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cAMP-induced modulation of the growth yield of *Saccharomyces cerevisiae* during respiratory and respiro-fermentative metabolism

Laurent Dejean, Bertrand Beauvoit *, Ana-Paula Alonso, Odile Bunoust, Bernard Guérin, Michel Rigoulet

Institut de Biochimie et Génétique Cellulaires du CNRS, Université Victor Ségalen-Bordeaux 2, 1 rue Camille Saint Saëns, 33077 Bordeaux cedex, France

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Abstract

The aim of this study was to investigate the effects of an overactivation of the cAMP/protein kinase A signaling pathway on the energetic metabolism of growing yeast. By using a cAMP-permeant mutant strain, we show that the rise in intracellular cAMP activates both anabolic and catabolic pathways. Indeed, different physiological patterns were observed with respect to the growth condition: (i) When cells were grown with a limiting amount of lactate, cAMP addition markedly increased the growth rate, whereas it only slightly increased the mitochondrial and cellular protein content. In parallel, the respiratory rate increased and the growth yield, as assessed by direct microcalorimetry, was not significantly modified by cAMP. (ii) Under conditions where the growth rate was already optimal (high lactate concentration), exogenous cAMP led to a proliferation of well-coupled mitochondria within cells and to an accumulation of cellular and mitochondrial proteins. This phenomenon was associated with a rise in the respiratory activity, thus leading to a drop in the growth yield. (iii) Under conditions of catabolic repression (high glucose concentration), cAMP addition markedly increased the fermentation rate and decreased the growth yield. It is concluded that overactivation of the cAMP/PKA pathway leads to uncoupling between biomass synthesis and catabolism, under conditions where an optimal growth rate is sustained by either a fermentative or a respiratory metabolism.

Keywords: cAMP; Mitochondria; Growth yield; Oxidative phosphorylation; Microcalorimetry; *Saccharomyces cerevisiae*

1. Introduction

In growing yeast cells, it has been previously shown that the respiratory capacity and the mitochondrial enzyme equipment simultaneously decrease in the late exponential phase, when the growth rate slows down [1–3]. Moreover, the latter phenomenon is correlated with the maintenance of growth yield, i.e. the amount of respiratory substrate assimilated into biomass as compared to that used for respiration [3]. Similar decreases in respiration have been observed for various cultured mammalian cells before confluence was reached [4–6] and when cells were deprived of serum [7] or nitrogen [6]. Therefore, during growth, a modulation of the mitochondrial content seems to be involved in a long-term regulation of oxidative phosphorylation. In this respect, the ccs1-1 yeast strain, mutated in the ira2 gene encoding for an activating protein of the Ras-GTPase activity, was characterized in the late exponential phase by a higher cellular respiration in parallel with a higher cytochrome content [8–10]. Moreover, this mutant exhibits a number of characteristics of cells overactivated for the Ras/cAMP pathway, i.e. a nutrient starvation sensitivity and a reduced glycogen accumulation. In total, these observations suggest that the Ras/cAMP/PKA pathway is involved at least in the regulation of the cellular mitochondrial content in the late exponential phase.

In the OL556 strain, it has previously been shown that the intracellular level of cAMP can be modulated. Hence, at the permissive temperature of 28 °C, the cdc25-5 mutation produced a sufficient level of cAMP to allow cell division while, in the presence of exogenously added cAMP, OL556 cells had a high intracellular cAMP level due to the rca1/pde2 mutation, and exhibited the same phenotypes as

Abbreviations: ClCCP, carbonyl cyanide *m*-chlorophenyl hydrazone; CR ratio, calorimetric–respirometric flux ratio; Y_H, enthalpy growth yield; J_H, heat production; J_O, rate of oxygen consumption (expressed as nanomoles of atoms per minute per milligram of proteins or dry weight); PKA, cAMP-dependent protein kinase; TET, triethyltin chloride

* Corresponding author. Tel.: +33-5-56999033; fax: +33-5-56999040.
E-mail address: bertrand.beauvoit@ibgc.u-bordeaux2.fr (B. Beauvoit).

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observed for yeasts overactivated for the Ras/PKA pathway [11]. This strain has mainly been studied in relation to the cAMP-induced changes in gene expression [12–14] and in the protein synthesis pattern [13–16] during the diauxic shift. In the present report, examining the effect of RAS/cAPK pathway overactivation on the cellular energetic metabolism, OL556 cells were aerobically grown in the presence of either a purely respiratory substrate (lactate) or a fermentable carbon source (glucose). It is concluded that overactivation of the Ras/cAPK pathway by exogenous cAMP activates the biosynthetic pathways. This modifies the cellular macromolecular composition and increases the mitochondrial biogenesis. Simultaneously, cAMP treatment increases the cellular ATP turnover, which, in turn, activates the oxygen uptake (lactate condition) or the fermentation rate (glucose condition). Finally, cAMP decreases the growth yield during respiratory and respiro-fermentative metabolism, thus demonstrating that the activation of protein kinases A is responsible for a decoupling between catabolism and biosynthetic reactions.

2. Materials and methods

2.1. Yeast strain, culture medium and growth condition

The yeast strain used was the diploid strain *Saccharomyces cerevisiae* OL556 (*a*/α, *cdc25-5/cdc25-5*, *his3/his3*, *leu2/leu2*, *rca1(pde2)/rca1*, *TRP1/trp1*, *ura3/ura3*) supplied by M. Jacquet (Orsay, France). Cells were grown aerobically at 28 °C on the following SCL medium (minimal medium): 0.17% Yeast Nitrogen Base without amino acids and ammonium sulfate (Difco), 0.1% potassium phosphate, 0.1% casamino acids (Merck), 0.5% ammonium sulfate, pH 5.5, containing either 0.2 or 2% (w/v) of D,L-lactate (Aldrich) and supplemented or not with 3 mM cAMP. The concentrations of auxotrophic requirements were: leucine (800 mg/l), histidine (300 mg/l) and uracil (100 mg/l). Yeast cells were harvested during the exponential growth period for resting cell experiments.

2.2. Growth determination

Growth was followed turbidometrically at 600 nm in a Phillips spectrophotometer. Dry weight determinations were performed on cell samples harvested throughout the exponential growth period and washed twice in distilled water. A coefficient of turbidity was obtained for OL556 cells grown in the presence or absence of 3 mM cAMP (0.17 ± 0.02 and 0.19 ± 0.01 mg dry weight/optical density unit, respectively). Enthalpy of combustion of biomass was calculated from the determination of the elemental composition of biomass ($CH_{1.89}O_{0.55}N_{0.2}$ and $CH_{1.91}O_{0.59}N_{0.16}$ for OL556 grown in the presence or not of cAMP, respectively), which was performed at the Ecole Nationale Supérieure de Chimie in Toulouse (Toulouse, France).

2.3. Preparation of spheroplasts and mitochondria

Yeast cells were harvested in the exponential growth phase and spheroplasts were obtained by enzymatic digestion of the cell wall with zymolyase (ICN Biochemicals) as described in Ref. [17]. Mitochondria were prepared after the plasma membrane rupture of spheroplasts in hypoosmotic medium [17]. Protein concentration was measured by the biuret method using bovine serum albumin as standard.

2.4. Calorimetry setup and heat measurement assay

The heat production rate (i.e. $P = dQ/dt$, expressed as μW/ml) was continuously monitored at sampling intervals of 10 s (growth conditions) or 2 s (resting cell conditions) with a multichannel microcalorimeter (Thermal Activity Monitor, TAM, Thermometric AB, Jarfalla, Sweden) in the flow-through mode. The effective volume of the flow cell is 0.6 ml [18]. Calibration and correction of the data was done by the Digitam software using the Tian equation and time constants [19]. The specific enthalpic flux J_H was subsequently calculated as the heat production rate, P, divided by the biomass concentration.

Cells were incubated in a water bath at 28 °C and were stirred by a magnetic stirrer at 300–400 rpm. Using a peristaltic pump (Gilson), they were transported via teflon tubings to the calorimeter (thermostated at 28 °C) and back to the flask. The flow rate of the pump was 2–3.5 ml/min, resulting in a total transport time of 0.5–1 min to avoid hypoxia. Cell cultures (200 ml) were performed under constant bubbling of sterile humidified air in 1-l flasks. Throughout the culture time, the cell suspension was sampled to measure optical density, metabolites in the culture medium and oxygen consumption rate.

2.5. Respiration assay

The oxygen consumption of cells and mitochondria was measured polarographically at 28 °C using a 'Clark-type' large diameter Orbisphere oxygen electrode in a 2 ml thermostatically controlled chamber (Oroboros Oxygraph, Paar, Graz, Austria). Data were recorded at sampling intervals of 1 s (DatLab Acquisition software, Oroboros, Innsbruck, Austria). Respiratory rates (J_O) were determined from the slope of a plot of O_2 concentration vs. time, divided by the biomass concentration. For cellular respiration assays, 2 ml of growing cell suspension were quickly transferred from the flask connected to the microcalorimeter to the respirometer.

Isolated mitochondria (0.15 mg proteins/ml) and spheroplasts (0.8 mg proteins/ml) were incubated in the following buffer: 0.65 M mannitol, 5 mM Tris/P_i, 0.36 mM EGTA, 0.3% bovine serum albumin, 10 mM Tris/maleate (pH 6.8), containing 4 mM NADH as respiratory substrate. Spheroplast permeabilization was carried out in the polarographic

chamber for 10 min in the presence of 20 μg nystatin/mg proteins, as described in Ref. [20].

2.6. Mitochondrial spaces, ATP and $\Delta\Psi$ measurements

Extra-mitochondrial ATP was measured in protein-free neutralized extracts by bioluminescence assay (Bio-Orbit, Finland) with a luminometer 1250 (LKB-Wallac). Extra-mitochondrial and matrix spaces were determined by the use of [^{3}H] H_2O and inner-membrane-impermeable [^{14}C] mannitol. Briefly, after equilibration (2 min), mitochondria (3.5 mg proteins/ml) were separated through a silicone oil layer (silicone AR200 fluid) [21]. Mitochondrial $\Delta\Psi$ was estimated using the fluorescence changes of rhodamine 123 as described in Ref. [22]. Briefly, mitochondria (0.35 mg proteins/ml) were incubated in the respiratory medium in the presence of 0.5 μg/ml of rhodamine 123 (Sigma) and fluorescence emission was continuously monitored with a Kontron SFM 25 fluorimeter at 525 nm, the excitation wavelength being 485 nm. The $\Delta\Psi$ value was calculated using the fluorescence signals and the calibration curve obtained in Ref. [22].

2.7. Determination of D,L-lactate, pyruvate and acetate contents of culture media

Samples of growing cell suspension were filtered for metabolite content determination in the culture medium. Pyruvate was measured enzymatically as described in Ref. [23]. D- plus L-lactate and acetate were measured by using enzyme combination kits (Biochemica Test Combination; Boehringer Mannheim).

2.8. D,L-Lactate dehydrogenase and citrate synthase assays

Samples of growing cells were centrifuged and pellets were resuspended and incubated for 30 min at 32 °C in a medium containing potassium phosphate 100 mM (pH 7.4), EDTA 2 mM, zymolyase (1 mg/ml) (ICN Biomedicals), Triton X-100 0.3% (v/v) and an anti-protease mixture (1 tablet for 10 ml solution) (complete™ EDTA-free; Boehringer Mannheim).

D-lactate plus L-lactate dehydrogenase (E.C. 1.1.2.3 and E.C. 1.1.2.4, respectively) and citrate synthase (E.C. 4.1.3.7) activities were determined spectrophotometricaly as described in Ref. [3]. One D,L-lactate dehydrogenase unit was equal to 1 μmol of 2,6-dichloro indophenol reduced per minute and one citrate synthase unit was equal to 1 μmol of 5-5′-dithiobis-2-nitrobenzoic acid reduced per minute.

2.9. Cytochrome content of cells

Cells were harvested, washed and concentrated to about 50 optical density units at 600 nm. Reduced minus oxidized difference spectra were obtained using a dual spectrophotometer Aminco DW2000 as described in Ref. [3].

2.10. Enzymes and chemicals

Carbonyl cyanide *m*-chlorophenyl hydrazone (ClCCP), triethyltin chloride (TET) and sodium azide (NaN_3) were purchased from Sigma, Merck and Boehringer Mannheim, respectively.

3. Results

3.1. Effect of cAMP on growth of OL556 strain in minimal lactate medium

OL556 cells were aerobically batch-grown with either 0.2% or 2% lactate as carbon and energy source. In the presence of 0.2% lactate, the addition of 3 mM cAMP increased the growth rate from 0.15 to 0.2 h^{-1}, the latter value being close to that measured in the presence of 2% lactate without cAMP (Table 1). However, in the presence

Table 1
Effect of cAMP on growth characteristics of OL556 cells on lactate[a]

	SC 0.2 % D,L-lactate medium		SC 2% D,L-lactate medium	
	− cAMP	+cAMP	− cAMP	+cAMP
Growth rate (μ) (h^{-1})	0.15 ± 0.02	0.2 ± 0.01	0.20 ± 0.01	0.20 ± 0.01
Protein content (mg/mg dry weight)	0.61 ± 0.02	0.65 ± 0.03	0.58 ± 0.03	0.88 ± 0.05
Protein synthesis rate (μg/min/mg dry weight)[b]	1.5 ± 0.2	2.2 ± 0.2	1.9 ± 0.2	2.9 ± 0.3
$J_{O\ growth}$ (nat. O/min/mg dry weight)	210 ± 20	300 ± 20	195 ± 20	350 ± 15
$Y_{lactate}$ (μmol/mg dry weight)	34 ± 5	42 ± 6	36 ± 4	69 ± 9
$q_{lactate}$ (nmol/min/mg dry weight)[b]	88 ± 16	139 ± 23	119 ± 15	230 ± 32
Enthalpic growth yield (Y_H) (%)[c]	55 ± 3	44 ± 5	55 ± 4	34 ± 3

[a] OL556 cells were grown aerobically in a minimal medium supplemented with 0.2% or 2% D,L-lactate as carbon source. When added in the culture medium, cAMP was 3 mM. Heat production rate, respiratory rate, biomass and metabolites in the culture medium were measured as described in Materials and methods throughout the entire exponential phase. Values are means ± S.D. of two independent experiments for each condition.

[b] The protein synthesis and the lactate consumption rates were calculated by multiplying the specific growth rate by the cellular protein content and the lactate yield, respectively (i.e. $q = \mu Y$).

[c] The enthalpic growth yield was calculated from the enthalpy balances of Fig. 1: $Y_H = \Delta_c H_X/(\Delta_c H_X + \Delta_r Q_X) \times 100$ with $\Delta_c H_X$, the enthalpy of combustion of biomass and $\Delta_r Q_X$, the heat production yield.

of 2% lactate as carbon source, this growth rate remained unchanged by cAMP. Moreover, for cells grown with 0.2% lactate, cAMP addition did not significantly change the cell protein content expressed as per dry mass (Table 1). In contrast, cAMP treatment led to a markedly high protein accumulation in cells grown with 2% lactate (Table 1). By multiplying the cellular protein content with the respective growth rate, it is possible to estimate the net protein synthesis rate under growth conditions. cAMP addition increased the protein synthesis rate of OL556 cells to about the same extent, regardless of the concentration of lactate in the culture medium (i.e. a 45% and 55% increase in the presence of 0.2% and 2% lactate, respectively; see Table 1). At the same time, cAMP addition significantly changed the respiratory activity of cells grown in the presence of 2% lactate (350 vs. 195 nat. O/min/mg dry weight) and to a lesser extent in the presence of 0.2% lactate (300 vs. 210 nat. O/min/mg dry weight) (Table 1), thus suggesting a global activation of the metabolism of these cAMP-treated cells.

The lactate consumption was measured in parallel to biomass formation, thus allowing us to calculate the amount of lactate needed to synthesize 1 g of biomass, i.e. the lactate yield (i.e. $Y_{lactate}$). Table 1 shows that the lactate yield was about the same regardless of the lactate concentration in the culture medium. However, addition of cAMP to the culture medium induced a 1.3- and 2-fold increase in $Y_{lactate}$ in the presence of 0.2% and 2% lactate, respectively (Table 1). Multiplication of the lactate yield with the respective growth rate allowed estimation of the lactate consumption rate (i.e. $q_{lactate}$). Indeed, cAMP treatment induced a large increase in the lactate consumption rate (i.e. with a factor of about 1.60 and 1.9 increase in the presence of 0.2% and 2% lactate, respectively), further emphasizing, together with the respiratory activity, the global activation of the oxidative metabolism by cAMP.

Since the first step of lactate oxidation is the complex IV-linked L+D lactate dehydrogenases [24,25], the oxygen uptake was only partially sensitive to complex III inhibitors (i.e. antimycin A and myxothiazol), but was very sensitive to the cytochrome *c* oxidase inhibitors, cyanide and azide (data not shown). By comparing the lactate consumption rate and the respective oxygen uptake listed in Table 1, it was possible to calculate that about half of the respiratory activity measured during growth was sustained by the sole complex IV-driven lactate to pyruvate oxidation (i.e. $42 \pm 9\%$ vs. $61 \pm 10\%$ for cells growing in the presence of 0.2% and 2% lactate, respectively). cAMP treatment did not significantly change this electron flux distribution (i.e. $46 \pm 8\%$ and $66 \pm 10\%$ for cells growing in the presence of cAMP and 0.2% or 2% lactate, respectively), despite the increase in the total respiratory activity of treated cells (see Table 1). Therefore, both the complex IV-driven lactate oxidation and the NADH-linked pyruvate oxidation were increased by cAMP treatment, regardless of the lactate concentration in the growth medium.

To determine the part of the lactate that is readily assimilated into biomass, i.e. the growth yield, the accumulation of by-products of the lactate catabolism was measured in the culture medium. Moreover, the heat production rate was continuously monitored by direct microcalorimetry, which allowed us to calculate the heat yield ($\Delta_r Q_X$) from the slope of integrated heat production vs. biomass formation [3,26,27]. In the exponential phase and regardless of the growth condition (e.g. in the presence of a limiting or nonlimiting amount of lactate in the medium), plots gave linear correlations. Therefore, under all conditions, the overall growth process can be considered as constant physiological states (data not shown). Enthalpy balances were subsequently constructed from the values of net substrate consumption (energy input) and net product formation (energy output) transformed into energy unit, and were normalized to one unit of biomass formed (kJ/g dry weight) [3,26,27]. Under each condition (0.2% and 2% lactate), recovery between energy input and output was closed to unity (Fig. 1A,B), so all major components were included in the balances. Of course, in these figures, the cAMP-induced changes in the amount of energy input corresponded to the respective changes in the $Y_{lactate}$ listed in Table 1. In parallel, cAMP treatment significantly increased the part of the energy output lost as by-products (i.e. pyruvate + acetate) in the presence of 2% lactate (36% vs. 22%; Fig. 1B) but not in the presence of 0.2% lactate (29% vs. 28%; Fig. 1A). Neither the part of the energy input lost as heat nor the part of the energy input stored as biomass (i.e. the growth yield) were affected by lactate concentration in the culture medium (compare Fig. 1A and B, without cAMP). Moreover, cAMP treatment in the presence of 0.2% lactate slightly increased the heat yield from 35% to 44% and decreased the biomass yield from 42% to 34% (Fig. 1A). Finally, a more pronounced effect of cAMP was observed in the presence of 2% lactate: i.e. an increase in the heat dissipation yield (41% vs. 33%) and a large decrease in the biomass yield (21% vs. 40%) (Fig. 1B). Since the by-product accumulation may significantly account for the cAMP-induced changes in the biomass yield (e.g. see Fig. 1A), we estimated the enthalpy growth yield (Y_H), i.e. the ratio between the enthalpy contained in the biomass and that of the substrate consumed minus that of the by-products formed (or alternatively the energy stored as biomass compared to that dissipated as heat) [28]. Table 1 shows that in the absence of cAMP, the enthalpic growth yield was nearly the same (about 55%; see also Refs. 3,18]), regardless of the lactate concentration in the culture medium. However, the effect of 3 mM cAMP on the enthalpic growth yield depended on the carbon source limitation: with 0.2% lactate, Y_H decreased from 55% to 44%, whereas Y_H dropped from 55% to 34% in the presence of 2% lactate (Table 1).

This large cAMP-induced decrease in the growth enthalpy yield was further assessed by measuring the calorimetric–respirometric ratio (CR ratio) in the presence of 2%

lactate, according to the procedure detailed in Ref. [18]. Fig. 2 shows a linear relationship between the heat flux and the oxygen uptake, when the respiratory activity was titrated using azide and regardless of the presence or not of cAMP.

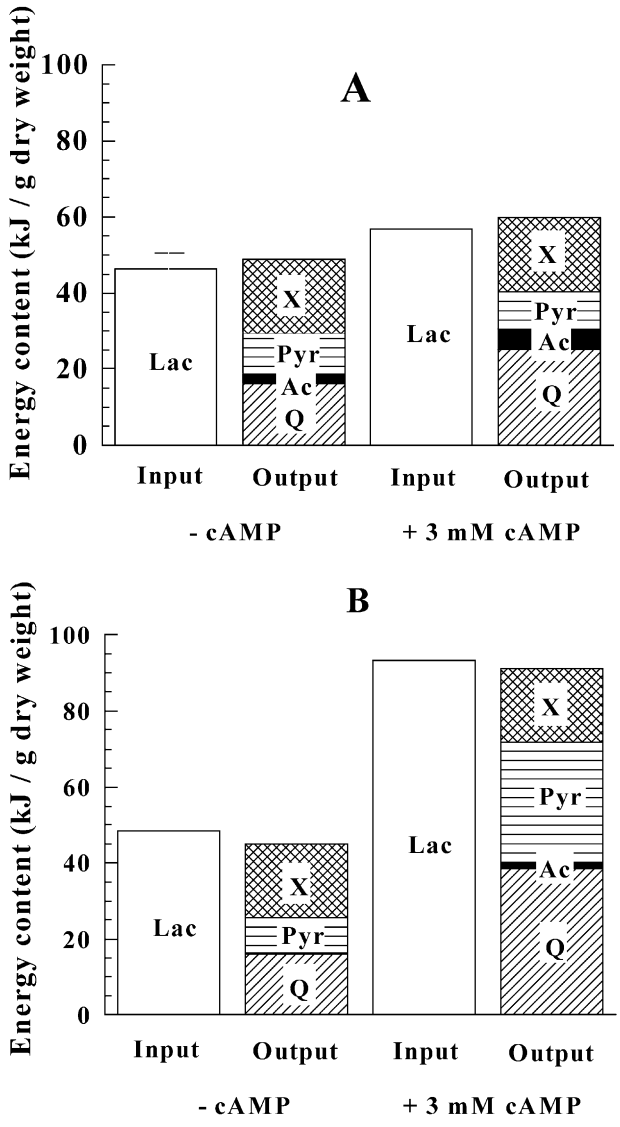

Fig. 1. Effect of cAMP and lactate concentration on the enthalpy balance of batch grown OL556. OL556 cells were grown aerobically in a minimal SC medium supplemented with 0.2% (A) or 2% D,L-lactate (B) as carbon source. When added in the culture medium, cAMP was 3 mM. Heat production, biomass and metabolites in the culture medium were measured as described in Materials and methods throughout the entire exponential phase. The cumulative biomass production vs. the lactate consumption, on one hand, and vs. the pyruvate and acetate accumulation, on the other, was determined as described in Ref. [3]. Heat yield was determined by plotting the integrated heat dissipated vs. the cumulative biomass production [3,26]. The elemental composition of biomass was nearly the same in the presence or not of cAMP (see Materials and methods), thus giving an enthalpy of combustion of biomass of 23.5 and 22.6 kJ/g dry weight (assuming the ash content to be 7.5% of the dry weight). The energy contents of substrates consumed and the product formed (kJ/g dry weight) are abbreviated as : X, biomass; Q, integrated heat; Lac, D+L lactate; Pyr, pyruvate; Ac, acetate. Values are means of two independent experiments.

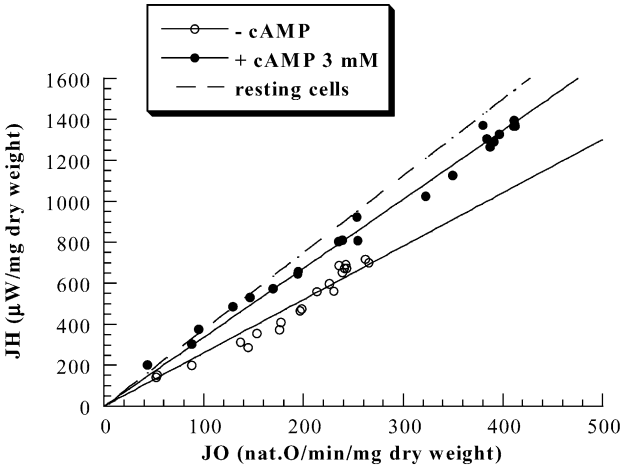

Fig. 2. Effect of cAMP on the relationship between heat production flux and respiratory rate of growing OL556 cells. OL556 cells were grown aerobically in minimal SC medium containing 2% D,L-lactate in the presence or not 3 mM cAMP in the culture medium. Heat production rate and respiratory activity were measured as described in Materials and methods. The cellular respiratory activity was modulated by NaN_3 titration up to 100 µM ($n=2$). Dashed line represents the flux–flux relationship in the absence of biomass synthesis (i.e. fully heat dissipative energy transformation), corresponding to an oxycaloric equivalent for lactate combustion of −440 kJ/mol O_2 [18].

For reasons of comparison, the relationship between the two fluxes during a complete oxidation of lactate to carbon dioxide and water was represented (Fig. 2). The discrepancy between the relationships observed under growth vs. the complete oxidation of lactate accounts for the endothermic biomass synthesis and, to some extend, for the byproduct accumulation [18]. As shown by Fig. 2, at the same respiratory rate, the heat flux was significantly higher in the presence of cAMP compared to the control condition, further demonstrating the lower growth yield in the presence of cAMP.

3.2. Effect of cAMP on growth of OL556 strain in minimal glucose medium

To analyze whether the respiro-fermentative metabolism was affected or not by cAMP treatment, OL556 cells were aerobically batch-grown with 2% glucose. These cells were characterized by a low respiratory activity regardless of the presence or absence of cAMP (see Table 2 and compare to Table 1). This is in agreement with the pattern of gene expression previously described for this strain during exponential growth on glucose with and without cAMP [15], and indicates that cAMP does not counteract the glucose repression of respiratory enzymes. During a 10-h period of exponential growth, glucose consumption and heat production rates were measured in parallel to biomass and by-product formation. Table 2 shows that cAMP addition induced a slight decrease in the growth rate (about 19%). In a previous report, a similar phenomenon was associated with an accumulation of cell proteins from 0.41 to 0.73 mg/

Table 2
Effect of cAMP on growth characteristics of OL556 cells during respiro-fermentative metabolism[a]

	YNBS 2% glucose medium	
	− cAMP	+ cAMP
$Y_{glucose}$ (mmol/g dry weight)	45 ± 5	80 ± 13
$Y_{ethanol}$ (mmol/g dry weight)	60 ± 2	104 ± 19
$Y_{glycerol}$ (mmol/g dry weight)	3.6 ± 1.2	11 ± 0.5
Heat yield (ΔQ_X) (kJ/g dry weight)	− 6.3 ± 0.5	− 10.8 ± 1.0
Growth yield (C-mol/C-mol)[b]	0.51 ± 0.06	0.31 ± 0.08
Growth rate (μ) (h^{-1})	0.32 ± 0.01	0.26 ± 0.01
J_O (μmol/min/g dry weight)	30 ± 10	27 ± 5
$q_{glucose}$ (μmol/min/g dry weight)	− 240 ± 28	− 350 ± 58
$q_{ethanol}$ (μmol/min/g dry weight)	320 ± 15	451 ± 84
$q_{glycerol}$ (μmol/min/g dry weight)	20 ± 6	48 ± 4

[a] OL556 cells were grown aerobically in a minimal medium supplemented with 2% glucose as carbon source. When added in the culture medium, cAMP was 3 mM. Heat production rate, biomass and metabolites in the culture medium were measured as described in Materials and methods during 10 h of exponential growth phase. Values are means ± S.D. of three independent experiments for each condition. Yields were calculated from the linear correlations of metabolite concentrations and integrated heat versus dry biomass formed during the same time interval. Carbon fluxes were calculated from the respective yields multiplied by the specific growth rate (i.e. $q = \mu Y$). Respiratory rate (J_O) was measured as described in Materials and methods.

[b] The C-molar growth yield, expressed as C-mol of biomass per C-mol of glucose consumed, was calculated by subtracting the C-mol of by-products formed and taking the same values of the mass of one C-mol of biomass as used in Fig. 1.

mg dry weight when cAMP was added to the culture medium (calculated from Table 1 of Ref. [15] in the presence of glucose 2%). Meanwhile, cAMP treatment induced an increase in the heat yield ($\Delta_r Q_X$, 70% increase), in the amount of glucose needed to synthesize 1 g of biomass ($Y_{glucose}$, 77% increase) and in the amount of ethanol ($Y_{ethanol}$, 73% increase) and glycerol ($Y_{glycerol}$, 3-fold increase) produced during the synthesis of biomass (Table 2). Accordingly, the C-molar growth yield was decreased upon cAMP addition from about 0.5 to 0.3 C-mol of biomass produced per C-mol of glucose consumed. Finally, calculation of the specific glucose consumption and ethanol production rates showed that exogenous cAMP increased the glycolytic flux ($q_{glucose}$, 46% increase and $q_{ethanol}$, 41% increase) (Table 2).

Taken together, these data suggest that the rise in the intracellular cAMP by exogenously added cAMP promotes a higher metabolic activity of OL556 cells during either a purely respiratory (lactate 2%) or a respiro-fermentative (glucose 2%) metabolism, thus leading, under both conditions, to a significant decrease in the growth yield.

3.3. cAMP effect on the oxidative phosphorylation of OL556 cells batch grown in the presence of lactate

Since OL556 cells were grown with lactate as the sole carbon and energy source, the decrease in the growth enthalpy yield could be somehow related to disturbances in the mitochondrial oxidative phosphorylation, leading to a higher respiration (see Table 1). Thus, the degree of coupling of mitochondrial ATP synthesis to respiration was studied for OL556 cells grown in the presence of 2% lactate with and without cAMP. This was achieved by measuring: (i) the part of oxygen uptake coupled to ATP synthesis under both conditions by using the cell permeable inhibitor of the F_1F_O ATP synthase, triethyltin (TET) [29]; and (ii) the maximal respiratory rate by means of the protonophoric uncoupler, ClCCP, to dissipate the proton electrochemical gradient across the innermembrane, and thereby to stimulate the respiratory rate to its maximum value sustainable by yeast cells. Table 3 shows that TET addition decreased the basal respiratory rate under both conditions (i.e. a 44% and 39% decrease with and without cAMP, respectively), thus indicating that the respiratory chain activity was coupled to ATP synthesis. However, the TET-insensitive respiration was significantly higher with cAMP than without (i.e. 195 vs. 120 nat. O/min/mg dry weight), which may eventually indicate a partial in situ uncoupling of mitochondria within overactivated cells. However, the ClCCP addition produced about a 50% increase in the basal respiratory rate under both conditions (Table 3). Finally, the ratio between the maximal oxygen uptake and the nonphosphorylating respiratory activity ($J_{O\ ClCCP}$ vs. $J_{O\ TET}$) did not vary significantly with respect to the cAMP treatment (i.e. about 2.6) (Table 3). This indicates that the respiratory activity is controlled by the proton electrochemical gradient to the same extent, thus making the in situ uncoupling of mitochondria unlikely.

3.4. Oxidative phosphorylation of mitochondria isolated from OL556 cells treated or not by cAMP

We further analyzed the effect of cAMP treatment on the functional properties of the mitochondria isolated from OL556 cells grown in the presence of 2% lactate. This was done on permeabilized spheroplasts on one hand, and on isolated mitochondria, on the other. The former approach has the advantage of preserving the structural and functional

Table 3
Oxidative phosphorylation regime of OL556 cells grown in the presence or not of cAMP[a]

	− cAMP	+ cAMP
$J_{O\ growth}$ (nat. O/min/mg dry weight)	195 ± 20	350 ± 15
$J_{O\ TET}$ (nat. O/min/mg dry weight)	120 ± 10	195 ± 15
$J_{O\ ClCCP}$ (nat. O/min/mg dry weight)	295 ± 20	535 ± 25
Cellular respiratory control ratio	2.5 ± 0.4	2.7 ± 0.3

[a] OL556 cells were grown aerobically in a minimal SC medium supplemented with 2% D,L-lactate as carbon source and containing or not 3 mM cAMP. The cellular respiratory rates were measured in the growth medium without ($J_{O\ growth}$) or with 100 μM of the F_0F_1 ATP synthase inhibitor, TET ($J_{O\ TET}$), as described in Materials and methods. The respiratory capacity of cells ($J_{O\ ClCCP}$) was measured in the growth medium after titration with the protonophoric uncoupler, ClCCP. Values are means ± S.D. of four independent experiments. The respiratory control is the ratio between $J_{O\ ClCCP}$ and $J_{O\ TET}$.

Table 4
Functional properties of mitochondria from OL556 cells grown in the presence or absence of cAMP[a]

	− cAMP	+cAMP
Permeabilized spheroplasts		
$J_{O\ state\ 4}$ (nat. O/min/mg proteins)	36 ± 3	76 ± 5
$J_{O\ state\ 3}$ (nat. O/min/mg proteins)	69 ± 4	182 ± 13
Respiratory control ratio	1.9 ± 0.3	2.4 ± 0.3
Isolated mitochondria		
$J_{O\ state\ 4}$ (nat. O/min/mg proteins)	221 ± 39	207 ± 20
$J_{O\ state\ 3}$ (nat. O/min/mg proteins)	488 ± 55	615 ± 90
$\Delta\Psi_{state\ 4}$ (mV)	170 ± 15	170 ± 20
$\Delta\Psi_{state\ 3}$ (mV)	110 ± 15	105 ± 15
ATP/O ratio	1.0 ± 0.2	1.1 ± 0.2
Respiratory control ratio	2.2 ± 0.6	3 ± 0.7

[a] Spheroplasts were obtained by the cell-wall digestion procedure from OL556 cells grown with 2% D,L-lactate and in the presence or not of cAMP. Mitochondria were isolated from intact spheroplasts as described in Ref. [17]. Respiratory rates of permeabilized spheroplasts and isolated mitochondria were measured in the presence of 4 mM NADH as respiratory substrate (state 4) as described in Materials and methods. Oxidative phosphorylation was induced by the addition of 1 mM ADP (state 3) and the ATP synthesis rate was measured as described in Materials and methods. The mitochondrial inner membrane $\Delta\Psi$ was determined by using a fluorescent probe as described in Materials and methods. Values are means ± S.D. of two to three independent experiments.

integrities of the mitochondrial compartment within permeabilized cells, even though permeabilization would lead to a significant loss of respiratory capacity [20]. Table 4 shows that the state 4 and the ADP-induced state 3 respiratory rates of mitochondria within permeabilized cells were about 2-fold higher for spheroplasts prepared from cells grown with cAMP than without. Moreover, the in situ respiratory control ratio of spheroplasts, i.e. the state 3 vs. state 4 ratio, was slightly higher for cells grown in the presence of cAMP (2.4 vs. 1.9) (Table 4). To characterize further the cAMP effect on mitochondrial compartment integrity, we isolated mitochondria from spheroplasts. It is noteworthy that the matrix volume of the isolated mitochondria was nearly the same for the two preparations (i.e. 1.9 ± 0.2 and 2 ± 0.3 μl/mg mitochondrial protein, with and without cAMP, respectively). Table 4 shows that the nonphosphorylating respiration was not significantly different for mitochondria prepared from cAMP-treated or untreated cells. Moreover, the value of the electrical potential difference across the inner membrane, $\Delta\Psi$, the major component of the protomotive force under our conditions [30], was not affected by cellular cAMP treatment (Table 4). Nevertheless, the ADP-induced state 3 respiration was significantly higher for mitochondria isolated from cAMP-treated cells (615 vs. 488 nat. O/min/mg protein), thus making the mitochondrial respiratory control 36% higher for cAMP-treated cells (3 vs. 2.2) (Table 4).

3.5. cAMP effect on the mitochondrial content of OL556 cells grown in the presence of lactate

Like the basal oxygen uptake, the TET-insensitive and the uncoupled respiratory rates of cells were significantly higher for cAMP-treated than control cells (i.e. a 60–80% cAMP-induced increase) (see Table 2), suggesting that cAMP treatment may have modified the mitochondrial content of cells. To further address this question, the maximal respiratory capacity of cells was estimated by titrating with ClCCP the respiratory activity in the presence of saturating amounts of respiratory substrates according to Ref. [3]. Table 5 shows that the respiratory capacity of OL556 cells was the same regardless of the concentration of carbon source in the medium (i.e. 325–345 nat. O/min/mg dry weight). Moreover, cAMP addition induced a 43% and 72% increase in the respiratory capacity of cells grown with 0.2% and 2% lactate, respectively (Table 5). These cAMP-induced changes also concerned the electron transfer components of the respiratory chain. In fact, cAMP addition

Table 5
Effect of cAMP on the mitochondrial content of OL556 cells grown on lactate[a]

	SC 0.2 % D,L-lactate medium		SC 2% D,L-lactate medium	
	− cAMP	+cAMP	− cAMP	+cAMP
Maximal respiratory capacity ($J_{O\ max}$) (nat. O/min/mg dry weight)	325 ± 15	465 ± 30	345 ± 20	595 ± 25
D,L-Lactate dehydrogenase (mU/mg dry weight)	95 ± 5	115 ± 10	100 ± 10	150 ± 10
Citrate synthase (mU/mg dry weight)	120 ± 15	260 ± 35	130 ± 25	430 ± 20
Cytochrome content (pmol/mg dry weight)				
c+*c*1	90 ± 15	200 ± 20	90 ± 10	195 ± 20
b	35 ± 5	70 ± 10	40 ± 5	80 ± 10
a+*a*3	15 ± 5	25 ± 5	10 ± 3	40 ± 5

[a] OL556 cells were grown aerobically in a minimal medium supplemented with 0.2% or 2% D,L-lactate as carbon source. When added in the culture medium, cAMP was 3 mM. The maximal respiratory capacity of OL556 cells ($J_{O\ max}$) was measured in the growth medium supplemented with saturating amounts of respiratory substrates (lactate 200 mM, ethanol 100 mM and glucose 20 mM) after titration with the protonophoric uncoupler, ClCCP [3]. Enzymatic activities and cytochrome contents were determined as described in Materials and methods. Values are means ± S.D. of three to four independent experiments.

increased the cellular content of cytochrome $c+c1$, b and $a+a3$ by a factor 2 and, with a lesser extent, the D+L-lactate dehydrogenase specific activity (Table 5). Finally, this increase in mitochondrial content was further analyzed by measuring the activity of citrate synthase, a mitochondrial matrix enzyme that has been shown to account for about 80% of the total activity of this enzyme within cells [31]. Table 5 shows that cAMP increased the cellular citrate synthase content by a factor 2.2 and 3.3 in the presence of 0.2% and 2% lactate in the culture medium, respectively.

4. Discussion

4.1. Exogenous cAMP activates anabolism and increases the mitochondrial content of derepressed yeast cells

By varying the lactate concentration in the batch culture medium from 0.2% to 2%, we first observed that the growth rate was kinetically controlled by the carbon substrate availability, i.e. the growth rate in the exponential phase was significantly lower in the presence of 0.2% lactate. Interestingly, in the presence of cAMP, the growth rate was optimal regardless of the lactate concentration in the medium, thus indicating that cAMP-signaling somehow relieved the kinetic constraints exerted by the low lactate concentration on the biomass synthesis. The kinetic control exerted by the lactate concentration on the growth rate could be shared among the individual enzymatic steps of D+L lactate assimilation into biomass. For instance, the lactate transport system of the yeast plasma membrane has been characterized as a proton-lactate symport [32], and therefore depends on the ΔpH value across the plasma membrane. Moreover, the first step of D+L lactate metabolism is the oxidation of lactate in pyruvate by two lactate dehydrogenases, which are connected to the respiratory chain between complexes III and IV (see for reviews in Refs. [24,25]). Regardless of the lactate concentration in the culture medium, cAMP addition increased the lactate consumption rate by a factor 1.6 (see q_{lactate}, Table 1) and the cellular lactate dehydrogenase content by a factor 1.2 (Table 5). However, cAMP addition induced a 3-fold increase in the puruvate+acetate accumulation in the culture medium when OL556 was grown with 2% but not with 0.2% lactate. This suggests that (i) in the presence of 0.2% lactate, growth is mainly controlled by the electron input to the respiratory chain and/or by the mitochondrial enzyme content; and (ii) in the presence of 2% lactate, growth is mainly controlled by the biosynthetic reactions downstream from the pyruvate branch point.

Simultaneously, cAMP treatment induced a stimulation of the protein synthesis rate, whose value increased by a factor of about 1.4–1.5 regardless of the lactate concentration in the medium (see Table 1 of this study), and by about 1.35 in the presence of glucose (calculated from Table 1 of Ref. [15]). This is in accordance with the activation by cAMP of the expression of nuclear genes encoding the translational machinery (e.g. ribosomal proteins), which has been demonstrated by using other exogenous cAMP-responsive strains [33,34]. Moreover, when cells were grown on a respiratory substrate such as lactate, cAMP addition led to an overall increase in the amount of mitochondrial enzymes (e.g. cytochromes, citrate synthase, D,L-lactate dehydrogenases), which in turn was responsible for a higher respiratory capacity of the cAMP-treated cells. It is worth noting that the cAMP effect was weaker for cells grown in the presence of 0.2% lactate than in cells grown with 2% lactate, regardless of the marker used to probe the cellular mitochondrial content (except for cytochromes b and $c+c1$, see Table 4). In the latter case, by comparing the enzymatic assays performed on cell lysates with those carried out on isolated mitochondria, we estimated that the relative mass fraction between mitochondrial protein and cell dry weight increased from 0.17 to 0.44 mg mitochondrial protein per milligram dry weight during cAMP treatment.

To summarize, during the exponential growth of OL556 on lactate, the cAMP-induced activation of the anabolism produced two different physiological patterns with respect to carbon source availability: (i) With a limiting concentration of lactate in the culture medium, the cAMP-induced activation of anabolism optimized the growth rate and, in turn, only slightly increased both the total protein content and the amount of mitochondria; (ii) In contrast, at a high concentration of lactate, the growth rate was already optimal, so anabolism activation by cAMP produced an accumulation of proteins within cells and markedly increased the mitochondrial content of the cAMP-treated cells.

4.2. Exogenous cAMP activates the catabolism and decreases the growth yield during fully respiratory metabolism and respiro-fermentative metabolism

In living cells, growth is the result of coupling between substrate catabolism and multiple anabolic reactions involved in the net biomass synthesis (Fig. 3). In these schemes, ATP cycling (in parallel to reducing equivalent cycling) plays a central role. When yeast cells are aerobically grown with a nonfermentable substrate as sole carbon and energy source, ATP synthesis mainly relies on electron transport phosphorylation in mitochondria (Fig. 3A), as compared to substrate level phosphorylation. In contrast, under conditions of glucose-repression, ATP synthesis mainly relies on substrate level phosphorylations (about 90%, see Table 2) that occur throughout the glycolytic pathway during glucose fermentation (Fig. 3B). According to Fig. 3, the growth yield represents the amount of carbon substrate assimilated into biomass as compared to the total amount of substrate utilized for all metabolic processes. Under steady-state conditions, the growth yield during fermentative or respiratory metabolism may depend on the fraction of ATP utilized for cell maintenance (e.g. ionic gradient, turnover of cell components, futile cycling) com-

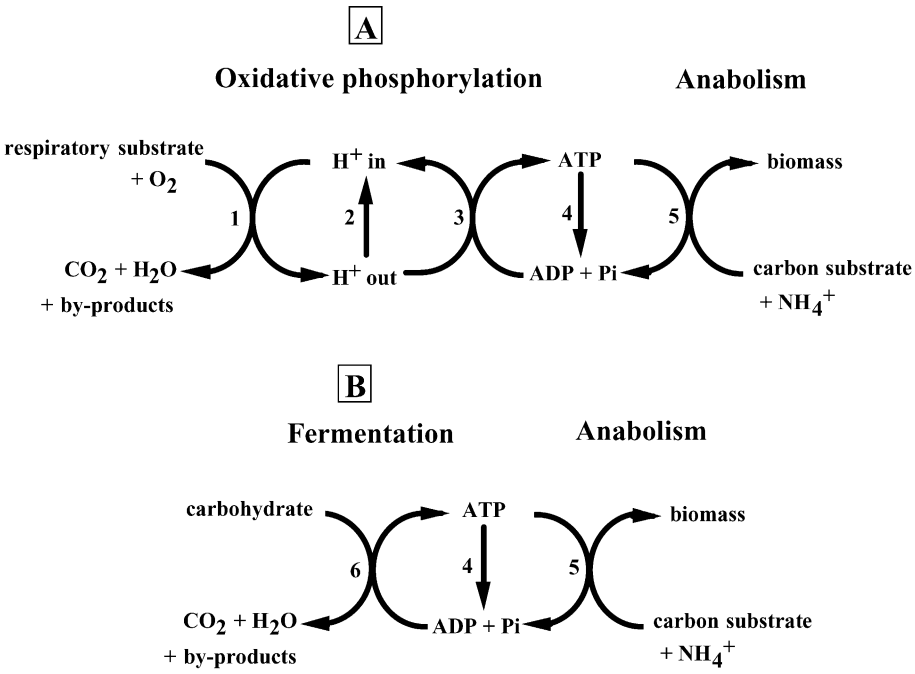

Fig. 3. Energy transformations during respiratory and fermentative growth of yeast cells. Energy transformations during growth are shown by the coupling between the net biomass synthesis (anabolism) and a purely respiratory catabolism (A) or a purely fermentative catabolism (B), assuming that all energy-transforming intermediates are kept at steady state (e.g. a complete ATP turnover occurs at constant concentration of ATP, ADP, Pi, at constant NAD/NADH ratio and at constant transmembrane electrochemical potential differences). Captions: 1 = respiratory chain; 2 = proton leak across the mitochondrial innermembrane; 3 = F0F1 ATP synthase; 4 = ATP utilization by maintenance reactions, which maintain homeostasis of ionic gradients and turnover of all cell components; 5 = ATP utilization by anabolic reactions during respiratory or fermentative growth process; 6 = substrate level phosphorylations of glycolysis.

pared to that used for biomass synthesis per se (Fig. 3A,B). However, energy-requiring metabolic processes are not easily attributed to either maintenance or anabolic non-maintenance processes [35]. Nevertheless, the maintenance ATP requirements, which are low on glucose-growing yeasts, have been found to increase considerably under conditions of external stresses (e.g. pH, ionic and osmotic stresses [36,37]). Moreover, because of the chemiosmotic coupling of oxidative phosphorylation, the growth yield of respiratory-competent yeast cells may eventually depend, in addition to the maintenance, on the amount of the respiratory substrate consumption driven by the proton leak across the inner membrane and by the redox proton pump slipping (Fig. 3A). For instance, the enthalpy growth yield has been shown to decrease during heterologous expression of the rat uncoupling protein 1 in yeast, according to the loss in the respiratory control ratio [18].

An important observation of our study is that an over-activation of the Ras/cAMP/protein kinase A signaling pathways induced a large increase in the catabolic activity of both fermentative yeast cells (see $q_{glucose}$ of 2% glucose repressed cells in Table 3) and respiratory competent cells (see $q_{lactate}$ of 2% lactate growing yeast in Table 1), in parallel with a slight decrease (Table 3) or no change (Table 1) in the growth rate. Indeed, under the former condition, we estimate that the ATP turnover increased by a factor of about 1.4 during the cAMP treatment (403 vs. 300 nmol ATP/min/mg dry weight in the presence or not of cAMP, respectively, calculated from $q_{ethanol}$ minus $q_{glycerol}$). Since the growth rate was slightly lowered by cAMP, this high ATP turnover is therefore a consequence of an increase in ATP requirements for cell maintenance and/or futile cycling, which, in turn, reduced the growth yield by a factor of about 1.6.

Similarly, when OL556 cells were grown with 2% lactate, cAMP addition increased both the respiratory activity and the lactate uptake by a factor 1.8–1.9 (Table 1), whereas cAMP induced a 40% decrease in the growth yield. Our data obtained on whole cells, permeabilized spheroplasts and isolated mitochondria, demonstrate that both the high respiration and the low growth yield of cAMP-treated cells are not due to an in situ uncoupling of mitochondria within cells, since the Respiratory Control Ratio values were similar under both conditions (see Tables 3 and 4). In fact, the higher respiratory activity of cAMP treated cells (in the presence of 2% lactate) results from two phenomena: (i) An increase in the respiration coupled to F0F1 ATP synthase by a factor 2 (i.e. $J_{O\ growth} - J_{O\ TET} = 155$ vs. 75 nat. O/min/mg dry weight in the presence or not of cAMP, see Table 3). Since the growth rate did not significantly change during cAMP treatment, this phenomenon is probably due to an increase in the ATP utilized for cell maintenance as compared to the growth-dependent ATP requirements, as mentioned above for glucose-repressed cells; (ii) A 1.6-fold increase in the oxygen uptake not coupled to ATP synthesis

(i.e. $J_{O\ TET}$ = 195 vs. 120 nat. O/min/mg dry weight in the presence or not of cAMP, see Table 3). This rise in non-phosphorylating respiration is caused by the increase in the mitochondrial enzyme content within the treated cells as compared to control cells (Table 5). The combination of both the increase in the ATP requirements for maintenance and futile cycling and the accumulation of electron-transport chain components lead to a large decrease in the growth yield.

This physiological pattern of overactivated cells is no longer valid in the case of growth in the presence of a limiting amount of lactate. Indeed, when OL556 cells were grown with 0.2% lactate, cAMP addition increased the respiratory activity and the lactate uptake, but only slightly decreased the growth yield. This is due to the fact that cAMP, under this particular condition, stimulates the growth rate together with the catabolic activity of cells. In this case, the protein kinase A overactivation plays a role in optimizing of the growth rate vs. the growth yield, by decreasing the kinetic control exerted by some unidentified enzymatic steps on the lactate assimilation into biomass.

In conclusion, our data demonstrate that the overactivation of the Ras/cAPK pathway by exogenous cAMP leads to a concomitant increase in cellular anabolism (e.g. protein synthesis, mitochondria biogenesis) and in cellular catabolism (e.g. oxygen consumption, glycolytic flux). Under conditions of optimal growth rate (e.g. growth in the presence of 2% glucose or 2% lactate), it is demonstrated that the higher catabolic activity of these overactivated cells is responsible for a metabolic decoupling between anabolism and catabolism. We provide strong evidence that the control of the growth yield in growing yeast relies on both: (i) the ATP requirements not linked to biomass synthesis, and (ii) the oxygen uptake not coupled to ATP synthesis. This reinforces our previous hypothesis that the ATP leakage, caused by maintenance and futile cycling, is not the only parameter controlling the growth yield [3]. In fact, the respiratory activity driven by proton leak and redox slipping, which depends on the amount of respiratory complexes within cells, is also a carbon substrate wasting system at the expense of the growth yield. It is worth noting that any overactivation of protein kinase A may have pleiotropic effects at different levels of the cell metabolism (e.g. protein inactivation or activation by cAMP-dependent phosphorylations; cAMP-dependent gene expression), so further studies are needed to understand the specific role played by some proteins in the optimization of growth yield vs. growth rate. The physiological relevance of the role played by the Ras/cAMP/protein kinase A signaling pathway might be established by studying the regulation of the mitochondrial enzyme content at the end of growth and during more prolonged starvation. In fact, it has been already shown that overactivativation of protein kinases A counteracts the down-modulation of the mitochondrial development that occurs during the transition from exponential to stationary phases (see, e.g. Refs. [3,8–10,38]).

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