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The calorimetric-respirometric ratio is an on-line marker of enthalpy efficiency of yeast cells growing on a non-fermentable carbon source

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Abstract

Although on-line calorimetry has been widely used to detect transitions in global metabolic activity during the growth of microorganisms, the relationships between oxygen consumption flux and heat production are poorly documented. In this work, we developed a respirometric and calorimetric approach to determine the enthalpy efficiency of respiration-linked energy transformation of isolated yeast mitochondria and yeast cells under growing and resting conditions. On isolated mitochondria, the analysis of different phosphorylating and non-phosphorylating steady states clearly showed that the simultaneous measurements of heat production and oxygen consumption rates can lead to the determination of both the enthalpy efficiency and the ATP/O yield of oxidative phosphorylation. However, these determinations were made possible only when the net enthalpy change associated with the phosphorylating system was different from zero. On whole yeast cells, it is shown that the simultaneous steady state measurements of the heat production and oxygen consumption rates allow the enthalpy growth efficiency (i.e. the amount of energy conserved as biomass compared to the energy utilised for complete catabolism plus anabolism) to be assessed. This method is based on the comparison between the calorimetric-respirometric ratio (CR ratio) determined under growth versus resting conditions during a purely aerobic metabolism. Therefore, in contrast to the enthalpy balance approach, this method does not rely on the exhaustive and tedious determinations of the metabolites and elemental composition of biomass. Thus, experiments can be performed in the presence of non-limiting amounts of carbon substrate, an approach which has been successfully applied to slow growing cells such as yeast cells expressing wild-type or a mutant rat uncoupling protein-1.

Keywords: Calorimetric-respirometric ratio; Oxidative phosphorylation; Mitochondria; Uncoupling protein-1; Yeast; Enthalpy efficiency; Growth yield

Abbreviations: CR ratio, calorimetric-respirometric flux ratio; η_H, enthalpy efficiency; Y_H, enthalpy growth yield; J_H, heat production rate; J_O, rate of oxygen consumption; J_{O2}, rate of dioxygen consumption; J_p, phosphorylation rate; J_a, anabolic flux; J_k, catabolic flux; $\Delta_r H_p$, enthalpy of net reaction of phosphorylation; $\Delta_r H_{redox}$, enthalpy of net reaction of oxidation; $\Delta_r H_a$, enthalpy of net reaction of biomass synthesis; $\Delta_r H_k$, enthalpy of net reaction of oxidative catabolism; ClCCP, carbonyl cyanide *m*-chlorophenyl hydrazone; TET, triethyltin chloride; UCP, uncoupling protein; $\Delta\tilde{\mu}_{H^+}$, proton electrochemical potential difference across the mitochondrial inner membrane; RC, respiratory control

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1. 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 cellular homeostasis (i.e. maintenance of ionic gradients, protein and nucleic acid turnover) [1,2]. For decades, the determination of mass yield ($Y_{X/S}$ in gram of biomass formed/gram of substrate consumed) and ATP yield (Y_{ATP} in gram of biomass formed/mole of ATP produced) was used to give insights into the degree of coupling between the catabolism and anabolism of microorganisms ([3,4]; see [5] for review). Hence, these variables were shown to depend on: (i) strain and carbon source metabolism, relying on the enzymatic equipment, (ii) futile cycling, uncoupling and overflow metabolism (by-product formation), and (iii) ATP requirements for cellular maintenance ([3,4]; see [5] for review). 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. Thus, by applying non-equilibrium thermodynamics, two parameters have been defined to assess for growth efficiency: thermodynamic Gibbs energy efficiency (η_G) and thermodynamic enthalpy efficiency (η_H) [5–8]. The estimation of the former parameter for microbial growth is still difficult due to the lack of knowledge about the Gibbs energy change of substrates, products and biomass [1,9]. In contrast, the quantification of enthalpy efficiency has been successfully achieved for microorganisms [10–13] as well as for cultured mammalian cells [14,15]. The latter 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 [2,16] for reviews). Indeed, a recent study showed that the part of the respiratory activity not coupled to ATP synthesis was the major factor affecting the enthalpy growth yield of fully aerobic yeast cells [17].

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 also been proposed for assessing metabolic efficiency [18,19]. Hence, a disproportional increase in heat production compared to oxygen uptake has been observed in isolated cells under conditions of enhanced futile substrate cycling [18,19] and uncoupling of oxidative phosphorylation [20]. 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 [21,22].

Although on-line calorimetry has been widely used to detect transitions in global metabolic activity during the growth of microorganisms ([13,23–26]; see [2] for review), the relationships between oxygen consumption flux and heat production rate are poorly documented (but see [27]). In the present work, we 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. Furthermore, the analysis has been extended to yeast cells expressing the wild-type brown adipose tissue uncoupling protein-1 (UCP1) and the mutant UCP1Δ9.

2. Materials and methods

2.1. Yeast strains, culture media, growth conditions and expression of rat UCP1

The haploid strain *Saccharomyces cerevisiae* W303-1a (α; *ade*2-10; *his*3-11; *trp*1-Δ1; *ura*3-52; *can*1-100) was grown aerobically at 28°C on the following medium (minimal medium): 0.17% yeast nitrogen base w/o amino acids w/o ammonium sulphate (Difco), 0.1% potassium phosphate, 0.5% ammonium sulphate at pH 5.5, containing growth limiting amount of D,L-lactate (0.2% w/v) (Aldrich). The concentration of auxotrophic requirements was 100 mg/l. Yeast cells were harvested throughout the exponential growth period for resting cells experiments.

The diploid strain *S. cerevisiae* W303 (a/α, *ade*2-10/*ade*2-10; *his*3-11, 15/*his*3-11, 15; *leu*2-3, 112/*leu*2-3, 112; *ura*3-52/*ura*3-52; *can*1-100/*can*1-100; *trp*1-Δ1/*trp*1-Δ1) was transformed with the vector pYeDP where the coding sequence of the wild-type UCP1 and the mutant UCP1Δ9 had been introduced [28,29]. The control yeast contained the same vector but without the insert [28,29]. Cells were grown

aerobically at 28°C on the SD minimal medium: 0.17% yeast nitrogen base w/o amino acids w/o ammonium sulphate (Difco), 0.1% casamino acids (Merck), 0.1% potassium phosphate, 0.5% ammonium sulphate at pH 5.5, containing D,L-lactate (2% w/v) (Aldrich). Auxotrophic requirements were 100 mg/l for adenine, histidine, tryptophan and 200 mg/l for leucine. In the exponential growth phase, vector expression was induced for 1 h by the addition of 0.4% galactose in the culture medium [28,29]. Prior to experiments, cells were either (i) diluted in fresh culture medium without galactose at 28°C and stirred by a magnetic stirrer at 300–400 rpm (growing cell experiments) or (ii) harvested and resuspended in a resting medium (resting cell experiments).

2.2. Growth determinations

Growth was followed turbidometrically at 600 nm in a Phillips spectrophotometer. Dry weight determinations were performed on samples of cells harvested throughout the exponential growth period and washed twice in distilled water. A coefficient of turbidity was obtained for W303-1a and UCP expressing strains (0.17 ± 0.01 and 0.23 ± 0.02 mg dry weight/optical density unit, respectively).

Elemental composition of biomass was determined using biomass combustion (C, H and N) and subsequent gas analysis [30]. O measurement was performed using biomass pyrolysis at 1100°C, in the absence of oxygen. In this case, subsequent gas analysis and mass difference between dry weight and ash content allow the determination of the O amount contained in the biomass. All these experiments were performed in the Ecole Nationale Supérieure de Chimie de Toulouse (Toulouse, France).

2.3. Preparation of mitochondria

The yeast strain used for preparation of mitochondria was the diploid strain *S. cerevisiae* Yeast Foam (PS194). Cells were grown aerobically at 28°C on the following medium: 1% yeast extract (Difco), 0.1% potassium phosphate, 0.12% ammonium sulphate at pH 5, supplemented with 2% (w/v) D,L-lactate (Aldrich) as carbon source. Yeast cells were harvested in the exponential growth phase and mitochondria were isolated from protoplasts as described in [31]. 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 (for balance determinations) or 2 s (for CR ratio determinations) with a multichannel microcalorimeter (Thermal Activity Monitor, TAM, Thermometric, Jarfalla, Sweden) in the flow-through mode [32]. Using the triacetin calibration procedure [33], we measured a flow cell effective volume of 0.6 ml under our experimental conditions. Calibration and correction of the data was done by the software (Digitam) using the Tian equation and time constants [34]. The specific enthalpy flux J_H was subsequently calculated as the heat production rate, P, divided by the biomass concentration (μW/mg protein or μW/mg dry weight). Under steady state conditions J_H is equal to:

$$J_H = \Sigma J_i \times \Delta_r H_i \quad (1)$$

where J_i is the flux throughout the reaction i and $\Delta_r H_i$ the molar reaction enthalpy of the respective reaction(s) [35].

Cells and mitochondria were incubated in a water bath at 28°C and stirred by a magnetic stirrer at 300–400 rpm. Using a peristaltic pump (Gilson), they were transported via teflon tubings to the calorimeter (thermostatted at 28°C) and back to the flask. The flow rate of the pump was 2 ml/min and 3.5 ml/min for experiments performed on isolated mitochondria and whole cells, respectively, 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 exponential phase, the cell suspension was sampled to measure optical density, metabolites in the culture medium and oxygen consumption rate [17]. For resting cell experiments, cells were harvested, washed twice with distilled water and resuspended in the following resting medium: 2 mM $MgSO_4$, 1.7 mM NaCl, 2 mM potassium phosphate, 50 mM MES (pH 6.0) supplemented with 0.2% D,L-lactate (Aldrich) as respiratory substrate. Mitochondria (2.5 mg proteins) were incubated in a 250 ml Erlenmeyer flask containing 50 ml of the following

buffer: 0.65 M mannitol, 3 mM Tris/P_i, 0.36 mM EGTA, 0.3% bovine serum albumin, 10 mM Tris/maleate (pH 6.8), containing 2 mM NADH as respiratory substrate.

2.5. Respiration assay

The oxygen consumption of cells and mitochondria was measured polarographically at 28°C using 'Clark-type' large diameter Orbisphere electrodes in a 2 ml thermostatically controlled chamber (Oroboros Oxygraph, Paar, Graz, Austria) [36]. 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 versus time, and expressed as nat.O/min/mg protein or nat.O/min/mg dry weight. For all assays, 2 ml of cells (growing or resting) or mitochondrial suspension were quickly transferred from the flask connected to the microcalorimeter to the respirometer cuvette.

2.6. Enzymes and chemicals

Hexokinase (EC 2.7.1.1) from baker's yeast and myokinase (EC 2.7.4.3) from rabbit muscle were purchased from Boehringer Mannheim. 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. Relationships between respiratory rate and heat production fluxes during oxidative phosphorylation of isolated yeast mitochondria

The specific heat production and oxygen consumption rates of isolated yeast mitochondria were simultaneously measured under various non-phosphorylating and phosphorylating conditions. Fig. 1 shows the typical determination of CR ratios of yeast mitochondria under different steady states (e.g. fully uncoupled, state 4 and ADP-induced state 3) when titrating the oxygen flux with azide, a complex IV inhibitor. The heat flux proportionally decreased as the respiratory rate was inhibited, regardless of the steady state condition (Fig. 1). The intercept of the linear regression of these plots was always close to zero, demonstrating that about all the heat produced by isolated yeast mitochondria was directly or indirectly linked to the respiratory activity. The CR ratio was subsequently calculated from the slope value of the best fits. Results are summarised in Table 1.

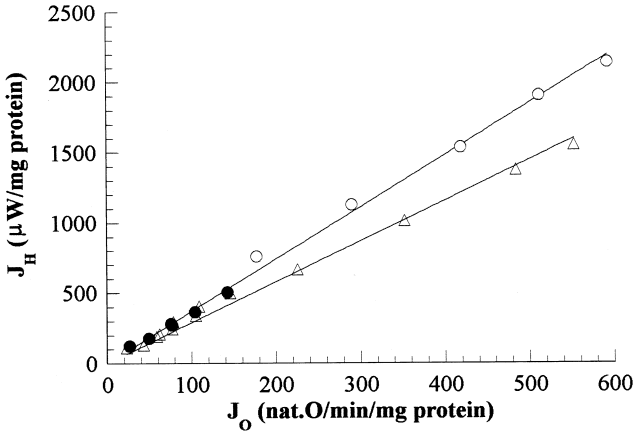

Fig. 1. Typical determination of the CR ratio of isolated yeast mitochondria under different steady states. Yeast mitochondria (0.05 mg protein/ml) were incubated in a flask connected to the microcalorimeter (see Section 2) in the presence of 2 mM NADH as respiratory substrate. Calorimetric and respirometric fluxes were measured as described in Section 2. The respiratory activity was titrated using NaN_3 concentrations up to 100 μM under different steady states: state 4 (●), uncoupled state in the presence of 25 μM ClCCP (○), ADP-induced state 3 (△). The corresponding CR ratios, determined from the slope of the linear regression of each titration, are listed in Table 1.

The CR ratio values determined at state 4 or uncoupled state were not significantly different, −439 vs. −429 kJ/mol O_2 (Table 1). Moreover, the CR ratio, determined when the respiratory rate was progressively stimulated by a proton leak increase (i.e. ClCCP titration), was not significantly different from the CR ratio obtained from the azide titration of fully uncoupled mitochondria (−439 vs. −430 kJ/mol O_2 (Table 1)). These findings differed completely from previously published data in which large discrepancies were observed between the CR ratio of rat liver mitochondria in state 4 vs. uncoupled states using different uncouplers and electron donors [38]. Our results rather indicate that the CR ratio of non-phosphorylating isolated yeast mitochondria does not depend on the extent of the proton electrochem-

Table 1
Calorimetric-respirometric ratio and thermodynamic enthalpy yield of isolated yeast mitochondria[a]

Condition	Phosphorylating systems and net reaction of phosphorylation	$\Delta_r H_p$ of the net reaction of phosphorylation (kJ/mol)	CR ratio (kJ/mol O_2)	η_H
State 4	none	none	-429 ± 6[b]	0
Uncoupled state	none	none	-439 ± 6[b]	0
			-430 ± 5[c]	
State 3	**ADP+P_i → ATP**	+30.9	-352 ± 6[a]	0.21 ± 0.03
Hexokinase ADP-regenerating system	ADP+P_i → ATP	−0.5	-442 ± 6[a]	-0.03 ± 0.03
	ATP+glucose → G6P		-451 ± 7[d]	
	glucose+P_i → G6P			
Myokinase ADP-regenerating system	ADP+P_i → ATP	+29.9	-361 ± 5[a]	0.19 ± 0.03
	ATP+AMP → 2 ADP			
	AMP+P_i → ADP			

[a]Isolated yeast mitochondria (0.05 mg prot./ml) were incubated in the respiratory medium supplemented with 3 mM P_i-Tris, 2 mM NADH as respiratory substrate. Heat production flux and oxygen uptake were measured as described in Section 2. When added (i.e. state 4 and uncoupled states), oligomycin was 10 μg/ml. The molar reaction enthalpy of the net reaction of phosphorylation ($\Delta_r H_p$) was calculated using the standard transformed enthalpy formation of different species according to [37]. The enthalpy efficiency η_H was calculated using Eq. (3) (see text).
[b]The CR ratio was calculated from a linear regression of J_H vs. J_O plots, obtained by titrating the respiratory activity with NaN_3 concentrations up to 100 μM. The direct slope value was multiplied by −120 to obtain the CR ratio expressed as kJ/mol O_2. For the uncoupled state, ClCCP was 25 μM. State 3 steady state was induced by 1 mM ADP addition. The myokinase system was composed of AMP 4 mM, ATP 2 mM in the presence of 1 mM $MgCl_2$ and 0.05 units/ml of myokinase. The hexokinase system was composed of glucose 10 mM, ATP 1 mM in the presence of 1 mM $MgCl_2$ and 0.075 units/ml of hexokinase.
[c]The CR ratio was calculated from a linear regression of J_H vs. J_O plots, obtained by increasing the proton leak using ClCCP concentrations up to 50 μM.
[d]CR ratio was calculated from a linear regression of J_H vs. J_O plots, by increasing the ATP synthesis rate with hexokinase from 0.015 to 0.075 units/ml, in the presence of 10 mM glucose, 1 mM ATP and 1 mM $MgCl_2$.

ical gradient across the inner membrane. Our mean value of -433 ± 6 kJ/mol O_2 is very close to previous measurements performed on rat liver submitochondrial particles (i.e. −454 kJ/mol O_2 [35,39]) but remains lower than the theoretical oxycaloric equivalent of NADH oxidation, which is about −515 kJ/mol O_2 [40,41]. However, as NADH respiration requires the donation of two protons by the buffer solution per molecule of O_2 reduced, the measured CR ratio may also include the enthalpy of deprotonation of the buffer mixture [38,39,42]. In fact, the discrepancy between the measured and theoretical values of the CR ratio is in agreement with the protonation enthalpy of Tris buffer, i.e. −47 kJ/mol H^+ [39].

The ADP-induced state 3 steady state was characterised by a lower CR ratio than in state 4 (Table 1), as already noted previously [38,42]. However, the CR ratio of phosphorylating yeast mitochondria varied significantly with regard to the nature of the extramitochondrial ADP-regenerating system. For instance, the CR ratio measured with the hexokinase system was close to that of state 4, whereas the CR ratio with the myokinase system was quite close to the ADP-induced state 3 value (Table 1). Using Eq. 1 and assuming steady state conditions of respiration, ATP synthesis and proton electrochemical gradient, the CR ratio of isolated mitochondria during oxidative phosphorylation may be expressed as:

$$\text{CR ratio} = (J_{O2} \times \Delta_r H_{redox} + J_p \times \Delta_r H_p)/J_{O2} = \Delta_r H_{redox} + (J_p/J_{O2}) \times \Delta_r H_p \quad (2)$$

where $\Delta_r H_{redox}$ is the molar enthalpy of the redox reaction, J_p the ATP synthesis flux, J_{O2} the respiratory flux, and $\Delta_r H_p$ the molar enthalpy of the net reaction of the phosphorylating system. Thus, the discrepancies between the CR ratio under phosphorylating conditions are due to differences in the net enthalpy change of the phosphorylating system. So,

by taking the $\Delta_r H_{redox}$ value measured on non-phosphorylating mitochondria (-433 ± 6 kJ/mol O_2) and the net enthalpy change associated with the ATP synthesis ($\Delta_r H_p$), it was possible to calculate an ATP/O ratio of 1.33. This value is in accordance with that of 1.25 for the ATP/O ratio of isolated yeast mitochondria using NADH as substrate and different methods to measure ATP synthesis [43].

Moreover, the enthalpy efficiency of oxidative phosphorylation is a function of the measured CR ratio, as follows:

$$\eta_H = -J_p \times \Delta_r H_p / J_{O2} \times \Delta_r H_{redox} = 1 - \text{CR ratio} / \Delta_r H_{redox} \quad (3)$$

The calculated enthalpy efficiency of oxidative phosphorylation, η_H, varied from a maximal value of 0.21 to about zero depending on the steady state (Table 1). In fact, zero enthalpy efficiency of transformation was reached when the net enthalpy change of the phosphorylating system was close to zero (e.g. hexokinase ADP-regenerating system) (Table 1). In Fig. 2, the effect of uncoupling on the enthalpy efficiency

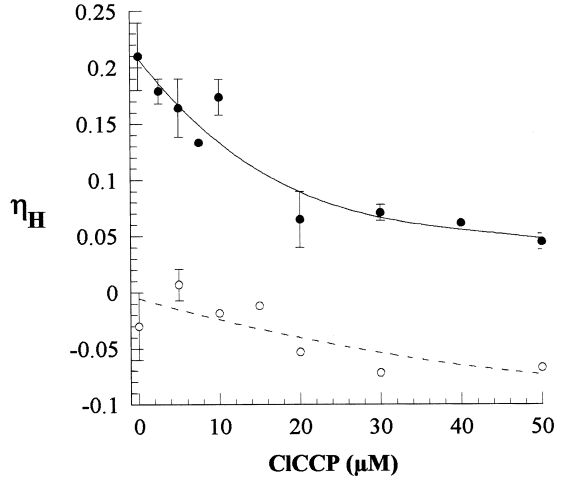

Fig. 2. Effect of uncoupling on the enthalpy efficiency of oxidative phosphorylation of isolated yeast mitochondria. Yeast mitochondria (0.05 mg protein/ml) were incubated in a flask connected to the microcalorimeter (see Section 2) in the presence of 2 mM NADH as respiratory substrate. ClCCP titrations were performed after ATP synthesis was induced either by the addition of ADP (●) ($n = 3$) or the addition of 0.05 units/ml of hexokinase in the presence of 1 mM ATP and 10 mM glucose (○) ($n = 2$). Calorimetric and respirometric fluxes were measured as described in Section 2. The enthalpy efficiency of oxidative phosphorylation, η_H, was calculated using Eq. 3 (see text).

of oxidative phosphorylation was investigated when ATP synthesis was induced by the addition of either ADP or hexokinase in the presence of glucose and ATP. Under the latter conditions (hexokinase system), η_H slightly decreased as the ClCCP concentration increased (Fig. 2). In contrast, under ADP-induced state 3 conditions, the η_H value progressively decreased from 0.2 without uncoupler to about 0.05 at a final concentration of 50 μM ClCCP (Fig. 2), a phenomenon corresponding to a decrease in the ATP/O ratio.

3.2. Heat dissipation and respiratory activity of yeast cells under resting and growing conditions

To perform experiments under resting conditions, W303-1a yeast cells were harvested in the exponential phase and subsequently incubated in a resting medium in the presence of 0.2% D,L-lactate. It is worth noting that net biomass synthesis was not detected during the entire rest period. Fig. 3 shows a correlation between the heat flux and oxygen consumption of resting cells harvested in the exponential growth phase. Moreover, a linear relationship between the two fluxes was obtained when the respiratory rate of resting cells was titrated using azide. The slope of the curve gives a CR ratio of -439 ± 10 kJ/mol O_2. To analyse the influence of the coupling between respiration and ATP synthesis on the cellular CR ratio, similar experiments were carried out in the presence of the cell permeant inhibitor of F_0F_1 ATP synthase, triethyltin (TET) [44]. Fig. 3 shows that TET addition did not significantly change the relationship between the two fluxes of resting cells. Thus, the azide titration of the TET-insensitive respiratory activity gave a linear relationship, with a corresponding CR ratio of -460 ± 14 kJ/mol O_2.

The heat dissipation associated with the aerobic growth of the W303-1a strain in a minimal medium containing a limiting concentration of D,L-lactate as energy and carbon source was measured recently [17]. Similarly, the total amount of lactate consumed and the amount of by-products and heat produced during the synthesis of 1 g of biomass was determined, and the data are summarised in Table 2. To compare these cumulative quantities, they were converted to enthalpy content using the respective enthalpy of combustion of substrate and products, in-

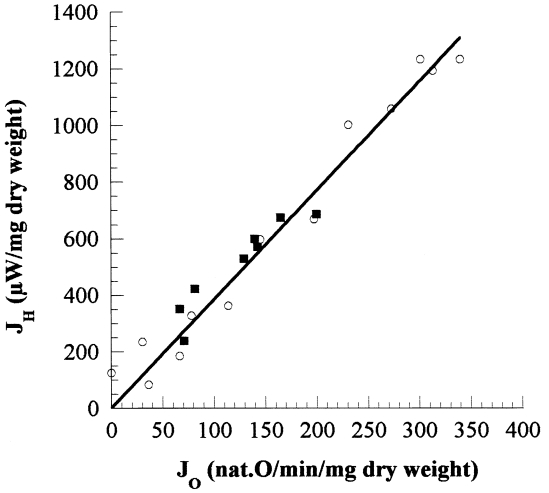

Fig. 3. Relationship between heat production flux and respiratory rate of resting yeast cells: effect of the cell permeant F_1F_0 ATP synthase inhibitor, TET. W303-1a cells were grown aerobically in minimal medium containing limiting amounts of D,L-lactate (0.2% w/v) as described in [17]. Cells were harvested in the exponential growth phase and incubated in a resting medium (0.1 mg dry weight/ml) supplemented with 0.2% (w/v) D,L-lactate, in the absence (○) or the presence of 50 μM TET (■). Under both conditions, the cellular respiratory activity was modulated by NaN_3 titration up to 100 μM ($n=5$).

cluding biomass [2,8,13]. Such determinations of enthalpy balance in the exponential phase are shown in Fig. 3 of [17]. If the enthalpy balance is satisfied, one can calculate the enthalpy growth yield (Y_H), which is 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 in the biomass compared to that dissipated as heat) [8]. Our results gave an enthalpy growth yield of 44 ± 3% (Table 2). Finally, by taking into account the latter value and the enthalpy change linked to the anabolic and catabolic reactions respectively (see Table 2), we estimated an overall enthalpy growth efficiency (η_H) for the W303-1a strain of about 10% (Table 2). Moreover, the simultaneous measurement of the heat production and the oxygen consumption fluxes gave an average value for the CR ratio during growth of about −378 ± 7 kJ/mol O_2 when respiratory activity was titrated with azide (Table 2).

It should be noted that the mean CR ratio of W303-1a cells was higher under resting than growing conditions (i.e. −439 vs. −378 kJ/mol O_2, respectively). The discrepancy between these values may be attributed to the endothermic enthalpy change associated with reactions of biosynthesis (see also [10,11]). For aerobically grown cells, the degree of biomass reduction is often close to that of the carbon source [2,16]. Therefore, the CR ratio of growing cells can be written as follows:

$$\text{CR ratio} = [Y_H \times \Delta_r H_a + (1-Y_H) \times \Delta_r H_k]/ [(1-Y_H) \times O_2/\text{Lac}] \quad (4)$$

where Y_H is the fraction of lactate transformed into biomass and $(1-Y_H)$ the fraction of lactate fully oxidised, $\Delta_r H_a$ and $\Delta_r H_k$ are the enthalpy change linked to the biomass synthesis and the oxidative catabo-

Table 2
Characteristics of the early exponential growth of W303-1a cells on lactate[a]

C-molar growth yield ($Y_{X/S}$) (%)	26 ± 2
C-molar by-products yield ($Y_{B/S}$) (%)	31 ± 3
Heat production ($\Delta_r Q_X$) (kJ/g dry biomass formed)	−24.7 ± 0.4
Enthalpy of combustion of biomass ($\Delta_c H_X$) (kJ/g dry biomass formed)	−19.2 ± 0.4
Enthalpy growth yield (Y_H) (%)[b]	44 ± 3
Enthalpy growth efficiency (η_H) (%)[c]	9.3 ± 1.3
CR ratio (kJ/mol O_2)[d]	−378 ± 7

[a]W303-1a haploid cells were grown in a minimal medium supplemented with 0.2% D,L-lactate. Heat production, metabolites in the culture medium, growth determination and oxygen uptake were measured as described in Section 2 and [17].
[b]Enthalpy growth yield was calculated using the following formula: $Y_H = \Delta_c H_X/(\Delta_c H_X + \Delta_r Q_X) \times 100$ (4).
[c]The enthalpy efficiency was calculated using the following formula: $\eta_H = Y_H/(1-Y_H) \times -\Delta_r H_a/\Delta_r H_k \times 100$ (5), where Y_H is the enthalpy growth yield, $\Delta_r H_k$ the enthalpy of the combustion reaction of lactate (i.e. −1323 kJ/mol lactate) and $\Delta_r H_a$ the enthalpy of the biomass synthesis from lactate (i.e. $C_3H_6O_3 + 0.57\ NH_3 \rightarrow 3CH_{1.96}O_{0.6}N_{0.19} + 0.14O_2 + 0.92H_2O$ with $\Delta_r H_a = +156$ kJ/mol lactate).
[d]The CR ratio during growth was calculated from the linear regression of J_H vs. J_O plots, obtained by titrating the respiratory activity with NaN_3 concentrations up to 150 μM.

lism, respectively, and O_2/Lac is the oxygen-lactate flux ratio of the complete lactate catabolism, which is equal to 3. The enthalpy growth efficiency is therefore a function of the measured CR ratio, so:

$$\eta_H = 1 - \text{CR ratio} \times (O_2/\text{Lac})/\Delta_r H_k \quad (5)$$

Note that the mean CR ratio measured for resting cells metabolising lactate (i.e. −439 kJ/mol O_2) was close to the theoretical oxycaloric equivalent for lactate combustion (i.e. $\Delta_r H_k/3 = -441$ kJ/mol O_2; calculated from [14,16,22]). Considering the CR ratio measured under growing conditions, we calculated an enthalpy growth efficiency of about 14%, a value which is quite close to that determined from the enthalpy balances (see Table 2).

3.3. Application to yeast cells expressing wild-type UCP1 and mutant UCP1Δ9

Recombinant protein expression in yeast has been widely used to characterise the different members of the uncoupling protein family (e.g. UCP1, UCP2, UCP3 and StUCP) at the cellular and mitochondrial level [28,29,45–50]. It is known that UCP1 catalyses an H^+ influx throughout the inner membrane of cold-adapted brown adipose tissue mitochondria, resulting in a dissipation of $\Delta\tilde{\mu}_{H^+}$ and therefore leading to an uncoupling of oxidative phosphorylation (see [51] for review). Moreover, it was previously shown that this activity can be downregulated by nucleotide addition (see [51] for review). In a previous study, a mutant of the brown adipose tissue UCP1, named UCP1Δ9, was obtained by disrupting the coding sequence of nine amino acids considered as a putative nucleotide recognition element [29]. During this work, coding sequences of wild-type UCP1 and mutant UCP1Δ9 were cloned in the pYeDP plasmid, thus allowing a conditional yeast cell expression of UCPs by galactose addition in the culture medium [28,29]. In isolated yeast mitochondria, whereas the uncoupling activity of UCP1 was prevented by GDP addition, this regulation was absent for the UCP1Δ9 protein [29,45,46]. In growing yeast cells, the loss of staining by a mitochondrial electrical potential-sensitive probe for the UCP1Δ9 strain, in contrast to wild-type UCP1 strain, indicated a constitutive uncoupling activity of the mutant protein [29]. Moreover, the generation time of yeast cells expressing UCP1 and UCP1Δ9 was increased compared to the control by a factor 1.4 and 3.5, respectively [29,46]. However, several reports clearly have shown that the decrease in the growth rate of yeast cells does not systematically imply that the growth yield decreases [8,17]. Hence, a change in the growth rate does not necessarily indicate a modification in the growth yield resulting from an uncoupling activity (i.e. due to UCP expression). Thus, in the present study, we first investigated the mitochondrial in situ uncoupling of the wild-type UCP1 and the mutant UCP1Δ9. For each strain, the part of oxygen uptake coupled to ATP synthesis was determined using TET, and the fully uncoupled respiratory activity of yeast cells was measured by means of the protonophoric uncoupler, ClCCP, to dissipate the proton electrochemical gradient across the inner mitochondrial membrane, and thereby to stimulate the respiratory rate to its maximum value. Fig. 4 shows that the residual oxygen uptake insensitive to TET was markedly higher for the mutant UCP1Δ9 and the UCP1 strain as compared to the control strain, whereas the fully uncoupled respiratory activity was about the same for the three strains. The optimal cellular respiratory control (RC), calcu-

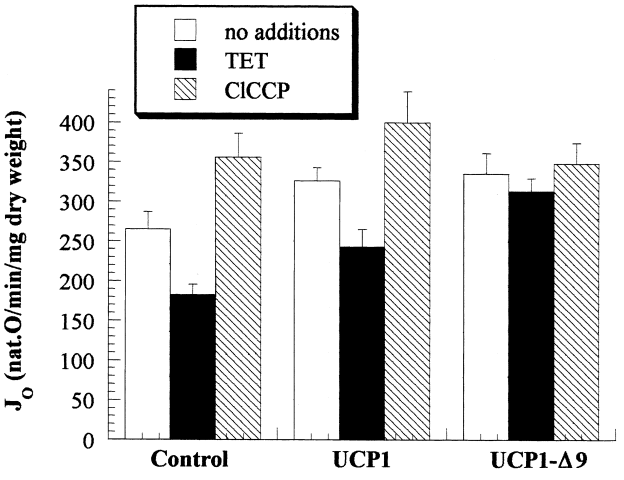

Fig. 4. Uncoupling effect of wild-type and mutant UCP1 expression on respiration of growing yeast cells. W303 cells were grown aerobically in minimal medium containing 2% (w/v) D,L-lactate. After galactose induction of wild-type and mutant UCP1 expression, cells were at least 10-fold diluted in fresh culture medium without galactose prior to measurements. Respiratory activity was measured as described in Section 2. When added, TET was 50 μM and ClCCP, 0.5–1 μM ($n = 3$ for each condition).

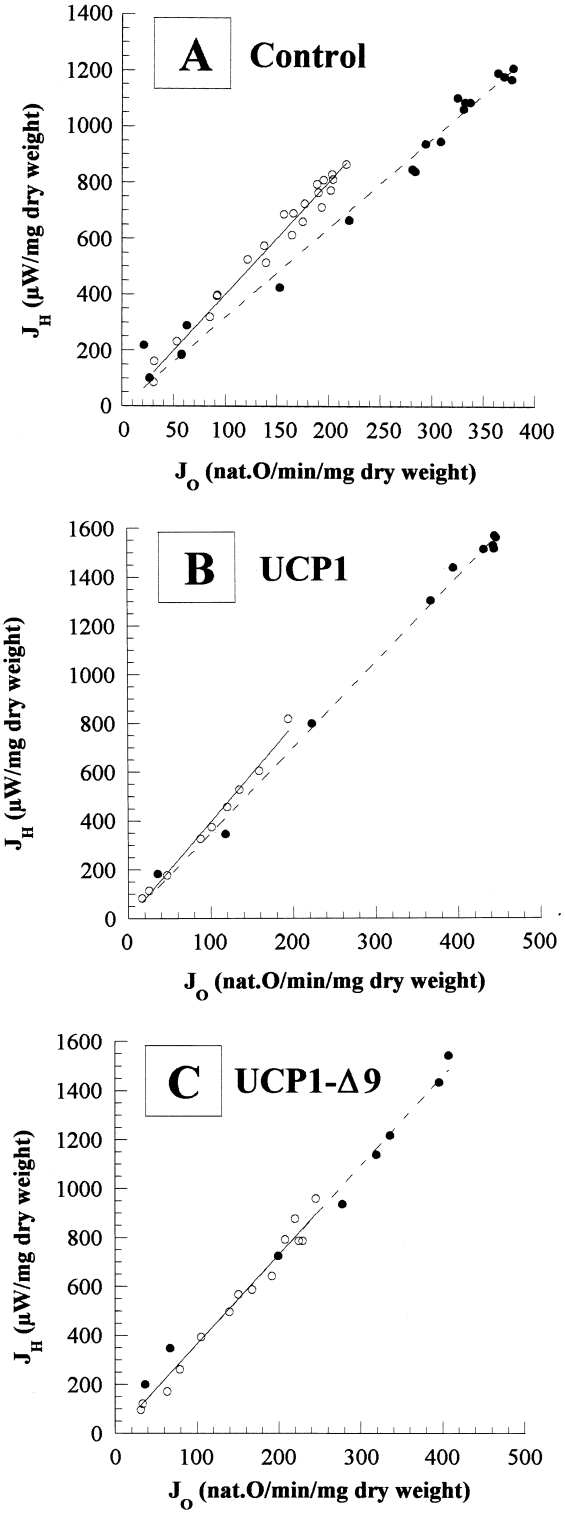

Fig. 5. Effect of wild-type and mutant UCP1 expression on the relationship between heat production flux and respiratory rate of yeast cells. W303 cells were grown aerobically in minimal medium containing 2% (w/v) D,L-lactate. After galactose induction of wild-type and mutant UCP1 expression, cells were either (i) diluted at least 10 times in fresh culture medium without galactose (●) or (ii) harvested and incubated in a resting medium (0.1 mg dry weight/ml) supplemented with 0.2% (w/v) D,L-lactate (○). Heat production rate and respiratory activity were measured as described in Section 2. Under both resting and growing conditions, the cellular respiratory activity was modulated by NaN_3 titration up to 100 μM ($n=2$ for each condition).

lated as the ratio between the fully uncoupled and the TET-insensitive respiratory activity, was lower for the UCP1 cells than for the control cells (i.e. 1.6 vs. 1.95, respectively), thus demonstrating a partial in situ uncoupling of the mitochondria during UCP1 expression. Moreover, the weak respiratory control of UCP1Δ9 cells (i.e. RC = 1.1 vs. 1.95 for UCP1Δ9 and control cells, respectively) indicated the complete in situ uncoupling of the mitochondria in UCP1Δ9 cells.

The CR ratio approach was then applied to analyse the effect of an in situ uncoupling of mitochondria on the enthalpy growth efficiency of UCPs expressing yeast cells. Fig. 5 represents the relationship between the heat production flux and the respiratory rate of yeast cells with respect to the expression or not of UCPs. During azide titration, the relationships were linear regardless of the strain and the metabolic state (growth vs. rest). For the control strain, as already mentioned above for the W303-1a strain (see Table 2 and Fig. 3), the heat production was about 20% lower at the same respiratory rate, under growing than under resting conditions (Fig. 5A). It should be noted that the discrepancy between growth and rest consistently decreased to about 10% for the UCP1 strain (Fig. 5B). Finally, for the UCP1Δ9 strain, no significant difference in the relationship between heat production and oxygen consumption was detected between rest and growth (Fig. 5C).

4. Discussion

Our data obtained with isolated yeast mitochondria demonstrate that the simultaneous measurement of heat production and oxygen consumption fluxes, and consequently the calculation of the CR ratio, can provide information on both the ATP/O yield

and the thermodynamic enthalpy efficiency of oxidative phosphorylation. There is a difference between the thermodynamic enthalpy efficiency (η_H) and the thermodynamic Gibbs energy efficiency (η_G) of energy transformation, which is defined as the free energy production rate ($-J_p \times \Delta_r G_p$) divided by the free energy consumption rate ($J_{O2} \times \Delta_r G_{redox}$) [5,6,52]. For instance, on isolated mitochondria, zero enthalpy efficiency and zero Gibbs energy efficiency obviously occur when the phosphorylation flux is null (i.e. in the presence of oligomycin). However, when ATP synthesis is induced by the hexokinase ADP-regenerating system, an efficient free energy transformation (e.g. $\eta_G = 0.33$–0.49 for isolated rat liver mitochondria respiring on various respiratory substrates [5,51]) can be achieved with a minimal enthalpy change (η_H close to 0; see Table 1) during the energy transformation. Therefore, any change in the ATP/O ratio of isolated mitochondria will be detectable unless the net enthalpy change linked to the energy output (i.e. phosphorylating system) is significantly different from zero (e.g. ADP-induced state 3, myokinase ADP-regenerating system).

At the cellular level, the CR ratio measured under aerobic resting conditions remained unchanged when the mitochondrial ATP synthesis was inhibited. This indicates that the steady state ATP turnover responsible for cellular maintenance under resting conditions (e.g. protein turnover, carbohydrate and polyphosphate store turnover, ATP-dependent ionic gradient homeostasis) operates in such a way that the final state equals the initial state, thus making the in vivo enthalpy efficiency of oxidative phosphorylation close to zero. It is worth noting that the mean CR ratio measured for resting cells metabolising lactate (i.e. -439 kJ/mol O_2) was close to the theoretical oxycaloric equivalent for lactate combustion (i.e. -441 kJ/mol O_2). This means that the incomplete oxidation of lactate linked to the accumulation of by-products such as pyruvate and acetate does not significantly interfere with the CR ratio. Moreover, during azide titration, the zero flux intercept was reached under resting conditions (see Fig. 3), indicating that the heat production was entirely linked to the respiratory activity of the cells. Therefore, neither polyphosphate hydrolysis [53,54] nor fermentation of the carbohydrate store occurs during resting conditions.

In the present study, we analysed the influence of biomass synthesis on the CR ratio during the growth of yeast cells on a non-fermentable carbon and energy substrate. The main observation was that the CR ratio was lower during growth as compared to rest. This phenomenon is in accordance with the endothermic contribution of anabolic reactions as compared to the exothermic contribution of catabolic reactions during growth (i.e. +156 kJ/mol lactate consumed and -1323 kJ/mol lactate consumed for biomass synthesis and catabolism, respectively (Table 2); see also [10,11]). Owing to the fact that the degree of reduction of the biomass is close to that of the carbon source and that the elemental composition of biomass is the same among yeast cells batch grown with respiratory substrate [2,16], the CR ratio is related to enthalpy growth efficiency, as shown by Eqs. 6 and 7. Indeed, from the CR ratios obtained, we estimated an enthalpy growth efficiency value which was only slightly higher to that obtained from the corresponding enthalpy balance, a difference which could be explained by an additional endothermic process due to a net polyphosphate synthesis during cell growth [55,56]. However, this last result indicated that the enthalpy growth efficiency of yeast cells growing on a non-fermentable carbon source could be well estimated using the CR ratio method. Moreover, in contrast to the enthalpy balance approach, this method does not rely on the exhaustive and tedious determination of the metabolites and elemental composition of biomass. Thus, experiments can be performed in the presence of non-limiting amounts of carbon source where substrate consumption can be difficult to assess. In these conditions, the CR ratio method is even more advantageous than the enthalpy balance approach for slow-growing cells. Hence, the determination of the CR ratio of UCP expressing yeast cells provides good evidence that the in situ uncoupling activity of UCP1 and mutant UCP1Δ9 decrease significantly (UCP1) or even cancel out (UCP1Δ9) the enthalpy growth efficiency of the respective strains. In the latter case, zero enthalpy efficiency indicates an absence of growth, equivalent to a resting state of the cells. Therefore, the residual growth of cells expressing UCP1Δ9 measured in a previous report [45] was likely due to a fermentative growth on galactose rather than to a respiration-linked growth. Finally,

it is to be noted that the decrease in cellular respiratory control is well correlated to the decrease in the respective enthalpy growth efficiencies determined for UCP expressing strains (see Figs. 4 and 5). Therefore, an increase in non-phosphorylating respiration compared to the basal respiratory rate leads to a parallel increase in heat production compared to biomass production. Moreover, this is in agreement with the fact that mitochondria by themselves are the major heat dissipative system when yeast cells are batch grown with a purely respiratory substrate, as described previously [17].

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