INCREASING MITOCHONDRIAL SUBSTRATE-LEVEL PHOSPHORYLATION CAN RESCUE RESPIRATORY GROWTH OF AN ATP SYNTHASE DEFICIENT YEAST*

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In a previous study, we have identified Fmc1p, a mitochondrial protein involved in the assembly/stability of the yeast F$_0$F$_1$-ATP synthase at elevated temperature. The Δfmc1 mutant was shown to exhibit a severe phenotype of very slow growth on respiratory substrates at 37°C. We have isolated ODC1 as a multicopy suppressor of the fmc1 deletion restoring a good respiratory growth. Odc1p expression level was estimated to be at least 10 times higher in mitochondria isolated from the Δfmc1/ODC1 transformant as compared to wild type mitochondria. Interestingly, ODC1 encodes an oxodicarboxylate carrier, which transports α-ketoglutarate and α-keto adipate or any other transported tricarboxilic acid cycle intermediate in a counter-exchange through the inner mitochondrial membrane. We show that the suppression of the respiratory-growth deficient fmc1 by the over-expressed Odc1p was not due to a restored stable ATP synthase. Instead, the rescuing mechanism involves an increase in the flux of tricarboxilic acid cycle intermediate from the cytosol into the mitochondria leading to an increase in the α-ketoglutarate oxidative decarboxylation resulting in an increase in mitochondrial substrate-level dependent ATP synthesis. This mechanism of metabolic by-pass of a defective ATP synthase unravels the physiological importance of intra-mitochondrial substrate-level phosphorylations. This unexpected result might be of interest for the development of therapeutic solutions in pathologies associated with defects in the oxidative phosphorylation system.

In the inner mitochondrial membrane, the F$_0$F$_1$-ATP synthase performs the late step of the oxidative phosphorylations. This hetero-oligomer uses the electrochemical transmembrane proton gradient generated by the respiratory chain to catalyze ATP synthesis from ADP and inorganic phosphate (1, 2, for review). It is composed of two distinct domains, a membrane integrated F$_0$ domain containing a proton channel and a hydrophilic peripheral F$_1$ domain bearing the catalytic sites for ATP synthesis.

This enzyme comprises about twenty different subunits for an overall molecular mass approaching 600 kDa. The genes encoding these proteins are located part in the nucleus and part in the mitochondrion itself. The subunits of nuclear origin are synthesized in the cytoplasm and then imported into the mitochondrion (3 for review), whereas the mitochondrial DNA encoded subunits are synthesized within the mitochondrion. This genetic compartmentalization makes the assembly of the ATP synthase complex a particularly intricate process. Studies in S. cerevisiae have shown that specific proteins, usually called assembly factors, not belonging to the final complex, are required in the different steps of the enzyme biogenesis. Three such proteins, Atp11p, Atp12p, and Fmc1p,
were shown to facilitate the assembly of the ATP synthase F1 component (4, 5 and 6), which is made of five different nuclear-encoded proteins in the \( \alpha_3 \beta_3 \delta e \) stoichiometry (7). The \( \alpha \) and \( \beta \) subunits alternate in an (\( \alpha \beta \)) hexamer harboring adenine nucleotide binding sites at the \( \alpha / \beta \) interfaces (8). Atp11p and Atp12p were shown to directly interact with the \( \alpha \) and \( \beta \) subunits respectively, presumably to prevent these two F1 subunits from non-productive interactions (4, 5). In the absence of either Atp11p or Atp12p, the \( \alpha \) and \( \beta \) subunits aggregate within large inclusion bodies in the mitochondrial matrix (9, 10). Similar defects in the assembly of the F1 domain were described in yeast lacking Fmc1p (6, 10). However, \( \alpha \) and \( \beta \) subunits aggregation occurs only when the fmc1 mutant is grown at 37°C, indicating that Fmc1p is involved in some assembly heat-sensitive step. The precise function of Fmc1p is still unknown. However, interestingly, \( \Delta fmc1 \) mutant is very efficiently rescued by an increase in the expression of Atp12p (6). Furthermore, Atp12p was found to accumulate very poorly in \( \Delta fmc1 \) cells grown at 37°C, indicating that the aggregation of \( \alpha \) and \( \beta \) subunits in the \( \Delta fmc1 \) mutant is caused by some failure in Atp12p.

The defects in the assembly of the ATP synthase caused by a lack in Atp11p, Atp12p or Fmc1p severely impair yeast growth on non-fermentable carbon sources (4, 5, 6). Interestingly, the \( \Delta \) fmc1 assembly mutants were also shown to grow very poorly in anaerobic conditions (11). Indeed, in the absence of respiration the F1 ATPase activity is required for a proper energization of the inner mitochondrial membrane and hence for a number of essential reactions to take place in the mitochondrion.

It is well known that yeast cells exhibiting mitochondrial dysfunctions - such as loss of the mitochondrial genome, loss of TCA cycle activities or respiration inhibition-underygo, through the so-called retrograde regulation, profound changes in nuclear genes expression resulting in key metabolic readjustments (12 for review). This inter-organelle signaling pathway aims at re-supplying mitochondria with intermediates of the TCA cycle, which can not operate properly in respiratory deficient cells. Indeed, succinate cannot be oxidized to fumarate, which limits the production of oxaloacetate (OAA), and in turn of \( \alpha \)-ketoglutarate, the direct precursor to glutamate. To compensate, the expression of many genes whose products function in anaplerotic pathways that would re-supply mitochondria with OAA and acetyl-CoA, is induced. In addition, the expression of the genes encoding the first three steps of the TCA cycle leading to the production of \( \alpha \)-ketoglutarate comes under the control of retrograde-specific regulatory genes, RTG1, RTG2 and RTG3 (13). These nuclear genes expression modifications result in a higher \( \alpha \)-ketoglutarate production and hence a sufficient glutamate supply in cells harboring dysfunctional mitochondria. However none of these responses have been reported to rescue the respiratory growth defect caused by mitochondrial dysfunction.

In this study, we report that the overexpression of Odc1p - the main form of the yeast oxodicarboxylate carrier (14) - can efficiently rescue the respiratory growth defect of the fmc1 mutant. We show that overexpression of Odc1p acts as a metabolic bypass suppressor for the loss of a functional ATP synthase through an increased intra mitochondrial ATP production via substrate-level phosphorylation. We will discuss this unexpected observation in relation to the retrograde metabolic response, which might contribute to the rescued of the ATP synthase deficiency by Odc1p over-expression.

**MATERIALS AND METHODS**

*Strains and Media* - Strains used in this study were MC1 Mat\( \alpha \) ade2-1 his3-11, 15 trp1-1 leu2-3, 112 ura3-1 [\( \Delta i E^8 O^6 \)] (Wild Type), MC6 Mat\( \alpha \) ade2-1 his3-11, 15 trp1-1 leu2-3, 112 ura3-1 \( \Delta fmc1 \) :HIS3 [\( \Delta i E^8 O^6 \) (\( \Delta fmc1 \))] described in (6), BY4742 Mat\( \alpha \), his3\( \Delta 1 \), leu2\( \Delta 0 \), lys2\( \Delta 0 \), ura3\( \Delta 0 \) (Euroscarf) and Y16480 Mat\( \alpha \), his3\( \Delta 1 \), leu2\( \Delta 0 \), lys2\( \Delta 0 \), ura3\( \Delta 0 \), ypl134c::kanMX4 (Euroscarf). \( \Delta atp11 \) and \( \Delta atp12 \) described in (4, 5) were a kind gift of S. Ackerman (Detroit). Yeast strains were grown either in complete media -YPGA, YPGALA and N3 as described in (6), YPLac (Yeast extract 1%, Bactopeptone 2%, Lactate 2%, KH\(_2\)PO\(_4\) 1%, pH 5.5)- or in minimal media lacking uracile composed with Casamino acids 1%, Yeast Nitrogen Base/ammonium sulfate 0.67% in which carbon sources were either glucose 2% or...
(CasGl), galactose 2% (CasGal) or glycerol/ethanol 2% (CasEG). Minimal media were supplemented with 40 µg.ml⁻¹ tryptophane and 20 µg.ml⁻¹ methionine. In all media, adenine was supplemented 60 µg.ml⁻¹.

Plasmids - pEMBLYe23 was a gift from D. Thomas (Gif-Sur-Yvette). pLL1 is a centromeric plasmid harboring a [HindIII-HincII] fragment containing FMC1 and its regulatory flanking sequences (6). Plasmids pLL9 and pLL11 were isolated in the Δfmc1 multicopy suppressor screen (see below) and contained two ORFs YPL134c and YPL135w. YPL135w was subcloned from pLL11 as a StyI-[HindIII] fragment containing YPL134c [HincI]. The resulting plasmid was named pLL14. A [StyI-Xmal] fragment from pLL11 containing YPL134c was cloned into pFL38L [XbaI-Xmal] to form pFL15 (CEN6). YPL134c was cut out of pLL15 as a [KpnI-SphI] fragment and inserted into pFL44L to give pLL16 (2µ).

Screen for multicopy suppressors of the Null allele Δfmc1 - The Δfmc1 strain, MC6, was transformed with a partial Sau3A digest of yeast chromosomal DNA cloned into the multicopy vector pEMBLYe23 (gift from D. Thomas, Gif-Sur-Yvette). About 100,000 transformants were selected on appropriate selective medium and screened for growth on respiratory medium (N3) at 37°C. Following a 5 days incubation, clones which exhibited a good growth were cured of the URA3 plasmids they harbored, on glucose rich medium supplemented with 0.1% 5-fluoroorotic acid and further tested for growth on respiratory medium N3 at 37°C. When growth in these conditions was found to be plasmid-dependent, the corresponding plasmid was recovered from E. coli and re-tested for suppression of the Δfmc1 phenotype. Confirmed suppressor plasmids pLL9 and pLL11 were further analyzed and the insert they contained sequenced.

Biochemical assays - Cells were pre-grown at 28°C overnight in glucose medium, diluted into CasEG and further grown for two days to enrich the pre-culture in rho⁺ cells. They were then used to set up cultures in CasGal, switched to 37°C and grown to log phase. Cells were harvested for mitochondrial purification according to the enzymatic procedure described in (15). Frequency of petite colonies was checked for every purification and did not exceed 11%. Protein concentration was measured according to Lowry et al (16). Cytochrome content was estimated as described in (17). Oxygen consumption rates were measured at 28°C in respiration buffer containing mannitol 0.65 M, EGTA 0.36 mM, Tris-Maleate 10 mM, pH 6.8, Tris-Pi 3 mM, pH 6.8 as described in (18), with either ethanol 100 mM or α-ketoglutarate 10 mM as substrate. Transmembrane potential variations (ΔΨ) in isolated mitochondria were assayed by monitoring the fluorescence quenching of Rhodamine 123 with a Kontron fluorescence spectrophotometer (19). ATP synthesis rates : mitochondria (0.3 mg.ml⁻¹) were placed in a 1.5 ml thermostatically controlled chamber at 28°C, in respiration buffer. The reaction was started by addition of respiratory substrate and 2 mM ADP and stopped by perchloric acid 7%/EDTA 25 mM. Samples were then neutralized to pH 6.5, by addition of KOH 2 N/MOPS 0.3 M. ATP was quantified by luciferin/luciferase assay (ThermoLabsystems) on a LKB bioluminometer. Participation of the F0F1-ATP synthase to ATP production was assessed by oligomycin (50 µg.mg protein⁻¹) addition. Adenylyl kinase (AK) activity was estimated by comparison of ATP synthesis rates in the presence of oligomycin (50 µg.mg protein⁻¹), antimycin A (0.5 mg.mg protein⁻¹) and arsenite (10 mM) with or without addition of AK specific inhibitor AP5A (5 mM). Oligomycin-sensitive ATPase activity was measured as described by Somlo (20). Spheroplasts were prepared as described in (21). Western Blots were performed as described in (22). Rabbit polyclonal antibodies raised against Odc1p -a kind gift of J. Velours (Bordeaux)- were used 1:4,000. Mouse monoclonal antibodies raised against porin were used 1:5,000 (Molecular Probes).

RESULTS

Isolation of ODCl as a multicopy suppressor of the null allele Δfmc1

To get insights on Fmc1p function, we looked for proteins whose over-expression would compensate the loss of Fmc1p. To this aim, the Δfmc1 strain MC6 was transformed with a yeast chromosomal DNA library (see Materials and Methods section).
Transformants were screened for suppression of the growth defect characterizing the Δfmc1 mutant at elevated temperature-37°C- on respiratory medium. Among the three different inserts which confirmed to be responsible for the rescuing of Δfmc1, the insert of plasmid pLL11 was isolated twice. It proved to contain a [Sau3A] 2,354bp fragment harboring two ORFs, YPL135w encoding Isu1p, a protein performing a scaffolding function during assembly of iron-sulfur clusters (23, 24), and YPL134c encoding the main isoform of the oxodicarboxylate carrier (14). Subsequent sub-cloning allowed us to confirm that ODC1 (14). Subsequent sub-cloning allowed us to confirm that ODC1 (YPL134c) was the sole ORF responsible for the compensation of Δfmc1 growth defect. Indeed pLL14 containing ISU1 (YPL135w) alone was not able to suppress Δfmc1 growth defect (Figure 1). To the contrary, a multicopy plasmid harboring ODC1 alone (pLL16) was able to restore growth of Δfmc1 on respiratory medium at 37°C (Figure 1), to a level that was similar to the one shown by the wild type control (not shown). This compensation was not seen when Δfmc1 was transformed with a centromeric plasmid harboring ODC1 alone (pLL15) indicating that this suppression was dose-dependent (data not shown). As a consequence, to decipher the mechanism of suppression by ODC1, we used the multicopy vector throughout the study. Furthermore, to ease the reading, the Δfmc1 strains transformed with plasmids harboring either FMC1, or ODC1 and the empty vector are referred to as WT, Δfmc1/ODC1 and Δfmc1 respectively.

Odc1p is over-expressed in the Δfmc1/ODC1 strain
To make sure that the oxodicarboxylate carrier (Odc1p) was indeed over-expressed in the Δfmc1/ODC1 strain, we performed western blot analysis on mitochondria purified from each strain grown at 37°C. As previously shown by Palmieri et al. (14), the polyclonal antibody raised against Odc1p revealed a doublet, in which the upper band corresponds to Odc1p as assessed by its absence in the mitochondria purified from the Δodc1 strain (Figure 2 lane 2). Figure 2 clearly shows that whereas a thin band appeared in both WT and Δfmc1 strains, Odc1p expression level was drastically increased in the Δfmc1/ODC1 strain. Indeed, it was at least 10 times higher in mitochondria purified from ODC1 transformed cells than in wild type cells. This confirmed that the suppression observed in the Δfmc1/ODC1 strain was related to Odc1p expression level.

The F0F1-ATP synthase activity is very low in the Δfmc1/ODC1 strain.
Since the over-expression of Odc1p was able to restore growth at 37°C on respiratory substrate in the Δfmc1 strain, we investigated whether this was due to the restoration of a functional F0F1-ATP synthase. Mitochondria purified from WT, Δfmc1 and Δfmc1/ODC1 strains grown at 37°C, were assayed for mitochondrial electrical transmembrane potential (ΔΨ) establishment via fluorescence quenching of the dye Rhodamine 123. Figure 3A (left panel) shows that when mitochondria were isolated from wild type cells grown at 37°C, as expected, ethanol addition induced an important fluorescence quenching due to the establishment of an electrical transmembrane potential. ADP addition induced a decrease of this quenching due to proton reentry through the F0F1-ATPSynthase and ΔΨ consumption by the ATP/ADP carrier. The transmembrane potential increased when most of the ADP had been transformed into ATP. As expected, further additions of KCN and CCCP to these mitochondria lead to a collapse of the membrane potential. As previously shown (6), the mitochondria isolated from the Δfmc1 strain, even though ethanol was able to establish a small electrical transmembrane potential, ADP failed to induce any decrease in ΔΨ (Figure 3A-center). Mitochondria isolated from the Δfmc1/ODC1 strain exhibited a behavior comparable to the one observed for the Δfmc1 mitochondria, even though the ΔΨ established by ethanol addition was higher (Figure 3A-right panel). These experiments indicated that in the Δfmc1/ODC1 strain, the F0F1-ATPSynthase had either no or a very low proton-pumping activity.

In order to further investigate the functionality of the F0F1-ATPSynthase in the Δfmc1/ODC1 strain, we measured the ability of this enzyme to establish a membrane electrical potential when hydrolyzing ATP (Figure 3-B). Mitochondria were first energized with ethanol. This ΔΨ establishment is required to remove IF1p, the natural
inhibitor of the mitochondrial ATPase (25). As expected in the wild type strain, KCN addition induced a collapse in the ΔΨ by inhibition of the respiratory chain (Figure 3-B left). ATP hydrolysis via the F0F1-ATPase induced a ΔΨ establishment, which was fully reversed after oligomycin addition reflecting the specific F0F1-ATPase activity. Further addition of CCCP did not have any effect indicating that the established ΔΨ was F0F1-ATPase dependent. In the Δfmc1 strain, we previously showed that ATP addition promoted a much decreased ΔΨ establishment almost insensitive to F0F1-ATPase inhibition (6). As Δfmc1/ODC1 and Δfmc1 exhibited a similar profile, Δfmc1/ODC1 alone is represented here. In these strains, we observed a very slight oligomycin-sensitive ΔΨ establishment due to ATP hydrolysis indicating that there probably was a very low proton-pumping activity.

These results were further confirmed by assessing the ATPase activity in the three considered strains. Oligomycin-sensitive ATP hydrolysis rates were 264 ± 29 nmol.min⁻¹.mg protein⁻¹, 8 ± 3 nmol.min⁻¹.mg protein⁻¹, 27 ± 3 nmol.min⁻¹.mg protein⁻¹ in WT, Δfmc1 and Δfmc1/ODC1, respectively. This assay revealed, in the Δfmc1 and Δfmc1/ODC1 strains, the presence of a small amount of functional ATPase accounting for around 3 and 10% of the total ATPase activity measured in the WT mitochondria. This result further confirmed what was observed in Figure 3 A and B, which implied that the Δfmc1/ODC1 strain was able to grow on non-fermentable substrate without a significant amount of functional F0F1-ATP synthase.

As described in (6), severe perturbations of the Fo domain were seen in the Δfmc1 strain, including the near-to complete absence of Atp6p. Western blot analysis of mitochondria purified from all three strains revealed that Atp6p accumulation was strongly reduced and hardly detectable in Δfmc1/ODC1 strain as it was in Δfmc1 strain as compared to WT strain (Figure 3-C). Moreover, a BlueNative-gel electrophoresis was performed and failed to show a significant band for the mature F0F1-ATP synthase complex in the Δfmc1 and Δfmc1/ODC1 mutants (not shown). This further confirmed that the growth restoration observed in the Δfmc1/ODC1 strain was not due to the restoration of the F0F1-ATP synthase assembly/stability.

**Respiratory chain function and content are partially restored in the Δfmc1/ODC1 strain**

To characterize oxidative phosphorylation in the mitochondria isolated from each strain, we investigated both phosphorylating respiratory rate and ATP synthesis rate with ethanol as substrate in the presence of a saturating amount of ADP (Figure 4-A). As previously shown, in the wild type strain, both respiratory and ATP synthesis rates were high and the ratio between them (ATP/O), representing the oxidative phosphorylation yield, was around 1.2 (26, see Figure 4-A). In the Δfmc1 strain, the phosphorylating respiratory rate is very low - only 12% of the wild type rate- and there was no detectable oligomycin sensitive ATP synthesis, consistent with previous published results (6) and the above-mentioned results of an absence of functional ATP synthase and a very low content in cytochromes (Figure 4-B). The latter is the sign of a down-regulation of the respiratory complexes expression in dysfunctioning mitochondria consistent with earlier reports (27, 28). In the Δfmc1/ODC1 strain, the phosphorylating respiratory rate was significantly increased as compared to the Δfmc1 strain, up to 42% of the wild type versus 12% for the Δfmc1. These data are consistent with an increase in the cytochrome content in the mitochondria isolated from this strain (figure 4-B). The oligomycin sensitive ATP synthesis rate in Δfmc1/ODC1 represented 20% of the one in the wild type. This was due to a small fraction (10%, see ATPase activities above) of the F0F1-ATP synthase able to properly assemble. Moreover, one might be puzzled by the important increase in cytochrome content in the Δfmc1/ODC1 strain. However, proper assembly of respiratory complexes and ATP synthase complexes require intra mitochondrial ATP (29, 30, 31, for reviews) which is produced in this strain at a much higher rate than in the Δfmc1 strain (see below).

**Substrate-level phosphorylation flux is increased in the Δfmc1/ODC1 strain**

Growth on respiratory substrate absolutely requires ATP synthesis in the mitochondria. Considering the low level of oxidative phosphorylation activity in the
\(\Delta fmc1/ODC1\) mitochondria, we investigated whether any other ATP synthesis occurred. The only alternative to oxidative phosphorylation to produce ATP in a cell is through substrate-level phosphorylation, which, at the mitochondrial level involves the \(\alpha\)-ketoglutarate oxidative decarboxylation. Our previous in vitro studies have shown that the velocity of substrate-level phosphorylation seems controlled only by the \(\alpha\)-ketoglutarate carrier (Odc1p) and the \(\alpha\)-ketoglutarate dehydrogenase complex (26). Since the over-expression of the \(\alpha\)-ketoglutarate carrier was able to restore growth of the \(\Delta fmc1\) mutant, this could imply that the kinetic control at the carrier level was decreased, allowing substrate-level phosphorylation to be carried out at a higher rate in the \(\Delta fmc1/ODC1\) strain, thus allowing ATP synthesis at a rate sufficient to compensate the \(\Delta fmc1\) ATP synthase defect. To test this hypothesis, we investigated substrate-level phosphorylation in mitochondria isolated from each strain grown at 37°C.

In order to assess the rate of these phosphorylations at the mitochondrial level, both respiratory and ATP synthesis rates were measured in the absence and presence of oligomycin with \(\alpha\)-ketoglutarate as substrate (Table 1). In the wild type strain, as previously shown (32), whereas the respiratory rate under phosphorylating conditions was lower than the one with ethanol as substrate (see Figure 4), the ATP synthesis rate was almost two times higher. Consequently the oxidative phosphorylation yield -ATP/O ratio- was 2.4 with this substrate in the absence of oligomycin (Table 1). In the presence of oligomycin, ATP can only be synthesized through substrate-level phosphorylation or adenylylate kinase. It was verified that adenylylate kinase activity did not account for the ATP synthesis rate measured in the presence of oligomycin (not shown). Table 1 shows that both oxygen consumption and ATP synthesis rates were of the same order of magnitude leading to an oxidative phosphorylation yield close to 1. In the \(\Delta fmc1\) strain, the respiratory rate was very low and the ATP synthesis was found to be mostly due to adenylylate kinase activity (i.e. inhibited in the presence of Ap5A, not shown, data are shown corrected of the AK activity). This indicated that in this strain, the mitochondria were not able to use \(\alpha\)-ketoglutarate for substrate-level phosphorylation. In the \(\Delta fmc1/ODC1\) strain, the respiratory rate with \(\alpha\)-ketoglutarate as a substrate, under phosphorylating conditions was higher than the one with ethanol as a substrate, indicating an efficient oxidation of this substrate in these mitochondria. In the absence of oligomycin, the oxidative phosphorylation yield was slightly over 1, which was most probably due to the presence of a few assembled F0F1-ATP synthases in these mitochondria (see ATPase activities above). In the presence of oligomycin, ATP can only be synthesized through substrate-level phosphorylation and both oxygen consumption and ATP synthesis rates were of the same order of magnitude leading to an oxidative phosphorylation yield close to 1. This clearly indicated that in the \(\Delta fmc1/ODC1\) strain, substrate-level phosphorylation was very efficient as opposed to the \(\Delta fmc1\) strain.

Moreover, it should be stressed that in the \(\Delta fmc1/ODC1\) isolated mitochondria, substrate-level phosphorylation flux was three times higher than ATP synthesis through the F0F1-ATP synthase (see Table 1).

**DISCUSSION**

In a previous paper, we showed that the \(FMC1\) nuclear gene, encoding a soluble protein localized to the mitochondrial matrix, is required for proper growth at elevated temperature in respiratory medium (6).

In order to better understand and characterize the function of Fmc1p, we looked for extragenic multicopy suppressors of the \(\Delta fmc1\) mutant growth defect at elevated temperature. As previously reported, we isolated Atp12p as a suppressor whose over-expression compensates the loss of Fmc1p via its chaperone role in the assembly/stability of the F1-ATP synthase (6). We thus proposed that Fmc1p is involved in the proper folding/stability or functioning of Atp12p in heat stress conditions. Surprisingly enough, in this same screen, we also isolated \(\text{ODC1}\), encoding the main isoform of the \(\alpha\)-ketoadipate carrier originally identified and characterized by Palmieri *et al.* (14). The main physiological role of Odc1p is probably to exchange \(\alpha\)-ketoadipate and \(\alpha\)-ketoglutarate from the mitochondrial matrix and the cytosol respectively (14). \(\alpha\)-ketoadipate is then used in lysine biosynthesis and \(\alpha\)-ketoglutarate enters
the Krebs cycle. The question arose to understand how the over-expression of this carrier could compensate the loss of a functionally assembled ATP synthase. We first showed that Odc1p over-expression did not compensate Fmc1p loss by restoring a sufficient amount of functional ATP synthase contrary to what was observed with Atp12p over-expression (6).

Characterization of the mitochondria isolated from the wild type strain, the Δfmc1 strain or the Δfmc1/ODC1 strain allowed us to show that in the Δfmc1 strain, the oxidative phosphorylations were not functional as assessed by the substrates oxidation and ATP synthesis rates, consistent with the measured cytochrome contents and previous published results (6). Lower respiratory chain contents were described as well in Δatp11 and Δatp12 mutants (33). This tends to indicate a retrograde signaling from mitochondria to nucleus involving a feedback loop in mitochondrial function. When oxidative phosphorylation functions are abolished, all the complexes seem to be downregulated. This observation is consistent with the genome-wide study of responses to mitochondrial dysfunction showing that the mRNA levels of several subunits of the cytochrome c oxidase i.e. Cox12p, Cox13p, Cox6p, decrease when mitochondria are incubated with oligomycin (27). Moreover, several mutants of the ATP synthase were shown to exhibit considerably reduced levels of NADH cytochrome c reductase and cytochrome oxidase (28).

Interestingly, respiratory rates were largely increased in the Δfmc1/ODC1 strain, indicating that Odc1p over-expression allowed a significant restoration of the amount of respiratory chain in the Δfmc1 mitochondria. In this strain, ATP production is greatly enhanced as described below allowing mitochondrial respiratory chain complexes biogenesis to occur. Indeed, crucial mitochondrial ATP-dependent processes such as those involving i) conserved proteases ensuring the quality control of mitochondrial proteins which controls essential steps in the mitochondrial biogenesis i.e. m-AAA proteases, Pim1p (29, 31, for reviews) or ii) Hsp70 family chaperones, mediating protein translocation i.e. Ecm10, proper import and folding i.e. Ssc1p and iron sulfur clusters assembly i.e. Ssq1 (34, 35 and 31 for review), can then occur properly.

Moreover, it appears that Odc1p over-expression allows the cells to grow on respiratory substrate thanks to substrate-level phosphorylation. Indeed, we have shown that when mitochondria isolated from the Δfmc1/ODC1 strain were incubated with α-ketoglutarate, they were able to produce ATP through substrate-level phosphorylation at a rate comparable to the wild type one. Furthermore, in the presence of saturating amounts of substrate, the rate of phosphorylation with α-ketoglutarate i.e. substrate-level phosphorylation, is three times higher than the one measured with ethanol as substrate i.e. oxidative phosphorylation.

The over-expression of Odc1p most likely decreases the kinetic control of this carrier on the α-ketoglutarate oxidation flux. This kinetic control is redistributed, mostly to the α-ketoglutarate dehydrogenase (26) which produces NADH (Figure 5). NADH has to be reoxidized through the respiratory chain. Unless this NADH was reoxidized at a sufficient rate, substrate-level phosphorylations rate would not be increased. This metabolic compensation was possible only because respiration rate and ATP synthase activity were not totally abolished in the Δfmc1 mutant. Indeed in mutants where ATP synthase activity is abolished or reduced to the point that it prevents any respiratory growth, such as Δb(not shown), E430 (not shown), a point mutant of the β subunit in which the ATP synthase is assembled (36) and Δatp12 (Figure 6-left), Odc1p over-expression did not restore any growth on respiratory substrate. Conversely, in the Δatp11 mutant, we observed a significant compensation of the very severe growth defect characterizing this mutant on a respiratory substrate (Figure 6-right). This mutant, Δatp11, harbors a very discrete leaky phenotype for growth on respiratory medium probably reflecting an extremely low ATP synthase activity. It should be stressed here that Δatp11 is considerably more affected in its respiratory growth than Δfmc1 is. However, the key point is the presence of a few functional ATPases in the mutant strain to start with. This condition was required for a potential biogenesis of respiratory chain through ATP-dependent processes to occur (see above and 29, 30, 31 for reviews). As a consequence over-expressing Odc1p in Δfmc1 lead to an increase
in respiratory chain content which in turn allowed a more efficient reoxidation of the NADH produced via α-ketoglutarate oxidation (Figure 5). The overall process induced, in a synergistic way, a high rate of ATP synthesis.

Moreover, ATP synthase and ATPase activities were slightly increased in the Δfmc1/ODC1 mutant. One could hypothesize that the increased production of ATP through substrate-level phosphorylation might allow another chaperone -ATP-dependent- to partially take over after Fmc1p to lead to a better assembly of the ATP synthase. The first candidate could be Atp12p whose over-expression, of even one sole extra copy, was previously shown to compensate Fmc1p loss (6). This hypothesis is consistent with the absence of compensation of Δatp12 by Odc1p over-expression.

For Δfmc1/ODC1 cells to be able to grow with a very low content of functional ATP synthase by producing ATP through substrate-level phosphorylation, they must be able to supply enough metabolites to the α-ketoglutarate/succinate by-pass (see Figure 5). Previous studies have shown that as cell oxidative phosphorylation function is impaired, numerous genes whose products are involved in the TCA cycle and the glyoxylate cycle CIT2 gene, switch from HAP control to control by three genes, RTG1, RTG2, and RTG3 (12). The RTG genes have been shown to control the retrograde pathway, defined as a change in the expression of a subset of nuclear genes, e.g. the glyoxylate cycle CIT2 gene, in response to changes in the functional state of mitochondria (36, 37, 12 for review). Liu and Butow (13) have proposed that in cells with compromised mitochondrial function, the RTG genes take control of the expression of genes leading to the synthesis of α-ketoglutarate to ensure that sufficient glutamate is available for biosynthetic processes and that increased flux of the glyoxylate cycle, via elevated CIT2 expression, provides a supply of metabolites entering the TCA cycle, sufficient to support anabolic pathways. Here, we provide an additive role for this increased α-ketoglutarate biosynthesis i.e. increase in the substrate-level phosphorylation when mitochondrial oxidative phosphorylations are deficient. However, this compensation requires an increase in the amount of Odc1p carrier in order to decrease the kinetic control of this carrier on the ATP production flux.

All in all, the observed respiratory growth of Δfmc1/ODC1 strain could be the result of synergistic effects combined to a balance of threshold effects. Δfmc1 mutation is sensed by the cell as a mitochondrial dysfunction, which most likely initiates the readjustments of carbohydrate metabolism through the retrograde regulation. These metabolic readjustments combined to Odc1p over-expression lead to an increased production of α-ketoglutarate (see Figure 5), and increase the flux of mitochondrial ATP produced through substrate-level phosphorylation. Increased mitochondrial ATP allows some assembly of the ATP synthase, possibly related to some compensation of Fmc1p absence by Atp12p, which in turn induces the up-regulation of the respiratory chain complexes biogenesis. Functional respiratory chain permits the reoxidation of NADH, which drives the flux through substrate-level phosphorylation.

This kind of compensation could be of interest for clinical research on pathologies associated with F0F1-ATP synthase dysfunctions. Within the last 15 years, F0F1-ATP synthase dysfunctions have been shown to be responsible for severe mitochondrial diseases (39, for review). In maternally inherited defects due to mutations in the mtDNA, namely in ATP6 (NARP syndrome, LHON disease…), the F0F1-ATP synthase is structurally and functionally altered, whereas defects due to nuclear genome mutations are characterized as a selective decrease of ATPase content of otherwise normal enzyme. The latter situation could be compared to the Δfmc1 nuclear mutant, in which at non-permissive temperature, only a very small proportion of F0F1-ATP synthase is properly assembled. In this case, ATP production is probably not the only element responsible for growth impairment on respiratory medium. The lack of sufficient functional ATP synthase leads to stringently reduced respiratory chain activity which is highly likely to increase the production of ROS. Here, we showed that when increasing the ATP supply by a process which indirectly allows the respiratory chain to function at a higher rate, close-to wild type growth on a respiratory substrate could occur. This could give way to new therapeutic approaches to mitochondrial diseases involving a lack of ATP production through F0F1-ATP synthase. Supporting this idea,
Weinberg et al. (40), showed that mitochondrial dysfunctions occurring in kidney proximal tubule cells during hypoxia/reoxygenation could be corrected by Krebs cycle metabolites (i.e. α-ketoglutarate ± Malate) generating ATP and maintaining $\Delta \Psi$ via respiratory chain complex I, which the authors showed was responsible for the maintenance of $\Delta \Psi$ in hypoxia/reoxygenation - rather than the F$_{0}$F$_{1}$-ATPase.

To our knowledge, this is the first report of yeast cells able to grow on respiratory substrate without a sufficient amount of functional ATP-synthase by producing, through substrate-level phosphorylation, enough ATP to sustain life.

REFERENCES

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**FOOTNOTES**

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1 Abbreviations: CCCP, carbonyl cyanide m-chlorophenylhydrazone; ΔΨ, transmembrane electrical potential; AP5A, P1,P5-di-(adenosine-5') pentaphosphate; IF1, endogenous F1-ATPsynthase inhibitor; TCA, tricarboxylic acid; mtDNA, mitochondrial DNA; ROS, Reactive Oxygen Species; NARP, Neuropathy Ataxia Retinis Pigmentosum, LHON, Leber’s hereditary optic neuropathy.
Table 1: Respiration and ATP synthesis rates with α-ketoglutarate as substrate. Purified mitochondria (0.3 mg.ml⁻¹) were incubated in respiration buffer with 10 mM α-ketoglutarate. State 3 respiration state and ATP synthesis rates were obtained by adding ADP (2 mM). Oligomycin was used at 50 μg.mg protein⁻¹. Data are means of at least 3 experiments performed with 2 different mitochondrial preparations.

Figure 1: Phenotype of the different strains at non-permissive temperature. pLL11 is the plasmid isolated from Δfmc1 transformant able to grow on non fermentescible medium at 37°C. ODC1 ORF alone was sub-cloned onto a multicopy plasmid pFL44L. The resulting plasmid was named pLL16. Cells were grown overnight at 37°C in glucose medium and serial dilutions were spotted onto either glucose or glycerol medium and incubated for three days at 37°C.

Figure 2: Odc1p steady-state level in mitochondria isolated from WT, Δfmc1 and Δfmc1/ODC1 strains. ΔODC1 is the Euroscarf deletion strain for YPL134c and WT(ODC1) its corresponding wild type strain (BY4742). Prior to mitochondria purification, WT(ODC1) and Δoec1 cells were grown in YPLac at 28°C, while other strains were grown in CasGal at 37°C as described in the Materials and Methods section. Differences in growth conditions explain the discrepancies in Odc1p level in the two WT strains. 50 μg of purified mitochondria were loaded onto each lane.

Figure 3: The F₀F₁-ATP synthase activity is very low in the Δfmc1/ODC1 strain. A and B- Rhodamine 123 monitored ΔΨ variations were assayed in isolated mitochondria (0.3 mg.ml⁻¹) of WT, Δfmc1 and Δfmc1/ODC1 strains (A and B). Additions were 100 mM ethanol (EtOH), 50 μM ADP, 200 μM KCN, 1 mM ATP and 3 μM CCCP. Transmembrane potential maintained proton pumping activity was visualized by addition of oligomycin (50 μg.mg protein⁻¹). C- Atp6p accumulation was visualized by western blot analysis on mitochondria isolated from WT, Δfmc1 and Δfmc1/ODC1 strains.

Figure 4: A- State 3 respiratory rate and oligomycin-sensitive ATP synthesis rate in mitochondria from all three strains with ethanol as substrate. Mitochondria (0.3 mg.ml⁻¹) purified from WT, Δfmc1 and Δfmc1/ODC1 strains were incubated in respiration buffer. State 3 respiration (closed bars) and ATP synthesis (open bars) rates were obtained after addition of ADP (2 mM). Oligomycin was used at 50 μg.mg protein⁻¹. Values are means determined from at least 3 different mitochondrial preparations. B-Cytochrome content of mitochondria purified from all three strains. Mitochondria (3 mg.ml⁻¹) purified from WT, Δfmc1 and Δfmc1/ODC1 strains were assayed in a double-beam spectrophotometer Amico DW2000 as in (16). Spectra for c+c1, b and aa3 hemes are presented as reduced minus oxidized spectra.

Figure 5: Scheme for by-pass suppression of Δfmc1 by Odc1p over-expression. In the whole cell, a citrate/malate or oxaloacetate counter-exchange mediated by Odc1p may occur. Citrate produced in the glyoxylate cycle and transported into the mitochondria by Odc1p (13), can enter the TCA cycle and lead to the production of succinate coupled to substrate-level phosphorylation. Either Malate or Oxaloacetate can then be transported to the cytosol via Odc1p and enter the glyoxylate cycle. This cycle of reactions can be self-maintained since acetyl-CoA is supplied through fatty acids degradation which is increased as peroxisomes proliferate dramatically in retrograde regulated cells (12, for review). RC stands for respiratory chain.

Figure 6: Odc1p over-expression partially restored growth on respiritory substrate of Δatp11 but
**not Δatp12.** Cells were grown overnight at 28°C in glucose medium and serial dilutions were spotted onto glycerol medium and incubated for eight to ten days at 28°C.
### Table 1

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Figure 1

Δfmc1/pLL16
Δfmc1/pLL11
Δfmc1/pFL44L
Δfmc1/pLL14

Glc

Gly

pLL16

pLL11

pFL44L

pLL14

ODC1

ISU1

ISU1
Figure 2

WT(ODC1) Δodc1 WT Δmec1 Δmec1/ODC1

--- Odc1p

--- Porin
Figure 4

A-

\[
\begin{align*}
\text{Δfmc1/ODC1} & \quad \text{Δfmc1WT} \\
\text{JO}_2 \text{ natO/min/mg protein} & \quad \text{Or} \\
\text{J}_{\text{ATP}} \text{ nmol/min/mg protein} & \quad \text{Or}
\end{align*}
\]

B-

[Graphs showing O.D. (A.U.) vs. λ nm for WT, Δfmc1, and Δfmc1/ODC1.]
Figure 6
Increasing mitochondrial substrate-level phosphorylation can rescue respiratory growth of an ATP synthase deficient yeast
Christine Schwimmer, Linnka Lefebvre-Legendre, Malgorzata Rak, Anne Devin, Piotr Slonimski, Jean-Paul di Rago and Michel Rigoulet

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