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The Importance of the Glycerol 3-Phosphate Shuttle During Aerobic Growth of *Saccharomyces cerevisiae*

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Maintenance of a cytoplasmic redox balance is a necessity for sustained cellular metabolism. Glycerol formation is the only way by which *Saccharomyces cerevisiae* can maintain this balance under anaerobic conditions. Aerobically, on the other hand, several different redox adjustment mechanisms exist, one of these being the glycerol 3-phosphate (G3P) shuttle. We have studied the importance of this shuttle under aerobic conditions by comparing growth properties and glycerol formation of a wild-type strain with that of *gut2Δ* mutants, lacking the FAD-dependent glycerol 3-phosphate dehydrogenase, assuming that the consequent blocking of G3P oxidation is forcing the cells to produce glycerol from G3P. To impose different demands on the redox adjustment capability we used various carbon sources having different degrees of reduction.

The results showed that the shuttle was used extensively with reduced substrate such as ethanol, whereas the more oxidized substrates lactate and pyruvate, did not provoke any activity of the shuttle. However, the absence of a functional G3P shuttle did not affect the growth rate or growth yield of the cells, not even during growth on ethanol. Presumably, there must be alternative systems for maintaining a cytoplasmic redox balance, e.g. the so-called external NADH dehydrogenase, located on the outer side of the inner mitochondrial membrane. By comparing the performance of the external NADH dehydrogenase and the G3P shuttle in isolated mitochondria, it was found that the former resulted in high respiratory rates but a comparably low P/O ratio of 1·2, whereas the shuttle gave low rates but a high P/O ratio of 1·7.

Our results also demonstrated that of the two isoforms of NAD-dependent glycerol 3-phosphate dehydrogenase, only the enzyme encoded by *GPD1* appeared important for the shuttle, since the enhanced glycerol production that occurs in a *gut2Δ* strain proved dependent on *GPD1* but not on *GPD2*.

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KEY WORDS — *Saccharomyces*; redox; glycerol; NADH; shuttle

INTRODUCTION

Maintenance of intracellular redox balance by controlling the cytosolic NADH/NAD^+ ratio is crucially important for sustained oxidation of cellular substrates. During growth of the yeast *Saccharomyces cerevisiae*, excess NADH is produced in the cytoplasm due to the formation of biomass and different by-products (Albers *et al.*, 1996; Nordström, 1968; Van Dijken and Scheffers, 1986). Glycerol formation is the only way of restoring the cytoplasmic redox balance under anaerobic conditions since ethanol production is a redox neutral process (Nordström, 1968; Van Dijken and Scheffers, 1986). Under aerobic conditions, on the other hand, excess of cytoplasmic NADH can be oxidized in an alternative way, by conveying its reducing equivalents to the mitochondrial electron transport chain. In *S. cerevisiae* this transfer can be achieved via external NADH-

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dehydrogenase facing the outer side of the inner membrane of the mitochondrion (von Jagow and Klingenberg, 1970) and/or via shuttle systems like the glycerol 3-phosphate (G3P) shuttle. There may also be additional shuttle systems present for oxidation of cytosolic NADH. However, the malate–aspartate shuttle present in higher eukaryotes seems to be non-functional and the fatty acid/malate–citrate shuttle non-important in yeast (de Vries and Marres, 1987).

In the G3P shuttle, cytosolic NADH reduces dehydroxyacetonephosphate (DHAP) and the resulting G3P passes through the permeable outer mitochondrial membrane where it is re-oxidized to DHAP by the FAD-dependent glycerol 3-phosphate dehydrogenase (GPD) located on the inner mitochondrial membrane. The DHAP produced then returns back to the cytosol by an unknown mechanism. Hence, the overall effect provides for mitochondrial oxidation of NADH that is produced in the cytoplasm. An irreversible cyclic process is achieved because the mitochondrial enzyme is a flavoprotein, the oxidation of which is strongly exergonic. In *S. cerevisiae* the FAD-dependent enzyme is encoded by the *GUT2* gene (Rönnow and Kielland-Brandt, 1993; Sprague and Cronan, 1977), while NAD^+-dependent GPD is encoded by two isogenes, *GPD1* and *GPD2* (Eriksson *et al.*, 1995; Larsson *et al.*, 1993). The two isoenzymes, Gpd1p and Gpd2p, seem to serve two different functions in the cell. Gpd1p is induced under osmotic stress in order to enhance the production of glycerol (André *et al.*, 1991; Larsson *et al.*, 1993), the main osmoregulator in *S. cerevisiae* (Blomberg and Adler, 1992). Gpd2p, on the other hand, appears to be under redox control since this isoenzyme is necessary for normal growth and glycerol production under anaerobic conditions (Ansell *et al.*, 1997; Björkqvist *et al.*, 1997).

The requirements for redox adjustment systems differs depending on growth conditions, e.g. availability of oxygen, type of carbon source, etc. For instance, ethanol oxidation probably leads to a high flux of cytosolic NADH generation due to the cytoplasmic localization of the isoform of alcohol dehydrogenase (ADHII) chiefly involved in ethanol oxidation (Wills, 1990; Figure 1). Indeed, it has been shown that the intracellular NAD(P)H level is much higher with ethanol than with glucose as the substrate under aerobic conditions (Beauvoit *et al.*, 1993), which might be a reflection of an increased production rate of NADH when using a reduced substrate such as ethanol. Growth on lactate results in a completely different situation. When lactate is oxidized by lactate: cytochrome *c*, oxidoreductase (LDH in Figure 1) the electrons are not transferred to NAD^+ but to ferricytochrome *c*, thus directly entering the electron transport chain (de Vries and Marres, 1987). In other words, there is no cytosolic NADH generation due to catabolic lactate oxidation. Similarly, catabolic oxidation of pyruvate should not result in cytoplasmic NADH formation, since this carbon source is oxidized through the citric acid cycle in the mitochondrion. Consequently, if the G3P shuttle is important to maintain the cytosolic redox balance, a high Gpdp activity would be expected during growth on ethanol. However, in batch cultures having ethanol as the carbon source, a continuous increase in Gpdp activity was observed (without concomitant increase in glycerol production) throughout the whole growth period with a maximum in the stationary phase (Blomberg *et al.*, 1988). During these experiments growth rate as well as external pH were subject to continuous changes which might have affected shuttle activity by for example influencing the cellular ATP requirement.

Although the necessary enzymes for the G3P shuttle are demonstrated in the appropriate cellular compartments of *S. cerevisiae*, experimental data assessing its significance are entirely lacking. The main objective of this study was to address this question by examining the metabolic importance of the shuttle under different conditions using mutants having defective NAD^+- or FAD-dependent GPD activities. To impose different demands on the capacity for redox adjustment by oxidation of cytosolic NADH, cells were cultured with carbon sources having various degrees of reduction, such as ethanol, glucose, lactate and pyruvate. We also performed studies to assess the relative importance of the two enzymes Gpd1p and Gpd2p for the shuttle activity. Furthermore, the influence of growth rate and external pH on shuttle activity was investigated by performing chemostat cultures with ethanol as the limiting substrate at different dilution rates and different external pH. The rationale for using different pH was to impose different energy demands on the cells and to study how this is reflected in the activity of the G3P shuttle. In this context we also measured, using isolated mitochondria, the performance of the G3P shuttle vs external NADH dehydrogenase in terms of respiratory rate and efficiency, i.e. P/O ratio.

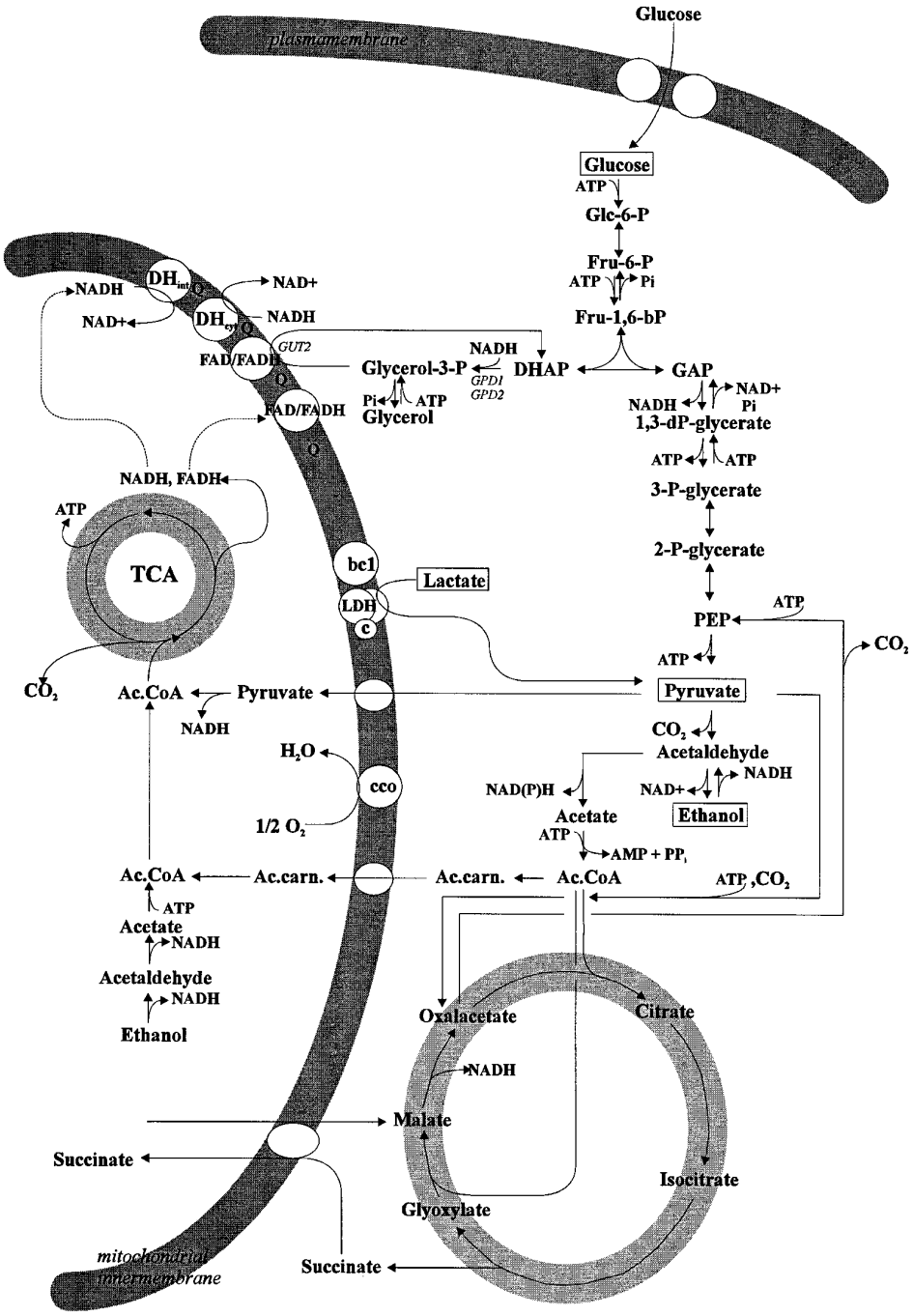

Figure 1. Overview of different pathways in *S. cerevisiae* involved in oxidation of glucose, ethanol, lactate and pyruvate (de Vries and Marres, 1987).

MATERIALS AND METHODS

Yeast strains and media

The *S. cerevisiae* strains used during batch cultures were all derived from W303-1A (*ade*2-1^0, *his*3-11, *leu*2-3, 112*trp*1-1a,*ura*3-1, *can*100^0) referred to as wild-type; *gut2Δ* (*ade*2-10, *his*3-11, *leu*2-3, 112*trp*1-1a,*ura*3-1, *can*100^0, *gut*2Δ::URA3); *gpd*1Δ*gut2Δ* (*ade*2-1^0, *his*3-11, *leu*2-3, 112*trp*1-1a,*ura*3-1, *can*100^0, *gpd1*Δ::TRP1, *gut*Δ::URA3); and *gpd*2Δ*gut2Δ* (*ade*2-1^0, *his*3-11, *leu*2-3, 112*trp*1-1a,*ura*3-1, *can*100^0, *gpd*2Δ::URA3, *gut*2Δ::URA3). During chemostat cultures the strains were: M1-2B(*ura*3, *trp*1) referred to as wild-type; and Bi1 (*ura*3, *trp*1, *gut*2Δ::URA3). The medium was yeast nitrogen base without amino acids (YNB, Difco) supplemented with glucose, ethanol, lactate or pyruvate at a concentration of 5 g/l (except for pre-cultures where 10 g/l of glucose was used). The concentration of the required bases and amino acids was 120 mg/l.

Growth conditions for batch cultures

The experiments were performed in 1-l Erlenmeyer flasks containing 200 ml of medium with an initial pH of 6·0 at 30°C on a rotary shaker at 130 rpm. The pre-culture was cultivated for 48 h under the same conditions except for the volume, which was 100 ml.

Growth conditions for continuous cultures

The chemostat experiments were performed in a Chemap, CMF 2-l fermenter (Chemap AG, Volketswil, Switzerland) with ethanol as the limiting substrate (reservoir concentration 5 g/l). The working volume was 1·5 l, the temperature 30°C, the stirring rate 700 rpm and the aeration rate 1 vvm. The pH was kept constant at 6·0, 4·5 or 3·5 by automatic addition of 1 M-NaOH. The total activity (rate of heat production) was continuously monitored by microcalorimetry. When steady-state was established samples were withdrawn for determination of substrate, product and biomass concentrations.

Measurements of oxygen consumption rate

The oxygen consumption rate was monitored by an acoustic carbon dioxide and oxygen monitor (type 1308; Brüel and Kjaer, Naerum, Denmark).

Microcalorimetry

The heat production rate (dQ/dt) was continuously monitored with a multichannel microcalorimeter (Thermal Activity Monitor, TAM, Thermometric AB, Järfälla, Sweden) in the flow-through mode.

Test for steady-state conditions

A constant heat production rate was used as the criterion for the establishment of steady-state conditions. The heat production rate can be used as a measurement of the total activity of the culture since all reactions in a cell result in a heat change (Belaich, 1980; Gnaiger, 1983; Gustafsson, 1991).

Dry weight determinations

Samples (2 × 5 ml) were centrifuged for 5 min at 3500 *g*, washed twice with water, and dried for 24 h at 110°C.

Preparation of extracellular samples for determination of substrate and product concentrations

Samples (2 × 1·5 ml) were centrifuged for 1 min at 15 000 *g*. The supernatants were frozen (− 20°C) until analysed.

Analysis of ethanol and glycerol concentrations

The analyses were made by using enzyme combination kits (Biochemica Test Combination; Boehringer Mannheim GmbH, Germany) except for analysis of glycerol concentrations with pyruvate as the substrate where HPLC was used. The HPLC analyses were performed according to Björkqvist *et al.* (1997).

Preparation of samples for Gpdp enzyme activity measurements

The cells were cultivated as batch cultures with ethanol as carbon source, harvested by centrifugation (5000 *g*, 5 min) at an OD (610 nm) of 2–3 and washed with TRED buffer (André *et al.*, 1991). The pellet was suspended in 5 ml TRED buffer, frozen in liquid nitrogen and stored at − 70°C.

Preparation of isolated mitochondria

A 3-l fermenter (Belach AB, Sweden) was used to culture the cells with a constant pH of 5·0 and a stirring and aeration rate that ensured a dissolved oxygen concentration above 60%. The cells were harvested by centrifugation (5000 *g*, 5 min) during exponential growth on ethanol. Subsequently mitochondria were isolated from protoplasts as described by Guerin *et al.* (1979). Mitochondrial

protein concentration was determined using Peterson's modification of the micro Lowry method (Sigma, protein assay kit).

Gpdp enzyme activity measurements in cell-free extracts

Enzyme activity was measured according to André *et al.* (1991). Protein concentration in crude enzyme extracts was determined using the Bradford method (Bio-Rad, protein assay kit).

Gut2p enzyme activity measurements

Gut2p enzyme activity was measured on isolated mitochondria by monitoring the phenazine methosulphate-mediated reduction of 3-(4,5-dimethylthiazolyl-2)-2,5 diphenyl tetrazolium bromide according to Larsson *et al.* (1993).

Determination of respiratory activity and P/O ratio of isolated mitochondria

Respiratory activity of isolated mitochondria was measured by using an Oxygraph respirometer (Anton Paar K.G. Graz, Austria). The experiments were performed at 30°C with a stirring rate of 500 rpm in 2·1 ml medium containing 0·65 5m-mannitol, 0·5 mM-EGTA, 10 mM-maleate and 10 mM-Tris/Pi, pH 6·7. The respirometer was connected to a computer and the results were evaluated by the DatGraf analysis software (Cyclobios, Grinzens, Austria). The mitochondrial concentration was 0·3 mg/ml and the substrate concentrations were NADH 5 mM; DL-G3P 5 mM; and ADP 1 mM. In order to determine the P/O ratio, duplicate samples were withdrawn at regular intervals, once before and on four occasions after ADP addition, and extracted using PCA. The ATP concentration was analysed by luminescence according to Larsson and Gustafsson (1987). The ATP formation rate was estimated from the linear slope and the P/O ratio was calculated by combining this with the measured oxygen consumption rate.

RESULTS

In *S. cerevisiae* oxidation of cytosolic NADH by reduction of DHAP results in formation of G3P which can be re-oxidized to DHAP via the mitochondrial FAD-dependent Gut2p dehydrogenase or dephosphorylated to glycerol, the main sink for reducing equivalents under anaerobic conditions. Assuming that a blocked oxidation of G3P at the level of Gut2p has to be compensated by conversion of G3P to glycerol, we assessed the importance and activity of the G3P shuttle by comparing growth properties and glycerol formation of a wild-type strain with that of an isogenic *gut2Δ* strain.

Function of the two isoforms of NAD$^+$-dependent GPD (Gpd1p and Gpd2p) in the G3P shuttle

To examine the contribution of Gpd1p and Gpd2p to the activity of the G3P shuttle under physiological conditions, a deletion of each of the corresponding genes was introduced into a *gut2Δ* background. The generated strains were assayed for glycerol production when cultured with glucose or ethanol as the carbon and energy source (Figure 2). The *gut2Δgpd2Δ* produced high amounts of glycerol. By contrast, deletion of *GPD1* resulted in a totally blocked glycerol production during growth on ethanol and only a very slight production during the respiro-fermentative phase when cultured on glucose. Hence, the results clearly demonstrate that the dehydrogenase catalysing the cytosolic reaction of the G3P shuttle is encoded by the *GPD1* gene, whereas the *GPD2* gene product plays no significant physiological role.

The influence of carbon source on the activity of the G3P shuttle

During growth on glucose, wild-type cells produce glycerol under the first respiro-fermentative glucose-consuming phase, whereas during the subsequent respiratory phase, part of the previously produced glycerol is consumed by the cells (Figure 3). It should be noted that glycerol formation is not a consequence of a blocked respiratory activity. In fact, the oxygen consumption rates were quite substantial also during growth on glucose with values ranging from 1 to 3 mmol/g per h (data not shown). The reason for the glycerol production during the first phase is most likely a blocked shuttle activity due to strong glucose repression of Gut2p (Sprague and Cronan, 1977). In agreement with this supposition, the *gut2Δ* mutant produces the same amount of glycerol as wild-type cells during respiro-fermentative growth on glucose, while the mutant continues to produce glycerol also when the cells start to respire the previously formed ethanol (Figure 3). These results indicate a strong shuttle activity in cells using ethanol as the carbon and energy source. This conclusion is corroborated by finding that virtually

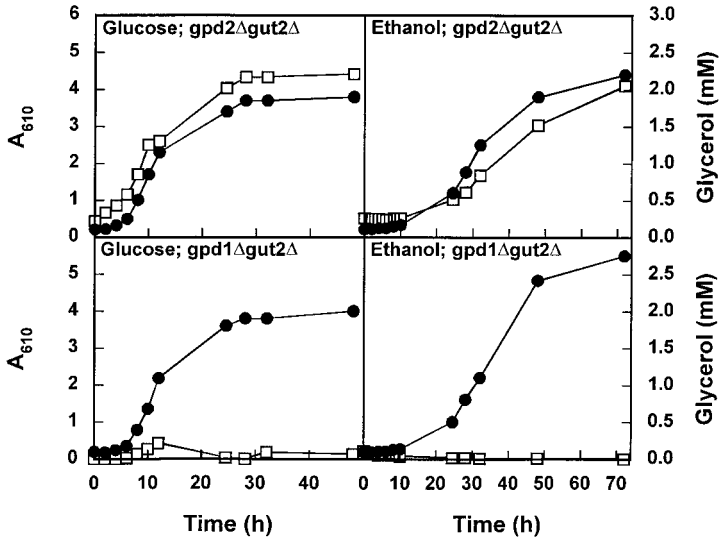

Figure 2. A comparison of growth and glycerol production between *S. cerevisiae*, *gpd1Δ gut2Δ* and a *gpd2Δgut2Δ* strain during aerobic batch cultures with glucose or ethanol as carbon and energy source. A_{610} (●), total glycerol (□).

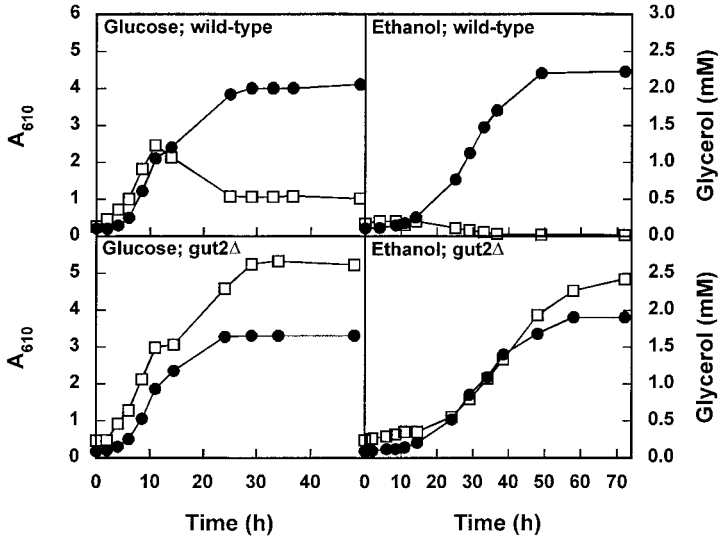

Figure 3. A comparison of growth and glycerol production between *S. cerevisiae*, wild-type and an isogenic *gut2Δ* strain during aerobic batch cultures with glucose or ethanol as carbon and energy source. A_{610} (●), total glycerol (□).

no glycerol was formed by wild-type cells cultured with ethanol as the sole carbon source, whereas the *GUT2* null mutant produced large amounts of glycerol under the same conditions (Figure 3). During growth on ethanol, it is evident that in the wild-type very few, if any, of the reducing equivalents transferred from NADH to G3P end up in glycerol; most of them are transferred to the mitochondrial electron transport chain via Gut2p. It should be noted, however, that there is no significant difference in growth rate or growth yield between wild-type and mutant strains when

Table 1. Comparison of growth yield and growth rate between *S. cerevisiae* wild-type and *gut2Δ* during aerobic batch cultures with glucose and ethanol as carbon and energy sources.

Strain	Carbon source	μ (h^{-1})	Y (g/g)
Wild-type	Glucose	0·301 ± 0·019 (2)	0·31 ± 0·04 (2)
Wild-type	Ethanol	0·095 ± 0·001 (2)	0·38 ± 0·00 (2)
gut2Δ	Glucose	0·303 ± 0·037 (3)	0·30 ± 0·05 (3)
gut2Δ	Ethanol	0·088 ± 0·004 (5)	0·37 ± 0·07 (5)

Values in parentheses show the number of experiments and ± indicates the standard deviation.

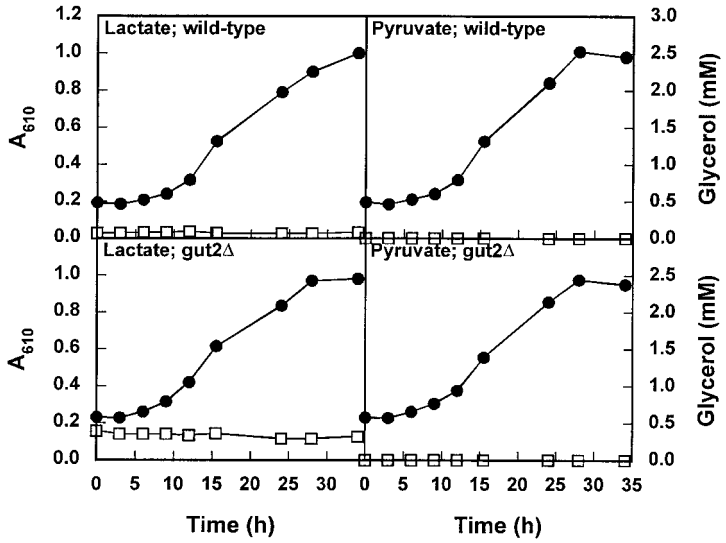

Figure 4. A comparison of growth and glycerol production between *S. cerevisiae*, wild-type and a *gpd2Δgut2Δ* strain during aerobic batch cultures with lactate or pyruvate as carbon and energy source. A610 (●), total glycerol (□).

cultured on either ethanol or glucose, indicating little physiological significance for the G3P shuttle when operating under these conditions (Table 1).

Using pyruvate or lactate as substrate resulted in poor growth and neither the wild-type strain nor the *gut2Δ* mutant formed any observable glycerol (Figure 4) (the slight glycerol content of the lactate cultures is the result of carry-over from inoculates). Obviously, with these substrates, there is no significant oxidation of NADH via reduction of DHAP, and the G3P shuttle is not operating.

Gpdp and Gut2p enzyme activity in wild-type and gpd1Δ *mutant during growth on ethanol or lactate*

There was no measurable NAD^+-dependent GPD activity in the *gpd1Δ* mutant while the wild-type and *gut2Δ* showed a high activity (Table 2) comparable to values previously reported for another strain of *S. cerevisiae* (Blomberg *et al.*, 1988). The Gut2p enzyme was very active in the wild-type irrespective of whether the carbon source was ethanol or lactate and the *gpd1Δ* mutant even showed a slightly higher value (Table 2).

Respiratory activity and P/O ratio of the external NADH dehydrogenase and the G3P shuttle

By external addition of either of two different substrates, NADH and G3P, to isolated mitochondria it is possible to distinguish between the respiratory activity and P/O ratio of the G3P shuttle and the external NADH dehydrogenase. When using NADH as a substrate, i.e. the external NADH dehydrogenase is operative, there was a

Table 2. Comparison of Gpdp and Gut2p activity between *S. cerevisiae* wild-type, *gpd1Δ* and *gut2Δ* mutants during aerobic batch cultures with ethanol or lactate as carbon and energy source.

Strain	Carbon source	Gpdp activity (mU/mg protein)	Gut2p activity (mU/mg protein)
Wild-type	Ethanol	24·3 ± 9·8 (4)	84·8 ± 9·9 (5)
Wild-type	Lactate	13·7 ± 4·6 (3)	71·7 ± 0·2 (2)
gpd1Δ	Ethanol	0·1 ± 1·7 (2)	120·8 ± 10·1 (3)
gut2Δ	Ethanol	28·3 ± 2·8 (2)	0[a]

Values in parentheses show the number of experiments and ± indicates the standard deviation.
[a]The activity never exceeded the blank values.

Table 3. Comparison of respiratory activity and P/O ratio of the Gut2p dehydrogenase and the external NADH dehydrogenase of *S. cerevisiae* using either NADH or G3P as substrates.

Substrate	Respiratory activity (nmol O/mg prot. per min)	P/O ratio (mol ATP/mol O)
NADH	259 ± 30 (2)	
NADH+ADP	820 ± 251 (2)	1·20 ± 0·05 (2)
G3P	166 ± 33 (2)	
G3P+ADP	189 ± 16 (2)	1·65 ± 0·07 (2)

Values in parentheses show the number of experiments and ± indicates the standard deviation.

high rate of respiration and a P/O ratio of 1·2 (Table 3). The performance of the G3P shuttle was distinctively different from that of the NADH dehydrogenase. In this case the respiratory rate was much lower, but on the other hand, the efficiency, i.e. the P/O ratio was significantly higher with a value of 1·7 (Table 3).

Effects of growth rate and external pH on the activity of the G3P shuttle

Wild-type and *gut2Δ* cells were cultured in ethanol-limited chemostat cultures in order to study the influence of growth rate and external pH on the G3P shuttle activity. The glycerol production was strongly enhanced in the mutant culture, which reached glycerol values considerably higher than in the wild-type culture at all dilution rates and external pH tested (Figure 5). These results point to a highly active shuttle during ethanol-limited growth in a well-aerated culture. It is also apparent that the G3P shuttle was most active at low dilution rates, and that this activity is sensitive to external pH since there was a lower activity with decreasing external pH, at least if the comparison is made at low or intermediate dilution rates (Figure 5).

DISCUSSION

It is well known that *S. cerevisiae* produces glycerol during anaerobic growth on glucose in order to maintain an intracellular redox balance (Albers *et al.*, 1996; Nordström, 1968; Van Dijken and Scheffers, 1986). This system is supposed to be the only way by which the cells can oxidize a surplus of cytoplasmic NADH anaerobically since ethanol formation is a redox neutral process. This supposition was corroborated by the coherence between theoretically calculated excess NADH and experimentally observed glycerol formation (Albers *et al.*, 1996). Under aerobic conditions the situation is less clear, but the cells apparently have different options for maintaining their cytoplasmic NAD/NADH balance by transfer of the reducing equivalents into the mitochondrial electron transport chain (Figure 1; de Vries and Marres, 1987).

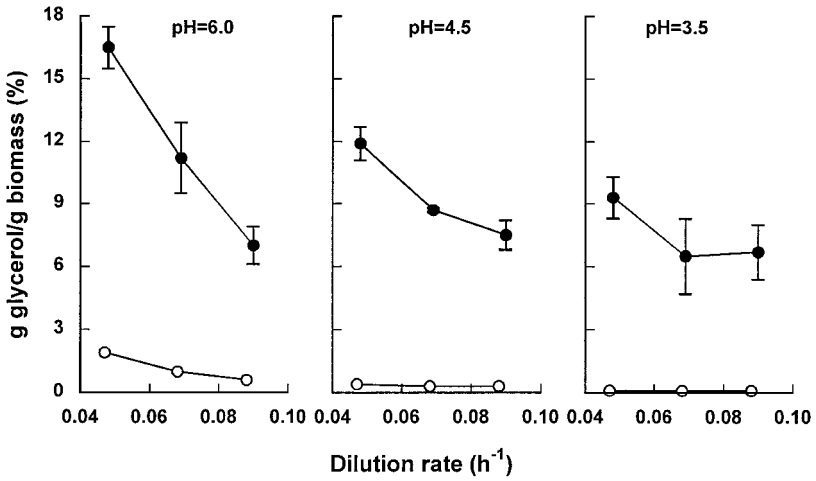

Figure 5. Glycerol production of *S. cerevisiae* M1-2B (wild-type) (○) and Bi1 (*gut2Δ*) (●), cultured in ethanol-limited chemostats at different dilution rates and external pH.

This can be achieved by irreversible cyclic processes or shuttle systems such as the G3P shuttle. There are several other potential shuttle mechanisms proposed but yeast cells appear to lack at least the malate–aspartate shuttle common among higher eukaryotes (de Vries and Marres, 1987). On the other hand, *S. cerevisiae* possesses an external NADH dehydrogenase, facing the outer side of the inner mitochondrial membrane, thus enabling direct oxidation of cytoplasmic NADH (de Vries and Marres, 1987; von Jagow and Klingenberg, 1970). The existence of this external NADH dehydrogenase might make the different shuttle systems superfluous in *S. cerevisiae*. However, it is clear from our results that *S. cerevisiae* does have a functional G3P shuttle and we have studied the activity and importance of the G3P shuttle under different conditions.

We have demonstrated that the shuttle is highly active during growth on ethanol (Figures 3 and 5), whereas no shuttle activity was detected with lactate or pyruvate as substrates (Figure 4), even though Gut2p has a high potential enzyme activity during growth on lactate (Table 2). The difference in shuttle activity is most likely a reflection of the lower requirement for cytoplasmic redox adjustment with lactate and pyruvate as substrates compared to ethanol (see Introduction). However, at least concerning the *gut2* mutant, there is a theoretical possibility that ethanol oxidation is performed via the mitochondrial alcohol dehydrogenase (ADHIII; Pilgrim and Young, 1987; Figure 1) thus avoiding cytoplasmic NADH production via this route. It should also be noted that when pyruvate oxidation is performed through the pyruvate dehydrogenase bypass (Flikweert *et al.*, 1996), the cells can still minimize cytoplasmic NADH production by using the NADP-linked acetaldehyde dehydrogenase (de Vries and Marres, 1987).

There was no detectable difference in growth rate or yield between wild-type and *gut2Δ*, even with ethanol as substrate (Table 1). The fact that the *gut2* mutant was able to grow at all with ethanol as the carbon source suggests that there are alternative systems for regeneration of cytosolic NAD^+. In contrast to growth on glucose, glycerol formation with ethanol as a substrate would not result in NADH consumption since glycerol production from ethanol is an oxidative process, i.e. generates NADH. In fact, it can be calculated from Figure 1 that formation of 1 mol of glycerol requires 2 mol of ethanol and the reaction yields 3–5 mol of NADH (depending on whether NAD or NADP is used as a cofactor). The fact that it is impossible to restore the redox balance by glycerol production during growth on ethanol means that the glycerol production rates observed for the *gut2Δ* mutant cannot be considered as a quantitative measure of the G3P shuttle activity in the corresponding wild-type strain. Another possibility to achieve NADH oxidation without delivering reducing equivalents to the respiratory chain would be to synthesize an end-product more reduced than ethanol, which is highly unlikely. A more probable alternative would be regeneration of NAD^+ via the mitochondrial

NADH dehydrogenase that faces the cytoplasm, giving direct access for cytosolic NADH to the electron transport chain. Obviously, the cells do not depend on a single NADH oxidizing system and it is likely that the external NADH dehydrogenase and the G3P shuttle operate additively, rather than in a mutually exclusive fashion, with a capacity to substitute for each other.

Two isoenzymes, Gpd1p and Gpd2p, which convert DHAP to G3P in the glycerol-producing pathway have been identified (Eriksson *et al.*, 1995; Larsson *et al.*, 1993). Of these enzymes, Gpd2p seems to be mainly responsible for redox adjustment under anaerobic growth (Ansell *et al.*, 1997; Björkqvist *et al.*, 1997). Deletion of *GPD1* did not appear to affect anaerobic growth, whereas a *GPD2* null mutant showed a severe reduction in growth rate and yield under anaerobic conditions (Ansell *et al.*, 1997; Björkqvist *et al.*, 1997). In this study, performed under aerobic conditions, the importance of the enzymes was quite the opposite. When *gpd1* or *gpd2* deletion mutants were combined with a *gut2* mutation, glycerol formation occurred only in the strain with an intact *GPD1* gene (Figure 2). Consequently, the isoform specifically required for reduction of DHAP to G3P with concomitant NADH oxidation under aerobic conditions is encoded by the *GPD1* gene and deletion of this gene is not compensated by an increased specific activity or increased expression of the enzyme encoded by the *GPD2* gene (Figure 2). The isoenzyme produced from *GPD2* appears to have a role that is specific for redox adjustments under anaerobic growth on glucose (Ansell *et al.*, 1997; Björkqvist *et al.*, 1997). It is also interesting to note that the activity of the Gut2p enzyme was higher in the *gpd1Δ* background compared to the wild-type (Table 2). This might indicate a regulatory role of the *gpd1* gene product on Gut2p activity which is relieved in the deletion mutant.

In comparison to the external NADH dehydrogenase, the respiratory activity when using the G3P shuttle was much lower, but on the other hand the P/O ratio was higher (Table 3). However, the external NADH dehydrogenase still has a higher capacity of producing ATP per unit time compared to the shuttle. The higher capacity is achieved at the expense of efficiency, i.e. more substrate is needed to produce the same amount of ATP. Hence, the combined activities of these two systems offer a possibility of regulating the respiratory activity and ATP-producing capacity in accordance with the different demands imposed on the cells under different conditions. This would also be compatible with the observation that the G3P shuttle becomes progressively less important at high dilution rates where high rates of ATP generation are needed (Figure 5). Furthermore, high rates of ethanol consumption challenge the cells with a requirement for rapid cytoplasmic NADH consumption. It was also found that the shuttle had a lower impact when the external pH was decreased (Figure 5). This could be explained by a higher maintenance requirement at low pH (Verduyn *et al.*, 1990) and thus a higher ATP production rate is needed. It might be that the shuttle is used only as a complement to the external NADH dehydrogenase, i.e. the shuttle only consumes a small fraction of the total cytoplasmic NADH consumption. If this is true it could also explain why a non-functional shuttle did not result in any reduction in biomass yield during growth on ethanol. Perhaps, the G3P shuttle is used also under 'normal' conditions but its real significance is not expressed until the cells are confronted with more challenging conditions. For instance the ability to survive starvation for long periods could be such a condition where an efficient ATP generation could be a critical parameter.

To summarize, the external NADH dehydrogenase and the G3P shuttle may be able to substitute for each other in order to keep a cytoplasmic redox balance. To what extent the two mechanisms are used may be governed by the cellular ATP requirements in combination with the need for redox adjustment.

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