Metabolic Control Analysis of the bc_1 complex of *Saccharomyces cerevisiae*: effect on cytochrome *c* oxidase, respiration and growth rate

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A number of strains varying in steady-state level of assembled bc_1 complex were used to test the conclusions from inhibitor titration experiments with isolated mitochondria that, in cells of *Saccharomyces cerevisiae* grown on non-fermentable carbon sources, the control coefficient of the bc_1 complex on the mitochondrial respiratory capacity equals 1 and the respiratory chain consists of supermolecular respiratory units [Boumans, Grivell and Berden (1998) J. Biol. Chem. 273, 4872–4877]. In addition, the control coefficient of mitochondrial respiration on the growth rate was determined. It was found that a reduced level of bc_1 complex is accompanied by an almost parallel decrease in steady-state level of cytochrome *c* oxidase. Since the linear relationship between level of active bc_1 complex and respiratory capacity still holds, it is concluded that cytochrome *c* oxidase has disappeared from respiratory units that are already deficient in the bc_1 complex and that the cytochrome *c* oxidase in a respiratory unit is destabilized when the bc_1 complex is deficient. The control coefficient of the bc_1 complex, and thus of mitochondrial electron-transfer capacity, on respiration of intact cells (without uncoupler added) is 0.20. Addition of uncoupler results in an increase in the

coefficient to 0.36. Thus changing the respiratory state changes the distribution of control, increasing the control coefficient of electron-transfer activity as the respiratory state goes towards State 3u. Rates of growth of the strains on different carbon sources were determined and subsequently fitted to calculate control coefficients of the bc_1 complex (and therefore of the respiratory capacity) on growth. Little variation was found between lactate-, ethanol- and glycerol-containing media, control coefficients being around 0.18 at pH 5. At pH 7 the control coefficient increased to 0.57, indicative of a higher dependence of the cell on ATP derived from oxidative phosphorylation. During growth on glucose-containing medium, the bc_1 complex has no control on the growth rate, as indicated by the fact that all strains, including a respiratory-deficient strain, grow as fast as the wild-type. However, the presence of respiratory capacity in the wild-type does result in a higher growth yield compared with the respiratory-deficient strain, indicating that, in contrast with what is generally assumed, in *S. cerevisiae* the 'Pasteur effect' is not restricted to special experimental conditions.

INTRODUCTION

The yeast *Saccharomyces cerevisiae* is a facultative anaerobic organism that is able to survive without a functional respiratory chain, by falling back on the fermentation of sugars to supply its energy demands. This latter mechanism is used preferentially, since when a combination of fermentable and non-fermentable carbon sources is available, respiration is greatly reduced and fermentation accounts for the major fraction of sugar catabolism. *S. cerevisiae* is thus able to rapidly adjust its metabolism to its environment and in particular to the availability of carbon sources.

To obtain more insight into the regulation of oxidative phosphorylation in *S. cerevisiae*, we applied the principles of Metabolic Control Analysis [1,2]. This approach was first used for mitochondrial respiration by Groen et al. [3], who found that, in mammalian mitochondria, control is shared by a number of steps and that distribution of control is highly dependent on the respiratory flux. In particular, proton leakage exhibited high control under State-4 conditions, whereas during State-3 respiration, most of the control was found at the adenine nucleotide carrier, dicarboxylate carrier and cytochrome *c* oxidase [3].

The respiratory chain is an example of a group-transfer pathway, in which the group transferred is an electron. The outcome of Metabolic Control Analysis applied to such a

pathway deviates from that obtained for a 'simple' metabolic pathway, the major difference being that the sum of the control coefficients of all enzymes on the flux can be as high as two [4,5]. This is because each carrier is involved in two processes, one transfer from the donor and one transfer to the acceptor (see Figure 1). When the coefficients are calculated from changes in the electron-transfer reaction rates, they add up to one. It was therefore proposed to use 'enzyme control coefficients' with respect to modulation of enzyme concentration and 'process control coefficients' when calculated from changes in reaction rates [6]. In this paper we will adhere to enzyme control coefficients.

The physiological significance of the distribution of control can be illustrated by the feedback mechanism of ATP on respiratory activity. Multiple binding sites for ATP have been found on cytochrome *c* oxidase (EC 1.9.3.1) in *S. cerevisiae* [7] and *Bos taurus* [8]. The reported finding that this enzyme is the major site of action for *in vivo* regulation of respiration by ATP [9] may explain its high control of respiration found by Metabolic Control Analysis [3,10].

The vast majority of studies on the control of respiration have been performed with mammalian mitochondria. The yeast *S. cerevisiae* has received much less attention. A study by Mazat et al. [10], which did not include the bc_1 complex (ubiquinol-cytochrome *c* oxidoreductase; EC 1.10.2.2), showed that for

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both flux of oxygen consumption and ATP synthesis a large portion of the control is located at the step of cytochrome *c* oxidase. However, our recent finding, based on inhibitor titrations with isolated mitochondria, that the respiratory chain in *S. cerevisiae* behaves as a single functional and physical unit, all respiratory complexes having a control coefficient of one on respiration [11], predicts that this value of unity will also be found when the concentration of the complexes is varied by genetic manipulation.

In the present study, we used a number of previously described yeast strains that all show a decreased steady-state level of the bc_1 complex without affecting the catalytic properties of this enzyme [12–14]. The different steady-state levels of the bc_1 complex provide a means of varying the activity of this respiratory complex in proportion to concentration. Using this approach, the enzyme control coefficient of the bc_1 complex on respiration and on the rate of growth of *S. cerevisiae* on different carbon sources was determined.

EXPERIMENTAL

Strains and media

S. cerevisiae strain DAL273(167) (*a*, *leu2*, *lys2*) [15] (derived from strain D273-10B) was grown on minimal medium [0.67% (w/v) yeast nitrogen base (Difco), 2% (w/v) glucose, 2% (w/v) agar] supplemented with leucine, lysine and uracil (20 µg/ml) and containing 0.5 g/l 5-fluoro-orotic acid to screen for *ura3*[−] cells [16], giving strain DALU273 (*a*, *leu2*, *lys2*, *ura3*). Disruption of the *QCR8* gene was performed as described previously [14], giving DALU80 (*a*, *ura3*, *lys2*, *qcr8::LEU2*).

S. cerevisiae strains DLL80 (α , *his3*, *ura3*, *qcr8::LEU2*) [13] and DALU80 were used for the transformation of plasmids. Transformation of yeast was performed as described by Klebe et al. [17]. Transformants were selected on minimal medium supplemented with histidine or lysine (20 µg/ml). All mutant *QCR8* genes including the wild-type were present on a plasmid, pRS316 [18], and were constructed as described previously [19].

Growth media used were: lactate medium [0.5% yeast extract, 0.2% (w/v) MgSO₄, 0.6% (w/v) ammonium phosphate, 1.4% sodium lactate, 1.0% lactic acid, pH 5]; glycerol medium [1% (w/v) yeast extract, 1% (w/v) bacto-peptone, 2% (w/v) glycerol]; ethanol medium [1% (w/v) yeast extract, 1% (w/v) bacto-peptone, 2% (w/v) ethanol]; and glucose medium [1% (w/v) yeast extract, 1% (w/v) bacto-peptone, 2% (w/v) D-glucose]. The pH of the bacto-peptone-based media was 7 and was adjusted with HCl to pH 5 when indicated.

Experimental set-up

The strains used in this study (Table 1) contain changes in the *QCR8* gene encoding subunit VIII. This subunit is required for

assembly of the bc_1 complex [14] and many mutations in this protein result in different levels of assembly. All chosen mutants display a reduced steady-state level of the bc_1 complex, but with specific activity the same as that of the wild-type. All strains have been described previously [12–14] apart from number 4, which contains two lysines instead of two arginines at positions 50 and 51 (R50K; R51K). In this strain also the specific activity of the bc_1 complex is the same as in the wild-type strain (not shown). As can be seen in Table 1, the steady-state levels of the bc_1 complex range from 100 to 0%. For each strain, the cytochrome *c* oxidase activity and the respiratory activity of isolated mitochondria with different substrates were also determined, as well as the rates of growth on different carbon sources. Measurements were plotted against bc_1 concentration and subsequently analysed.

Growth-rate measurements

Growth rates were determined in flask-batch in a 300 ml Erlenmeyer flask containing 40 ml of the medium of interest. Further increases in the ratio Erlenmeyer volume/medium volume did not alter the growth rates. Medium was inoculated with exponentially growing cells to give a starting D_{600} of 0.05. Cultures were grown at 30 °C up to a maximum D_{600} of 0.3 to avoid oxygen-limited growth at higher cell density. During growth, samples were taken at different time points to measure D_{600} .

Cellular and mitochondrial oxygen-consumption measurements

Oxygen-consumption rates were measured at 30 °C with a Clark oxygen electrode (Gilson) in a 1.8 ml thermostatically controlled chamber. Rates were determined from the slope of a plot of oxygen concentration versus time.

Cells were taken from an exponentially growing culture, centrifuged and washed three times with water. They were then suspended in the cellular respiratory buffer containing 1% yeast extract, 0.1% KH₂PO₄, 0.12% (NH₄)₂SO₄, pH 4.5 [20]. During this preparation, cells were kept on ice at all times.

Mitochondria (0.5 mg of mitochondrial protein) were incubated in the mitochondrial respiratory buffer containing 0.6 M sorbitol, 25 mM potassium phosphate, pH 7.0, 1 mM EDTA, 1 mM MgCl₂ and 4 µM S13 as an uncoupler. Mitochondria were preincubated with specific inhibitors (antimycin A, myxothiazol, KCN) for 2 min. Substrates (0.5 mM NADH, 5 mM succinate, 0.5% ethanol, 5 mM ascorbate + 0.1 mM *N,N,N',N'*-tetramethyl-*p*-phenylenediamine) were added directly to the reaction chamber.

Spectral analysis and bc_1 assay

Spectral measurements were carried out at room temperature in an Aminco dual-wavelength spectrophotometer model DW2000. Concentrations of cytochromes were determined in isolated mitochondria using the following absorbance coefficients and wavelength pairs for the reduced-minus-oxidized proteins: 24.0 mM^{−1}·cm^{−1} at 603–625 nm for cytochrome *aa₃* [21], 20.1 mM^{−1}·cm^{−1} at 550–540 nm for cytochromes *c* + *c₁* [22] and 28.5 mM^{−1}·cm^{−1} at 562–575 nm for cytochrome *b* [22].

The ubiquinol-cytochrome *c* oxidoreductase (bc_1) assay was performed spectrophotometrically at 30 °C by measuring the reduction of 18 µM horse heart ferricytochrome *c* at 550–540 nm by 25 µM 2,3-dimethoxy-5-methyl-6-geranyl-1,4-benzoquinol. The buffer used contained 2 mM EDTA, 0.5 mM KCN and 20 mM potassium phosphate, pH 7.4, to obtain maximal activity with horse heart cytochrome *c* as acceptor [23].

Table 1 Relative bc_1 concentration and activity of the yeast strains used in this study

Strain	Number	[bc_1] (%)	bc_1 activity (%)	Reference
DL1 (wild-type)	1	100	100	[14]
⁵⁹ -FASAA- ⁶³	2	79	76	[12]
<i>S. pombe</i> VIII	3	50	44	[13]
⁵⁰ -KK- ⁵¹	4	31	30	This study
⁵⁹ -SASAA- ⁶³	5	5	6	[12]
DLL80 (<i>QCR8</i> ⁰)	6	0	0	[14]

Figure 1 Part of the respiratory chain depicted as a group-transfer pathway

Electron-transfer reactions from ubiquinol to cytochrome *c* oxidase are shown and are numbered 1–3. The redox state of the carriers is indicated by ox for oxidized and red for reduced. As can be seen, the *bc₁* complex is involved in reactions 1 and 2. Q, ubiquinone; *bc₁*, *bc₁* complex; cco, cytochrome *c* oxidase.

Analysis of the concentration of carbon compounds in the growth medium

Concentrations of carbon compounds (glucose, ethanol and glycerol) were determined by HPLC analysis using an Aminex HPX87H organic acids column of Bio-Rad at 65 °C. The column was eluted with 5 mM H₂SO₄. Detection was by means of a 2142 refractive index detector (LKB) and SP4270 integrator of SpectraPhysics.

Determination of flux control coefficients

The extent to which the *bc₁* complex controls the flux *J* through the biochemical pathway of interest, the flux control coefficient of the *bc₁* complex, can be described as the relative change in that flux on an infinitely small fractional change in the concentration of the *bc₁* complex:

$$C_{bc_1}^J = \frac{dJ/J}{d[bc_1]/[bc_1]}$$

The flux *J* in this definition can be, for example, the activity of the respiratory chain (*J_{resp}*) or of all processes in the yeast cell reflected by the growth rate (*J_{growth}*). A more general definition of flux control coefficient refers to enzyme activity rather than enzyme concentration, since the activity is the relevant parameter. In our case, however, the activity of the enzyme is proportional to its concentration, since the specific activity (turnover per cytochrome *b*) is not changed in the mutants. Since we in fact modify the enzyme concentration, we prefer to define the control coefficient in relation to concentration of enzyme and not activity.

When the concentration of the *bc₁* complex is varied, without change in the specific activity of the complex, two distinct processes are affected, namely oxidation of ubiquinol and reduction of cytochrome *c* (processes 1 and 2 in Figure 1). This is a general characteristic of a group-transfer pathway and, as a consequence, the sum of the control coefficients of carriers of that pathway is theoretically two. Since in this paper the concentration of the *bc₁* complex and not specific activity is changed, control will be described in terms of enzyme control coefficients, as suggested by Kholodenko and Westerhoff [6].

The relationship between the concentration of the *bc₁* complex and the growth rate of the corresponding strain was fitted according to the Michaelis–Menten equation:

$$J_{\text{growth}} = J_{\text{growth}}^{\text{max}} \times \frac{[bc_1]}{[bc_1] + K_m}$$

in which *J_{growth}* is the growth rate, *J_{growth}*^{max} the maximal growth rate at infinitely high [*bc₁*] and *K_m* the concentration of *bc₁* complex at which the growth rate equals 50 % of the maximum growth rate. The control coefficient can be calculated for any [*bc₁*] by taking the normalized slope of the plot, which can be calculated according to:

$$(J_{\text{growth}})' = \frac{J_{\text{growth}}^{\text{max}} \times K_m}{(K_m + [bc_1])^2} \times [bc_1] / J_{\text{growth}} = \frac{K_m}{K_m + [bc_1]}$$

RESULTS

Relationship between the amount of *bc₁* complex and respiration

The strains used in this study (described in the Experimental section and Table 1), have previously been characterized with respect to properties of the *bc₁* complex. The chosen strains carry mutations in subunit VIII and all exhibit similar effects: the steady-state level of the *bc₁* complex is reduced, but specific activity of the mutant enzymes is the same as that of the wild-type ([12–14] and Table 1). The respiratory capacity of uncoupled mitochondria of the *bc₁* mutants grown on lactate medium was measured in an oxygraph using ethanol as substrate. When the concentration of the *bc₁* complex is plotted against respiration, a linear relationship is found (Figure 2A). Since ethanol oxidation was found to be slower than NADH oxidation (see e.g. [20]), not all control on electron transfer with ethanol as the substrate is exerted by the cytochrome chain. The linear relationship is in line with our previous finding, based on inhibitor titrations, that the control coefficient of the *bc₁* complex for uncoupled substrate oxidation equals one, and the respiratory chain of *S. cerevisiae* grown on lactate is organized into a physical unit [11] (see also the Discussion). In terms of Metabolic Control Analysis, the linear relationship means that the enzyme control coefficient *C_{bc₁}*^{*J*} of [*bc₁*] on respiration is one at all *bc₁* concentrations measured, and that, whenever the control coefficient of [*bc₁*] is considered for a system containing the respiratory chain as one of the components, the control coefficient of the process of electron transfer through the respiratory chain is in fact determined.

Pleiotropic effect of *bc₁* mutants on the steady-state level of cytochrome *c* oxidase

Since spectral analysis indicated that, in addition to the *bc₁* complex, the *a*-type cytochromes were also diminished in the mutants, we further characterized this aspect by measuring the cytochrome *c* oxidase activity in mitochondria isolated from the mutants after growth on lactate medium using ascorbate + *N,N,N',N'*-tetramethyl-*p*-phenylenediamine as the electron donor. As shown in Figure 2(B), the mitochondria from these strains not only have a different amount of *bc₁* complex, but different cytochrome *c* oxidase activity as well. The activity corresponds to the level of *a*-type cytochromes found in the spectral analysis, indicating that the reduction in cytochrome *c* oxidase activity is not due to a change in the specific activity, but to a decrease in the amount of enzyme. The extent of this decrease almost parallels the effect on the *bc₁* concentration.

The finding, reported above, that the relation between respiration and the *bc₁* complex is still linear, as expected from the value of one for the control coefficient [11], despite the concomitant partial decrease in cytochrome *c* oxidase, indicates that the coupling between the steady-state levels of the *bc₁* complex and cytochrome *c* oxidase is related to the fact that the respiratory chain in *S. cerevisiae* appears to exist as a single physical and functional unit [11]. We will further corroborate this conclusion in the Discussion.

Figure 2 Relationship between the concentration of bc_1 complex and cytochrome c oxidase activity or ethanol oxidation of isolated mitochondria

The steady-state concentration of the bc_1 complex in mitochondria from the mutant strains relative to the parent strain was plotted against: (A) the respiratory activity using ethanol as substrate and against (B) the cytochrome c oxidase activity, measured oxygraphically using ascorbate + N,N,N',N' -tetramethyl- p -phenylenediamine as substrate. All activities are depicted as percentages of the wild-type activity.

Figure 3 Effect of the bc_1 complex on ethanol oxidation of intact cells

Ethanol oxidation of intact cells of different strains was measured oxygraphically in the absence (○) or presence (□) of 4 μ M uncoupler S13 and subsequently plotted against the steady-state level of the bc_1 complex. Data were fitted according to the Michaelis–Menten equation (see the Experimental section). ———, Relationship between respiration of isolated uncoupled mitochondria and the concentration of the bc_1 complex as depicted in Figure 2.

Control on respiration of intact cells

Ethanol oxidation was also measured using intact cells prepared according to standard procedures (see the Experimental section) and in both the absence and presence of uncoupler (Figure 3). In the former case, a plot of respiration versus $[bc_1]$ was fitted according to the Michaelis–Menten equation (see the Experimental section), providing the control coefficient of $[bc_1]$, and thus of electron-transfer activity, on coupled respiration of 0.12 at wild-type level of the complex. This value depends to some extent on the previous history of the cells used. For wild-type cells taken directly from exponentially growing cultures, respiratory activity was found to be higher (121%) than that of ‘prepared’ wild-type cells, and the control coefficient of $[bc_1]$ on coupled respiration was found to be 0.20 (results not shown).

The difference found may be explained by assuming that, when cells are kept on ice during the preparation, growth is arrested, thereby changing the respiratory state. Growth arrest probably causes ATP hydrolysis to be significantly lowered, shifting the respiratory state towards State 4. Thus the method used to prepare cells for oxygraph measurements has an effect on the respiratory state, thereby affecting the distribution of control.

In the presence of uncoupler S13, a control coefficient of 0.30 was found (Figure 3). This value becomes slightly higher when corrected for the NADH–cytochrome c oxidoreductase activity (see below), but is much lower than the control coefficient of 1.0 for $[bc_1]$ on uncoupled respiration of isolated mitochondria. We speculate that addition of uncoupler to intact cells does not result in complete uncoupling of mitochondria, indicated by the relatively small stimulation of respiration (respiratory coefficient of 1.8, whereas for isolated mitochondria normally a coefficient of 3–4 is found (see e.g. [24]), and therefore resulting in a control coefficient of the bc_1 complex on respiration that is significantly smaller than unity. Alternatively, it is possible that, in contrast with isolated mitochondria, ethanol added to intact cells does not result in saturating substrate concentration. This would result in increased control at steps other than the electron-transport chain (e.g. alcohol dehydrogenase) and therefore a smaller control coefficient of the bc_1 complex. Such a relationship between control coefficient and substrate concentration was found between adenine nucleotide translocator and the concentration of 2-oxoglutarate in isolated rat heart mitochondria [25]. Use of carbonyl cyanide m -chlorophenylhydrazone, for which a respiratory coefficient of 2.6 has been reported in the literature [26], did not prove to be any better than S13. The control coefficient of $[bc_1]$ on uncoupled respiration in intact cells is thus smaller than found in isolated mitochondria, presumably because in intact cells under these conditions more reactions than electron transfer are implicated.

Contribution of the NADH–cytochrome c oxidoreductase pathway to total NADH oxidation

In addition to the NADH dehydrogenases bound to the mitochondrial inner membrane, yeast contains a third enzyme, bound

Figure 4 Effect of the *bc₁* complex on the growth rate

Rates of growth of the mutants on lactate medium, pH 5 (□) and glycerol medium, pH 7 (○), were determined. Growth rates are plotted as percentage of the growth rate of the wild-type strain against the $[bc_1]$ fitted according to the Michaelis–Menten equation.

to the mitochondrial outer membrane [27]. This enzyme is overexpressed in a ubiquinone-deficient mutant of *S. cerevisiae*, which may indicate the presence of a regulatory mechanism of induction of this activity under conditions unfavourable for normal respiration [28]. Since this pathway also bypasses the *bc₁* complex, the NADH–cytochrome *c* oxidoreductase activity was measured as the oxygen consumption, using NADH as substrate in the presence of both antimycin A and myxothiazol. It was found to be the same for all mutants and the wild-type strain (results not shown), and thus had little effect on the observed control coefficient of the *bc₁* complex on NADH respiration.

We have further investigated the effect of the level of the *bc₁* complex on NADH–cytochrome *c* oxidoreductase by comparing the DAL273 wild-type strain with DALU80, which lacks *bc₁* activity, both grown on galactose medium. The same strain and the same medium were used in the studies on the ubiquinone-deficient strain, making direct comparison possible. However, even with these strains no difference in NADH–cytochrome *c* oxidoreductase activity was observed, indicating that the regulatory mechanism suggested for the ubiquinone-deficient strain [28] does not apply for the *bc₁*-deficient strain. The previously suggested role for NADH–cytochrome *c* oxidase as a ‘by-pass’ pathway under conditions unfavourable for normal respiration therefore seems unlikely [28].

Subtraction of the contribution of NADH–cytochrome *c* oxidoreductase activity from total ethanol oxidation of isolated mitochondria has only a minor effect on the calculated control of $[bc_1]$ in intact cells: the coefficient increases from 0.30 to 0.36 when corrected for the NADH–cytochrome *c* pathway.

Control of the *bc₁* complex on growth rate

Rates of growth of the strains were determined as described in the Experimental section using different carbon sources (Figure 4), and the data empirically fitted according to the Michaelis–Menten equation (see the Experimental section). The control coefficient determined from the slope of the curve at the wild-type $[bc_1]$ ranged from 0.16 to 0.23 on lactate-, ethanol- and glycerol-containing media at pH 5 (Table 2).

On glycerol-containing medium at pH 7, a control coefficient of 0.57 is found (Table 2). This higher value is indicative of a higher dependence of the cell on ATP derived from oxidative phosphorylation, presumably because the conditions for the plasma-membrane ATPase are energetically less favourable [29].

Table 2 Control coefficient of the *bc₁* complex on the growth rate on different carbon sources

n.a., Not applicable.

Carbon source	pH of medium	Control coefficient
Lactate	5	0.16
Ethanol	5	0.17
Glycerol	5	0.23
Glycerol	7	0.57
Glucose	n.a.	0.0

Table 3 Glucose consumption and ethanol and glycerol production in wild-type and respiratory-negative strain

Exponentially growing cells were inoculated in glucose medium to give a starting D'_{600} of 0.2, and growth was continued to $D_{600} = 1.0$. Cells were centrifuged and 1 ml of supernatant was used for analysis of concentrations of carbon compounds. The growth yield for the wild-type strain is 16.1 g dry weight/mol of glucose consumed and 11.4 for the respiratory-negative strain.

Strain	Glucose consumption (mM)	Ethanol production (mM)	Glycerol production (mM)
DL1 (wild-type)	6.9	10.2	0.4
DLL80 (QCR8 ⁰)	9.7	14.4	1.2

The increase in control coefficient of the *bc₁* complex may be due to either an increase in ATP consumption by the plasma-membrane ATPase (which can be as high as 40% of total ATP production [30]) or alkalization of the cytosol [31], thereby directly affecting the membrane potential across the inner mitochondrial membrane.

Control coefficient for glucose medium

The maximum growth rate of all strains grown on glucose medium was found to be the same as that of wild-type cells ($\mu_{\max} = 0.49 \text{ h}^{-1}$). Without using any fitting procedure, it can be directly deduced from this that the control coefficient of respiration for growth on glucose is 0.0. Under these conditions, only a small fraction of the available glucose is fully oxidized via respiration, since fermentation to ethanol is the major pathway of glucose metabolism in yeast [32]. To determine the percentages of carbon used in the different pathways, strain DL1 was grown in a batch-fermenter under fully aerobic conditions, with excess glucose as carbon source (J. Blom, personal communication). It was found that 73% of the carbon was fermented to ethanol (consistent with values of 70–80% found in the literature [32]), and 7% was fully oxidized to CO_2 via the respiratory-chain pathway (slightly higher than previously published values). Taking into account that the P/O ratio in *S. cerevisiae* is smaller than the theoretical value of 2.0 (reported values are 0.95 [33] and 1.5 [34]), it can be calculated that respiration accounts for about 30–50% of the total ATP production in the DL1 strain. The fact that strain no. 6, which lacks a functional respiratory chain, grows as fast as the wild-type strain suggests that the glucose consumption rate is significantly higher in this mutant and, as a consequence, the growth yields significantly lower. Table 3 compares glucose consumption as well as ethanol and glycerol production between this strain and the wild-type grown in flask-batch. The ratio between glucose consumption and ethanol

production indicates that these results are consistent with the data from the fermenter-grown cells. (In addition, the maximum growth rate of batch-wise fermenter-grown cells was found to be almost identical with that of flask-batch-grown cells.) Both glucose consumption and ethanol production in strain no. 6 are 40 % higher than in the wild-type, corresponding to 30 % of total ATP production by respiration. The growth yield for the wild-type strain is 16.1 g dry weight/mol of glucose consumed and 11.4 for strain no. 6. Glycerol production is increased even more, by a factor of 3, in accordance with the role of glycerol in maintaining redox balance. We conclude that an increase in glycolytic flux compensates for the absence (or decreased activity) of respiration in the mutants, resulting in the same maximum growth rate and a corresponding decrease in growth yield.

DISCUSSION

A number of bc_1 -mutant strains that contain a reduced steady-state level of this complex without any change in specific activity have been used to calculate enzyme control coefficients on respiration and cell growth. We find that the reduced steady-state level of the bc_1 complex in the mutants, a consequence of distorted assembly by mutations in subunit VIII, is accompanied by a reduced steady-state level of cytochrome *c* oxidase. Extrapolation to a $[bc_1]$ of zero in Figure 2(A) suggests a 'basal' level of cytochrome *c* oxidase of around 15 % of the total amount. The idea of a 'basal' level of this enzyme is further supported by comparing respiratory rates of mitochondria isolated from strains 1 and 6 (wild-type and *QCR8*-null) after growth on glucose: the cytochrome *c* oxidase activity of strain 6 was the same as that of strain 1, despite the absence of respiration in strain 6 because of the lack of bc_1 activity. Since the level of respiratory-chain complexes under glucose-repressed conditions is a factor of 7–10 lower than under conditions in which expression is induced, it would appear that this 'basal' level of cytochrome *c* oxidase (representing 10–15 % of the amount under induced conditions) is not regulated in response to diminished levels of bc_1 complex. An effect of bc_1 deficiency on the level of cytochrome *c* oxidase under inducing conditions, but not under repressing conditions, has been noted previously [18]. The data reported by Coppée et al. [35] indicate that during growth on the non-repressing substrate galactose also a deficiency in the level of the bc_1 complex results in a lowering of the level of cytochrome *c* oxidase.

The coupling between the steady-state levels of the bc_1 complex and cytochrome *c* oxidase indicates the existence of a regulatory mechanism that balances the respiratory-chain complex stoichiometry. Such a mechanism is consistent with our recent finding that the respiratory chain in *S. cerevisiae* grown on non-fermentable carbon sources behaves as one unit [11]. The implication of organization into one unit is that the different respiratory complexes physically interact. A decreased level of the bc_1 complex thus would result in 'free' cytochrome *c* oxidase when no co-regulation exists.

The respiratory chain is a group-transfer pathway, the group transferred being an electron. It has been shown that the sum of enzyme control coefficients on the flux through a group-transfer pathway can be as high as two [4–6]. This value is not necessarily constant, but may vary, depending on the concentrations of the enzymes involved, as was found in the case of another group-transfer pathway, the phosphotransferase system [36]. This concentration-dependence arises from the fact that the sum of enzyme control coefficients depends on whether enzymes of the pathway form complexes that are stable longer than the transit time of the metabolite [4–6]. As the concentration of the enzymes

increases, the lifetime of complexes between them increases and the sum of control coefficients decreases. Metabolic Control Analysis predicts that, if the complexes are long living, the sum of the control coefficients should be one, but, in that case, there is in fact only one enzyme. Thus the organization of the respiratory chain of *S. cerevisiae* into a single unit would imply that the chain is one supermolecular entity with a control coefficient for respiration of one. In contrast, for the respiratory chain in higher eukaryotes, when the respiratory chain complexes are found to be freely diffusible in a dynamic organization, this sum is predicted to be two. Thus Metabolic Control Analysis of group-transfer pathways provides a method of determining differences in molecular organization of the respiratory chain in *S. cerevisiae* compared with higher eukaryotes.

We found a linear relationship between the $[bc_1]$ and substrate oxidation rate, implying a control coefficient of one, as was previously found using inhibitor titrations [11]. Organization into one unit predicts that the same control of unity is exerted by any step within the whole chain. Thus the control coefficients of the bc_1 complex and cytochrome *c* oxidase on electron-transfer activity, when determined separately, should both be unity. The decrease in cytochrome *c* oxidase in response to the deficiency of the bc_1 complex complicates the matter. If we assume that the respiratory chain consists of randomly colliding entities, ubiquinone and cytochrome *c* showing pool function kinetics (as is observed in mammalian mitochondria), the relation between respiration, especially succinate oxidation, and the level of bc_1 complex should be non-linear (less than proportional). This also applies when the oxidase content is lowered, since the activity of succinate dehydrogenase is very much lower than that of the bc_1 complex and cytochrome *c* oxidase. If, on the other hand, the respiratory chain is organized into units, a concomitant decrease in oxidase content may occur in two different ways: random or linked to the bc_1 deficiency. If deficiency is random, the relation between respiration and bc_1 content should be more than proportional, since, in addition to the units without bc_1 complex, the units without cytochrome *c* oxidase are also inactive. If, however, the deficiency of oxidase is linked to the deficiency of bc_1 complex, the relationship between respiratory activity and bc_1 complex remains proportional, since oxidase deficiency only occurs in units that are already inactive because of the lack of bc_1 complex. The experimental data are in agreement with this latter case, and we may therefore conclude that the absence of bc_1 complex from a unit destabilizes the cytochrome *c* oxidase in that unit or prevents incorporation of cytochrome *c* oxidase into such a unit, resulting in a decreased steady-state level of this enzyme. Since the ratio between $[bc_1]$ and [cytochrome *c* oxidase] is different in different yeast strains, our conclusions on the required interaction between two enzymes cannot be extrapolated to other yeasts without specific measurements.

A number of reports have shown that the distribution of control of mitochondrial respiration is strongly dependent on the respiratory state. When respiration is in State 4, most of the control is located at the step of proton leakage across the inner membrane [37]. In agreement with this, we find that the enzyme control coefficient of the bc_1 complex on the growth rate is very small when cells are kept on ice before respiratory activity is measured. Such cells are growth arrested, and ATP hydrolysis is likely to be greatly reduced, so that respiration approaches State 4. However, since ATP hydrolysis will never be completely absent in intact cells, it can be assumed that State 4 cannot be completely reached. As a consequence, the enzyme control coefficient of the bc_1 complex in this state will be even lower than 0.12. Our data do not permit us to conclude whether the bc_1 complex, and therefore also electron transfer, will have any

control under State 4 conditions, but they do show that intact cells prepared according to this generally applied procedure do not reflect metabolic processes in their physiological state. Furthermore, we observed with intact cells that, as respiration shifts towards State 3u, more control becomes located at the *bc₁* complex. This is in agreement with previous findings with isolated mitochondria from yeast [10], mammals [3] and plants [38].

The flux control coefficient of the *bc₁* complex (and thus of electron transfer) on the rate of growth on lactate medium was found to be 0.16 and, assuming that respiration in intact cells directly correlates with the ATP synthesis rate, the control coefficient on the ATP synthesis rate in intact cells was found to be 0.20. (This assumption may not be entirely true, since the proton-motive force is also the immediate source of free energy for a large number of carriers that transport a variety of ions and charged metabolites across the mitochondrial inner membrane.) The two control coefficients of the *bc₁* complex, on ATP synthesis and on the growth rate, can (indirectly) provide the flux control coefficient of ATP synthesis on the growth rate. This relationship becomes clear when the top-down, or modular, approach to control analysis is applied as described by Brand and co-workers [39,40]. In this approach, all steps of a certain flux are first divided into blocks, which in turn consist of several steps. ATP synthesis can be considered to be one block of the growth flux, whereas electron transport is a step within this block. The flux control coefficient of ATP synthesis on the growth rate thus equals $0.16/0.20 = 0.80$. It should be noted, however, that small errors in the control coefficients found for the *bc₁* complex on the growth rate and ATP synthesis (0.16 and 0.20) introduce rather large uncertainties into the calculated control coefficient of ATP synthesis on growth. Nevertheless, these data suggest that, although ATP synthesis is the major controlling process for growth, not all control is exerted at this step.

The identical maximum growth rates on glucose for all strains indicate that respiration has no control on the growth rate of *S. cerevisiae* on this medium. This is in line with earlier findings that both growth yield and maximum growth rate for (wild-type) *S. cerevisiae* are similar under aerobic and anaerobic conditions [41]. The fact that the respiratory-deficient strain no. 6 has a lower growth yield than the wild-type suggests that this rather surprising equivalence is due to an increased consumption of ATP under aerobic conditions [42], rather than to errors in estimates for the fraction of ATP produced by respiration in aerobiosis.

The finding that the growth yield of wild-type yeast under anaerobic and aerobic conditions is similar [41] has led to the conclusion that *S. cerevisiae* grown in batch culture does not show the so-called 'Pasteur effect', i.e. inhibition of glycolytic flux by respiration [42]. It has long been believed that the Pasteur effect can only be observed under special conditions such as in chemostat cultures when glucose is limiting for growth [43], or in resting yeast cells [44]. However, our results show that respiration in wild-type cells causes a decrease in the glucose-consumption rate in comparison with the respiratory-negative cells, thus implying that in batch cultures also *S. cerevisiae* shows the Pasteur effect.

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