GROWTH YIELD HOMEOSTASIS IN RESPIRING YEAST IS DUE TO A STRICT MITOCHONDRIAL CONTENT ADJUSTMENT

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Running title : Cell growth homeostasis through mitochondrial adjustment

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Abstract :
In living cells, growth is the result of coupling between substrate catabolism and multiple metabolic processes taking place during net biomass formation and cell property maintenance. A crucial parameter for growth description is its yield i.e. the efficiency of the transformation from substrate consumption to biomass formation. By using numerous yeast strains growing on different respiratory media, we show that the growth yield is identical whichever the strain, the growth phase and the respiratory substrate used. This homeostasis is the consequence of a strict linear relationship between growth and respiratory rates. Moreover, in all conditions tested, the oxygen consumption rate is strictly controlled by the cellular content of respiratory chain compounds in such a way that, in vivo, the steady state of oxidative phosphorylation is kept constant. Thus the growth yield homeostasis depends on the tight adjustment of the cellular content of respiratory chain compounds to the growth rate. Any process leading to a defect in this adjustment allows an energy waste and consequently an energy yield decrease.

Introduction
In living cells, growth is the result of coupling between substrate catabolism and multiple metabolic processes which can be assumed to take place during net biomass formation and maintenance processes (i.e. maintenance of ionic gradients, protein, lipid and nucleic acid turnover; 1, 2). During the last two decades, the complete thermodynamic description of growth processes has been obtained by establishing
the balanced chemical reactions for anabolism and catabolism. A crucial parameter for growth evaluation is its yield, i.e. the efficiency of the transformation processes (from substrate consumption to biomass formation). The quantification of enthalpy efficiency (the energy converted into biomass divided by the energy input) has been successfully achieved for microorganisms (3-6) as well as for cultured mammalian cells (7, 8). This approach is based on the continuous measurement of heat production and on the exhaustive determination of substrates and by-products, thus allowing the construction of enthalpy balances (see 2 and 9 for reviews). Using this approach, we have previously shown that the enthalpic growth yield remained constant during any growth phase in yeast on respiratory substrate (i.e. exponential and transition phase, see 10). This constant enthalpic growth yield is owed to a tight adjustment of mitochondrial enzyme content to cellular energy demand (10). This indicates that molecular mechanisms are involved in the growth yield homeostasis, which allows a constant growth yield all throughout growth for a considered strain. However, when one is able to by-pass the mitochondrial enzyme content adjustment, for example through overactivation of the Ras/cAMP pathway, the growth yield is largely decreased (11). This led us to wonder whether any and every yeast strain has its own growth yield. In this paper, we describe a new and very simple method to assess the enthalpic growth yield through the amount of oxygen consumed to generate 1 mg of biomass, which allowed the study of numerous strains. The main result of this study is that the growth yield is identical whichever the strain and the growth phase, clearly pointing to a homeostasis process. We also show that the cellular respiratory rate is always strictly controlled by the respiratory chain content. Thus the growth yield homeostasis is based on the tight adjustment of the cellular content of respiratory chain compounds to the growth rate. Any process leading to a defect in this adjustment allows an energy waste and consequently an energy yield decrease.

**Experimental procedures**

**Strains used in this study:**

1. **W303-1A**: Mata leu2-3, 112 ura3-1 trp1-1 his3-11,15 ade2-1 can1-100 GAL SUC
2. **YSH652**: Mata leu2-3, 112 ura3-1 trp1-1 his3-11,15 ade2-1 can1-100 tps1::TRP1 tps2::LEU2 GAL SUC
3. **YSH286**: Mata leu2-3, 112 ura 3-52 trp1-92 GAL SUC
4. **YSH672**: Mata leu2-3, 112 ura3-1 trp1-1 his3-11,15 ade2-1 can1-100 tps2::LEU2 GAL SUC
5. **BY4742**: MATa his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 tpk1:: kanMX4 (euroscarf)
6. **Y11261**: MATa his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 tpk1:: kanMX4 (euroscarf)
7. **Y11089**: MATa his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 tpk2:: kanMX4 (euroscarf)
8. **Y15016**: MATa his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 tpk3:: kanMX4 (euroscarf).
9. **OL556**: a/a, cdc25-5/cdc25-5, his3/his3, leu2/leu2, rca1(pde2)/rca1, TRP1/trp1, ura3/ura3
10. **W303**: a/a ade2-10/ade2-10 his3-11,15/his3-11,15; leu2-3, 112/leu2-3/112 ; ura3-52/ura3-52; can1-100/can1-100; trp1-Δ1/trp1-Δ1. +pYeDP-UCP1 11
11. **BD12-3B**: a met-, ura3-8, ccs1-1
12. **SDC6**: Mata leu2-3, 112 ura3-1 trp1-1 his3-11,15 ade2-1 can1-100 GAL SUC atp16::kanMX4 + pSDC13
13. **Yeasts foam**

**Growth media:**

a. **YPL2%**: 1% yeast extract, 1% bactopeptone 0.1% KH₂PO₄, 0.12 ammonium sulfate, 2% lactate, pH 5.5
b. **YPEG**: 1% yeast extract, 1% bactopeptone, 50mM KH₂PO₄, 80mg/ml adenine, 3% glycerol, 2% ethanol pH 6.2
c-SML2% : 0.17% Yeast nitrogen base w/o aminoacids w/o ammonium sulfate, 0.1% KH₂PO₄, 0.5% ammonium sulfate, 2% lactate pH 5.5. The concentration of auxotrophic requirements was 100mg/l

*d-SML0.2% : 0.17%, Yeast nitrogen base w/o aminoacids w/o ammonium sulfate, 0.1% KH₂PO₄, 0.5% ammonium sulfate, 0.2% lactate pH 5.5. The concentration of auxotrophic requirements was 100mg/l

e-SCL2% : 0.17% Yeast nitrogen base w/o aminoacids w/o ammonium sulfate, 0.1% KH₂PO₄, 0.5% ammonium sulfate, 2% lactate pH 5.5. 1g/l casein hydrolysate. The concentration of auxotrophic requirements was 100mg/l

f-SCL0.2% : 0.17% Yeast nitrogen base w/o aminoacids w/o ammonium sulfate, 0.1% KH₂PO₄, 0.5% ammonium sulfate, 0.2% lactate pH 5.5. 1g/l casein hydrolysate. The concentration of auxotrophic requirements was 100mg/l

Respiratory rate measurements:

The oxygen consumption of cells was measured polarographically at 28 °C using a Clark oxygen electrode in a 1ml thermostatically controlled chamber. Respiratory rates (JO₂) were determined from the slope of a plot of O₂ concentration versus time. Respiration assays of growing cells were performed in the growth medium, except in the case of uncoupled respiration rate which was performed after cells were harvested in the following buffer: 2 mM magnesium sulfate, 1.7 mM sodium chloride, 10 mM potassium phosphate, 10 mM glucose and 100 mM ethanol, pH 6.8.

Determination of biomass over oxygen ratio

Growth was measured spectrophotometrically in different media by assessing the turbidity at 600 nm. Dry weight determinations were performed on samples of cells harvested throughout the growth period and washed twice in distilled water. For each strain the relationship between dry weight and optical density was determined.

The biomass over oxygen ratio (1/QO₂) was the inverse of the integrated value of oxygen consumption by cells during the time necessary to increase the biomass of 1 mg dry weight. It was expressed as mg dry weight/µat O.

Cytochrome content determination

The cellular content of c+c, b, and a+a hemes was calculated as described in Dejean et al (10) taking into account the respective molar extinction coefficient values and the reduced minus oxidized spectra recorded using a dual beam spectrophotometer (Aminco DW2000).

Results:

Growth yield homeostasis

During the aerobic metabolism of mammalian cells, the calorimetric-respirometric ratio (i.e. CR ratio, defined as the ratio of heat production flux to oxygen consumption flux) has been proposed for assessing metabolic efficiency (12, 13). A disproportional increase in heat production compared to oxygen uptake has been observed in isolated cells under conditions of enhanced futile substrate cycling (12, 13) and uncoupling of oxidative phosphorylation (14). However, such high CR ratio values have been reinterpreted as a consequence of an increase in glycolysis under aerobic conditions rather than as a change in metabolic efficiency per se (15, 16). Although on-line calorimetry has been widely used to detect transitions in global metabolic activity during the growth of microorganisms (6, 17-
20 and see 2 for review), the relationships between oxygen consumption flux and heat production rate are poorly documented (but see 21). We have previously developed a respirometric and calorimetric approach to determine the enthalpy efficiency of respiration-linked energy transformations of isolated yeast mitochondria and yeast cells under resting and growing conditions (22). In contrast to enthalpic balance approaches, this method does not rely on the exhaustive and tedious determination of the metabolites and elemental composition of biomass. Moreover during the complete combustion of purely respiratory substrate such as lactate each thermodynamic step corresponding to the consumption of one atom of oxygen corresponds to the same variation in enthalpy (21). Since calorimetric-respirometric ratio assessment is a technique which requires heavy and rare equipment (microcalorimeter) we envisioned the possibility that the measure of both oxygen consumption and biomass would allow us to indirectly assess the enthalpic growth yield.

When yeast cells are grown on a purely respiratory substrate, biomass generation is entirely connected to substrate oxidation through oxidative phosphorylation and hence to oxygen consumption. In a previous study, we have shown that either uncoupling or overactivation of the Ras/cAMP pathway lead to a decrease in the enthalpic growth yield through uncoupling between biomass synthesis and catabolism (10, 11). These peculiar conditions gave us a broad range of enthalpic growth yields, which were connected to the biomass over oxygen ratio under the same experimental conditions. Figure 1 clearly shows that there is a unique linear relationship between enthalpic growth yield and the biomass over oxygen ratio. This ratio is defined as the inverse of the amount of oxygen necessary to generate 1mg dry weight of biomass. This validates our hypothesis and by measuring both respiratory and growth rates, we have a good estimate of the growth yield. Moreover, this method which has the advantages of being quick and simple allowed us to investigate (i) whether the fact that enthalpic growth yield does not vary during growth is a general rule whichever the yeast strain and the respiratory medium; (ii) whether the growth yield is a parameter which varies as a function of the yeast strain considered and/or the respiratory medium or a general feature which would be constant regardless of the strain.

We measured the spontaneous respiratory rate as well as the growth rate of numerous yeast strains in different growth phases and in different respiratory media. Figure 2 shows that there is a linear relationship between these two parameters on a broad range of values (respiratory rate varied from 60 to almost 350 natO/min/mg dry weight). This shows that there is a tight adjustment between growth and respiratory rates, which is further confirmed by the fact that the growth yield expressed as the biomass over oxygen ratio remains almost constant. This shows that there is an actual homeostasis of growth yield in yeast.

**Loss of growth yield homeostasis**

However, from figure 1 one can see that under peculiar conditions (uncoupling, overactivation of the Ras/cAMP cascade), the enthalpy growth yield is decreased, thus indicating that this homeostasis in growth yield can be lost. Obviously, when the coupling between respiratory chain and ATP synthase activities is altered, the growth yield must be affected by the increase in proton permeability of the inner mitochondrial membrane. This is clearly shown in figure 3: when uncoupling of oxidative phosphorylation is achieved either by means of UCP1 expression (22) or controlled depletion of the δ subunit of the ATPsynthase
(23), cells have a higher respiratory rate than control cells which is not associated with a concomitant increase in growth rate. Consequently, there is a loss in growth yield homeostasis for these cells, the biomass over oxygen ratio is significantly decreased compared to wild type cells. Furthermore, previous work from our laboratory has shown that overactivation of the Ras/cAMP cascade induces a deregulation between the mitochondrial enzyme content and the growth rate (11, 24). Indeed, these mutants present an increase in the amount of mitochondrial enzyme content, which does not lead to a concomitant increase in growth rate. Depending on the kind of overactivation induced (Ras2val19, cAMP addition), the increase in respiratory rate can be more or less important. This increase in respiratory rate from 180 to 340 natO/min/mg dry weight do not lead to any increase in growth rate, thus under these conditions the relationship between respiratory rate and growth rate reaches a plateau (Figure 3). Consequently, there is an exponential decrease in the biomass over oxygen ratio (see figure 3). It is noteworthy that whatever the means (uncoupling of oxidative phosphorylation or overactivation of the Ras/cAMP cascade) by which the tight adaptation between growth rate and respiratory rate is lost, there is the same relationship between either respiratory rate and growth rate or respiratory rate and biomass over oxygen ratio. This is indeed noteworthy since the increase in respiratory rate has very different origins in the two situations: (i) UCP1 expression or controlled deletion of the ATP synthase δ subunit induce an increase in respiratory rate by inducing an increase in mitochondria inner membrane proton leak; (ii) in the case of the overactivation of the Ras/cAMP cascade, the increase in respiratory rate originates in an increase in the amount of mitochondrial enzymatic content. In this case the mitochondria have been shown to be well differentiated and coupled (not shown but see 11).

Control of the cellular respiratory rate

We then investigated the molecular basis of the homeostasis described above. Under conditions where yeast is grown on purely respiratory substrates, ATP synthesis mostly occurs within oxidative phosphorylation. A constant growth yield indicates that the part of ATP turnover involved in either biosynthesis or maintenance is constant. Yeast cells growth is characterized by two phases : an exponential one and the transition phase. We have shown that during the last one the decrease in growth rate is tightly linked to a decrease in mitochondrial enzyme content in such a way that the enthalpic growth yield is kept constant (10). The question which is now raised is whether the fact that the growth yield is the same for any studied strain implicates that there is a tight adjustment between growth rate and the mitochondrial enzyme content. The amount of respiratory chains within a cell can be estimated through the cellular amount of mitochondrial cytochromes. We thus assessed the mitochondrial cytochrome content in the different strains under consideration, in different respiratory media and growth phases. If one plots the respiratory rate in these cells against the amount of each kind of cytochrome, one can clearly see that there is a linear relationship between these two parameters indicating that respiratory rate in this case is mostly controlled by the amount of respiratory chain (figure 4). Since homeostasis is due to an adjustment between respiratory and growth rates, this implies that the mitochondrial enzyme content controls the growth rate. The question then arises to understand what controls the respiratory rate under conditions where the homeostasis is lost. We considered the loss of homeostasis
due to overactivation of the Ras/cAMP pathway since in this case the oxidative phosphorylation remained well coupled. Figure 4 shows that in this case, the relationship between respiratory rate and cytochrome content is identical to the one obtained in the case of homeostasis. Therefore, cellular respiratory rate is always tightly controlled by cytochrome content.

Discussion

In this paper, we unambiguously show that the determination of the total amount of oxygen consumed to generate 1 mg cell dry weight during growth on purely respiratory substrate allows an easy assessment of the growth yield. We studied multiple strains and showed that the growth yield is maintained constant (i) regardless the strain considered (ii) regardless the growth phase (iii) regardless the respiratory substrate. In any wild type yeast strain, there is an actual homeostasis of growth yield which is due to a tight adjustment of respiratory rate to the growth rate leading to a constant energy waste. Whichever the pathway(s) allowing such an adjustment it is clear that this process involves a good adjustment between the amount of cytochromes and the growth rate.

In previous studies, Boumans et al (25) using a number of strain varying in steady state levels of assembled bc1 complex have shown that there is a linear relationship between the level of bc1 complex and the respiratory rate of growing cells (see also 26). Moreover, they observed that a reduced level of bc1 complex is associated with a parallel decrease in steady state amount of cytochrome oxidase. They proposed that the respiratory chain in yeast behaves as a single functional unit (24 see also 27 and 28). Indeed, Schägger has shown that Complex III and IV are assembled into large supramolecular complexes. On the same line but using another approach, we have shown that there is a very specific competition between electrons to enter the respiratory chain which is also in favor of a supramolecular organization of the respiratory chain, in which the relative ratio between any cytochrome remains constant (28). This is in close agreement with the results presented in this paper since we observed a parallel variation in all the cytochromes for the different strains considered. It should be stressed that for any of the cytochromes, there is a linear relationship between the cellular respiratory rate and the cytochrome amount crossing through the origin (see figure 4). This clearly indicates that respiratory rate is fully controlled by each component of the respiratory chain. Such a fact indicates that there are respiratory chain units in which the electrons are channeled along the respiratory chain complexes. This is a strong argument in favor of a supramolecular organization of the mitochondrial respiratory chain in yeast which is a hypothesis that clearly accounts for the full kinetic control of the oxygen consumption flux by each of the electron carriers.

One of the pathways involved in the tight adjustment between the growth rate and the amount of cytochromes has been shown to be the Ras/cAMP pathway which is involved in nutrient sensing. Whenever the activity of this pathway is increased, Ras2Val19 strain or OL556 strain in the presence of cAMP, the energy waste is increased due to the loss of adjustment of the respiratory rate to the growth rate in such a way that respiratory rate is increased whereas growth rate remains constant. Indeed, in these mutants, the relationship between the amount of cytochromes and the respiratory rate is identical to the one obtained in various wild type strains (see figure 4). Consequently, the oxidative phosphorylation stationary state remains constant i.e. the amount of oxygen consumed per minute and per unit of respiratory chain is constant. It is worth-noting that there are physiological advantages...
for the cell to keep a constant stationary state in oxidative phosphorylation at about 70% of the maximal rate of respiration observed in presence of uncoupler. Indeed, higher respiratory rate requires a decrease in phosphate potential (i.e. the free energy in ATP synthesis) which would impair the cell functions; but to lower the respiratory rate leads to an increase in protonmotive force which is well known to induce an increase in mitochondrial generation of reactive oxygen species. Indeed this last situation has been observed when yeast cells enter the stationary phase and lead to a large enhancement of protein oxidation (29).

The fact that there is a unique relationship between respiratory rate and cytochrome content implies that respiratory rate and consequently ATP synthesis do not adjust to cellular energy demand. Indeed, in mutants overactivated in the Ras/cAMP pathway, both cytochrome content and respiratory rate increased whereas the growth rate did not increase leading to a decrease in growth yield which implies an increase in energy waste. Consequently, the homeostasis in growth yield is due to the ability of cells to adapt the mitochondrial enzyme content to the growth rate. Even though the precise molecular mechanisms of this process are unknown to this day, it is clear that the Ras/cAMP pathway plays an important role in this regulation.

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Legends to figures:

**Figure 1:** *Relationship between the enthalpic growth yield and the biomass/QO_2 ratio.*
Enthalpic growth yield was determined as described in (22). Biomass and QO_2 were determined as described in the experimental procedures section. Experimental values correspond to the following conditions: ● OL556 strain, SCL2%; ○ OL556 strain, SCL0.2% cAMP 3mM; ■ OL556 strain, SCL2% cAMP 3mM, ♦ W303+pYeDP-UCP1 SCL0.2%; * W303+pYeDP-UCP1Δ9 SCL0.2%.

**Figure 2:** *Relationship between either growth rate and respiratory rate or growth yield and respiratory rate.* Biomass and QO_2 were determined as described in the experimental procedures section. Numbers and letters correspond to the strain and the growth medium respectively (see experimental procedures). * corresponds to the transition phase (between the exponential and the stationary phase). □ Growth yield; ■ Growth rate.

**Figure 3:** *Relationship between either growth rate and respiratory rate or growth yield and respiratory rate in mutants of the Ras/cAMP pathway and partially uncoupled strains.* Biomass and QO2 were determined as described in the experimental procedures section. Numbers and letters correspond to the strain and the growth medium respectively (see experimental procedures). When added cAMP was 3mM. * refers to the Ycp50-Ras2val19 plasmid (30). 0.25 and 0.5 refer to the amount of doxycycline added to the growth medium (µg/ml). Dots represent strains with an overactivation of the Ras/cAMP pathway whereas diamond represent strains in which oxidative phosphorylation are mildly uncoupled. ○○ growth
yield; ⚫ growth rate. Dotted lines refer to figure 2 (growth rate and growth yield evolution as a function of respiratory rate).

**Figure 4: Relationships between respiratory rate and cytochrome content.** Respiratory rate and cytochrome content were determined as described in the experimental procedures section. Numbers and letters correspond to the strain and the growth medium respectively (see experimental procedures). Open symbols refer to mutants of the Ras/cAMP cascade. Closed symbols refer to wild type strains.
Figure 1
Figure 2
Figure 3
Figure 4
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