exists primarily in an aggregated state, different from UCP1 or the ADP/ATP carrier.

A striking feature of the isolation of mitochondria from the very high UCP3 expressing cells was the appearance of a white viscous layer in the centrifugal fraction usually reserved for the collection of mitochondria. It was distinct from the brown mitochondrial pellet and surpassed it in size. The small size of the brown pellet contrasted to the increase of the UCP3 content in the total fraction. Obviously aggregate of UCP3 is contained in the viscous pellet which indicates that UCP3 is deposited primarily in extramitochondrial inclusion body like aggregates. The high sensitivity of UCP3 in these fractions towards proteolytic attack point to an at least partially unfolded state of most of the UCP3. In contrast, UCP1 even when highly overexpressed was insensitive to this proteolysis, reflecting the native state of UCP1.

The results supplement the conclusion drawn in the previous paper (1), that UCP3 is incorporated into mitochondria largely in a deranged state, which allows for an unregulated uncoupling. Only apparently minute amounts of UCP3 are in a native state and can catalyse regulated, i.e., fatty acid dependent and nucleotide inhibited uncoupling. Both these in the mitochondria incorporated forms of UCP3 are represented

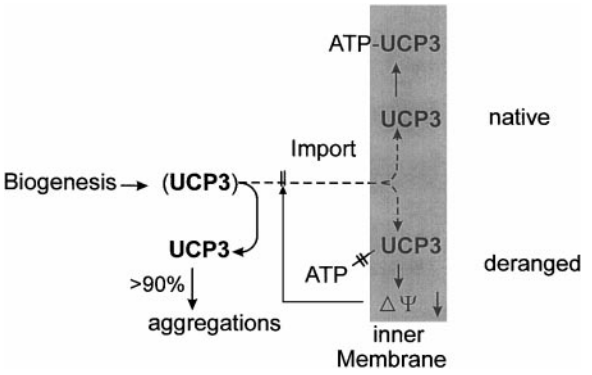

FIG. 6. Scheme depicting the suggested incorporation stages in mitochondria of UCP3 expressed in yeast. On induction initially some UCP3 is imported into the inner membrane of mitochondria. A small part is folded into the native state and blocked by cytosolic ATP, similar to all of UCP1 expressed in yeast. Another portion is, after insertion in the inner membrane, in a deranged conformation which cannot bind ATP. It causes uncontrolled H^+ leakage and thus uncouples the mitochondria in yeast cells, even without activation by fatty acids, different from UCP1. As a result the low $\Delta\psi$ prevents import of further synthesised UCP3 so that the majority of UCP3 (>90%) aggregates outside the mitochondria.

by the very small Triton X100 soluble portion. Thus we may define three portions of UCP3 in yeast cells, which are correlated as depicted in the scheme (Fig. 6). A small amount of UCP3 is incorporated into mitochondria and out of this pool only a fraction reaches the native state, competent for regulated uncoupling, i.e. it can bind nucleotides. In the yeast cell it is complexed with ATP, similar as UCP1 and therefore it does not uncouple. Another fraction of the imported UCP3 remains in a deranged, partially unfolded state where it is unable to bind ATP. Here it causes unregulated uncoupling which lowers the $\Delta\psi$ and thus prevents the incorporation of the further synthesised UCP3. The continuously produced UCP3 is rejected from entering the mitochondria and forms extramitochondrial inclusion body type aggregates. In contrast, all of the translated UCP1 in yeast cells is incorporated into mitochondria in a native state. Therefore UCP1 exhibits only regulated uncoupling. In the yeast it is mostly complexed with ATP and thus does not uncouple. Renold *et al.* (23) reported, using an in vitro system, that UCP3 is incorporated into yeast mitochondria. However in this case, the incorporation assayed by ^{35}S-methionine corresponds to only trace amounts of UCP3, in line with our finding. These experiments obviously do not reflect the in vivo biosynthesis of bulk UCP3. Recently we reported (13, 14) that H^+ transport by UCP1, 2 and 3 reconstituted from inclusion bodies in *E. coli* requires CoQ. It might be expected that UCP3 from yeast which should be devoid of CoQ after reconstitution into vesicles can also be reactivated by CoQ addition.

What is the reason for the divergent incorporation of UCP3 and UCP1? It should reside in the different

structures, as UCP3 has only 57% homology to UCP1. The particular amino acid sequence of UCP3 may cause some instability of the transmembrane helices within the foreign environment of the yeast mitochondrial membranes. The critical element may be one helix which cannot be stabilised in the native transmembrane configuration by interaction with the yeast phospholipides. Transmembrane instability of only one helix is described for the cystis fibrosis Cl^- channel (24). The variability of the derangement is evident from the comparison of human and murine UCP3 which differ by a few residues only. mUCP3 expression in yeast is less deleterious for the growth and correspondingly the mitochondria are found to exhibit a higher $\Delta\psi$. But still mUCP3 cannot be inhibited by nucleotide and thus even the less drastic uncoupling does not yet permit incorporation of UCP3 in mitochondria.

These results do not only substantiate the previously drawn conclusion (1) that UCP3 expressed in yeast is mostly in a deranged state but they also provide a simple method, the solubility in a nonionic detergent such as Triton X100, for assaying the incorporation state of the UCPs and also of other mitochondrial membrane proteins heterologously expressed in yeast. Only when UCPs, whether UCP2, 3, 4, etc., are Triton soluble, the function of the UCPs in the yeast cells and mitochondria can be conclusively studied. Thus, it will be important to extend this test to other in yeast expressed uncoupling proteins (5–7, 21).

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