Distinct Intracellular Localization of Gpd1p and Gpd2p, the Two Yeast Isoforms of NAD⁺-dependent Glycerol-3-phosphate Dehydrogenase, Explains Their Different Contributions to Redox-driven Glycerol Production*

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During anaerobiosis *Saccharomyces cerevisiae* strongly increases glycerol production to provide for non-respiratory oxidation of NADH to NAD⁺. We hereby report that respiratory-deficient cells become strictly dependent on the Gpd2p isoform of the NAD⁺-linked glycerol-3-phosphate dehydrogenase (Gpd). The growth inhibition of respiratory incompetent cox18Δ cells lacking GPD2 is reversed by the addition of acetoin, an alternative sink for NADH oxidation. Growth is also restored by addition of lysine or glutamic acid/glutamine, the synthesis of which involves production of mitochondrial NADH. Lysine produced a stronger growth stimulating effect than glutamic acid consistent with an up-regulated expression of the IDP3 gene for peroxisomal synthesis of the glutamate precursor α-ketoglutarate. Gpd2p is known to be a cytosolic protein but possesses a classical mitochondrial precursor, which we show is sufficient for mitochondrial targeting. A partial mitochondrial localization of Gpd2p will provide for establishment of intramitochondrial redox balance under non-respiratory conditions. Gpd1p, the other Gpd isoform, is partly cytosolic and partly peroxisomal and becomes more strictly peroxisomal in respiratory-deficient mutants. The different cellular distribution of Gpd1p and Gpd2p thus appears to be the main reason Gpd1p cannot substitute for Gpd2p in cox18Δgpd2Δ cells, despite similar kinetic characteristics of the two iso-enzymes.

Like other organisms, yeasts derive energy from controlled oxidation of molecular substrates using NADH as the major carrier of the generated reducing equivalents. The limiting amounts of this coenzyme (1) require that it is rapidly re-oxidized so that the cellular redox reactions do not become restricted by lack of NAD⁺. Since NADH cannot pass the mitochondrial inner membrane (2), it has to be re-oxidized in the compartment where it is produced by transfer of the reducing equivalents to a suitable acceptor molecule. Under aerobic conditions *Saccharomyces cerevisiae* has several mechanisms to re-oxidize NADH, thereby allowing metabolism to proceed (3). Cytosolic NADH can be transferred into mitochondria by two mechanisms, (i) by the mitochondrial external NADH dehydrogenase Nde1p/Nde2p or (ii) by the glycerol-3-phosphate (G3P)1 shuttle in which Gut2p, an FAD-linked glycerol-3-phosphate dehydrogenase, which is located in the inner mitochondrial membrane, oxidizes G3P to dihydroxyacetone phosphate in the intermembrane space, delivering the reducing equivalents to the respiratory chain (4–8). In *S. cerevisiae* the NADH formed in the mitochondrial matrix can be oxidized by the internal NADH dehydrogenase, encoded by the NDI1 gene (9, 10).

Under non-respiratory conditions when yeast cells are entirely dependent on fermentation for biomass and energy, they require alternative mechanisms for re-oxidation of NADH. Although the fermentation of glucose to ethanol is redox neutral, synthesis of biomass and organic acids generate net production of NADH (11–13). Carbon and redox balance studies have demonstrated that yeast cells achieve redox balance under anaerobic conditions by NADH-dependent reduction of DHAP to G3P and subsequent production of glyceral (11, 14, 15). The observation that mutants with a blocked glycerol pathway are unable to grow under anaerobic conditions (16–18), confirm the supposition that glycerol production is an exclusive sink for reducing equivalents under non-respiratory conditions. Hence, one of the physiological roles of glycerol production is to serve as an outlet for surplus NADH. Glycerol can serve this function also under aerobic conditions, e.g. on growth at high glucose concentrations, which repress respiration and reduces the respiratory capacity to oxidize NADH. In addition, glycerol has another important physiological function; it serves as an osmo-regulator and is produced and accumulated in cells subjected to hyperosmotic stress to prevent loss of water and turgor of the cells (19–21). The isoform of NADH-dependent glycerol-3-phosphate dehydrogenase that has a prime role in anaerobic production of glycerol is encoded by the GPD2 gene (17, 18), while the other isoform of the enzyme, Gpd1p, is used for osmstess-induced glycerol production (21, 22). Gpd1p also appears to be the isoform responsible for the glycerol-3-phosphate dehydrogenase activity in the G3P shuttle (8). While the osmstress-induced expression of the GPD1 gene has been extensively studied (reviewed in Refs. 23 and 24), the regulation of the GPD2 gene remains uncharcterized. It is reported that GPD2 expression is induced in cells shifted to anaerobic conditions (17, 25) and in cells, which have a respiratory activity that has been indirectly or directly inhibited (17, 26, 27).

It is estimated that up to 50% of the NADH that is generated in amino acid biosynthesis is produced in the mitochondrial matrix (12). A substantial part of this production stems from

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¶ This abbreviation used are: G3P, glycerol 3-phosphate; Gpd, glycerol-3-phosphate dehydrogenase; GFP, green fluorescent protein.
synthesis of the glutamate precursor, α-ketoglutarate (13). It is, however, not clear how surplus NADH in the mitochondrial matrix is re-oxidized to NAD⁺ under anaerobic conditions. It has been suggested that a putative ethanol-acetaldehyde shuttle has a role in the process (2, 3). According to this model, acetaldehyde diffuses from the cytosol into the mitochondria where it is reduced to ethanol by the NADH linked mitochondrial alcohol dehydrogenase (Adh3). Subsequent diffusion of the produced ethanol to the cytosol will remove redox equivalents from the mitochondrial matrix, thereby contributing to redox balance.

We here report on the role of Gpd1p and Gpd2p in cellular redox control in respiratory sufficient and insufficient yeast strains grown under controlled conditions. To avoid nutritional supplementation that may affect cellular redox conditions (28), we have used prototrophic yeast strains whenever possible. We observed that respiratory insufficiency strongly enhances requirement for GPD2 to relieve the intracellular redox imbalance that appears to arise in the mitochondrial matrix. The reason that Gpd1p cannot substitute well for Gpd2p in this function seems due to important differences in the cellular localization of the two iso-enzymes.

EXPERIMENTAL PROCEDURES

Yeast Strains and Media—S. cerevisiae strains utilized in this study are listed in Table I. TheYPD medium used contained 1% (w/v) yeast extract (Merck), 2% (w/v) Bacto-peptone (Merck), and 2% (w/v) glucose (Merck). The YNB medium used was composed of 0.17% (w/v) yeast nitrogen base without amino acids (Difco), 0.5% (w/v) ammonium sulfate (Merck), and 2% (w/v) glucose. For ethanol agar plates, glucose was replaced by 2% (w/v) ethanol. The pH of all media was adjusted to 6.0. Solid media contained 2% (w/v) agar (Merck). Cell densities of liquid cultures were determined from OD measurements at 610 nm using a Novaspec II spectrophotometer (Amersham Biosciences).

DNA Manipulation, Deletion Techniques, and Transformation Methods—Standard DNA techniques were performed as described elsewhere (29, 30). Strains having a deleted COX18 gene were constructed using a the cox18Δ::kanMX4 cassette generated by PCR amplification of DNA isolated from the BY 4741 strain, using the oligonucleotides 5'-GTGCGCTACTAGCAGCAACCGGAGGAGCAGGACGACCCGATGAGCTGAGAGGATGG-3' and 5'-ACCTGAGCTTCTTTGTTATGGACCTGGGAG-3'. Transformants were selected on YPD plates containing 200 μg/ml geneticin (G418, Sigma-Aldrich) and integration was confirmed using PCR. To analyze for effects of GPD gene overexpression relevant strains were transformed with the multicopy plasmid YEplac112 carrying the GPD1 gene. To generate a GPD-tagged version of the putative N-terminal targeting sequence of Gpd2p, oligonucleotides with attached Clai sites were used to PCR amplify a GPD2 fragment spanning the region from −1000 bp to −51 bp. The PCR primers used were 5'-ATCGATAGCCCGAAAGAGTTATCGTTA-3' and 5'-ATCGATATCTTCATGTAGATCTAATTCTT-3'. The resulting fragment was ligated into the 2.1-TopoVector (Invitrogen), and transformed into E. coli Top10F (Invitrogen). Plasmids were isolated using a commercial kit (Qiagen), cleaved with Clai (Roche Applied Science) and purified by gel extraction (Qiagen). The fragment was inserted into the Clai site of a GFP (green fluorescent protein) carrying pRS426 plasmid (kindly provided by Dr. P. Sunnerhagen). The construct was controlled by digestion with Clai (Roche Applied Science) and HindIII (Roche Applied Science). GPD1 was GFP-tagged using 5'-ATCGATAGCCCGAAAGAGTTATCGTTA-3' and 5'-ATCGATATCTTCATGTAGATCTAATTCTT-3' including flanking Clai sites. The resulting PCR fragment contains 1000 base pairs from the native promoter of GPD1 and the complete open reading frame. The fragment was cloned into the pRS426 GFP plasmid using the same procedure as for GPD2 (above). Fragment orientation of GPD1 was confirmed using Sall (Roche Applied Science).

Growth Conditions for Controlled Batch Cultures—Liquid precultures were grown at 30 °C with shaking (200 rpm) in 1.5 × YNB medium containing 1% (w/v) glucose. Batch cultures were grown in duplicates in SARA BR 0.5 bioreactors (Belach Biotechnik) containing 750 ml of the same medium, using air (aerobiosis) or oxygen-free nitrogen gas (anaerobiosis) as the gas phase at a flow rate of 30.0 liters/h (Bronkhorst Hi-tech). Medium for anaerobic cultures contained ergosterol, Tween 80 (a source of oleic acid) and antifoam as previously described (31, 32). For cultures growing with an artificial redox sink, acetoin (1 g/liter) was added prior to inoculation. The temperature was maintained at 30 °C, pH 5.0, and the stirring rate was kept at 500 rpm for anaerobic cultures and 800 rpm for aerobic cultures. The heat production rate was monitored by a multichannel microcalorimeter (2277 Thermal Activity Monitor, Thermometric). Oxygen concentration and CO₂ production rate were followed using a gas analyzer (Innova).

Growth Conditions for E-flash Cultures—To study whether overexpression of GPD genes could reverse the growth arrest of the redox-inhibited gpd2Δcox18Δ strain, the auxotrophic gpd2Δcox18Δ, gpd2Δcox18ΔΔYEpplac112-GPD1, and gpd2Δcox18ΔΔYEpplac112-GPD2 were cultured in E-flasks at 30 °C with shaking (200 rpm) in 1.5 × YNB medium adjusted to pH 6.0, containing 1% glucose and appropriate amino acids and nucleotides. To study whether additions of growth factors could reverse the growth arrest of the prototrophic gpd2Δcox18Δ strain, the minimal medium was provided with additions of amino acids or nucleotides at a concentration of 120 μg per ml.

Determination of Glucose and Glycerol Concentrations—Samples for metabolite analyses were withdrawn from batch cultures and heat-treated at 100 °C for 5 min before centrifugation at 5,000 × g for 5 min. The obtained supernatants were stored at −20 °C until analyzed. The glycerol and glucose concentration were analyzed using commercial kits, following the manufacturer’s protocol (Biochemica Test Combination ‘Yeast’, Roche Applied Sciences).

Northern Analysis—Samples for Northern blot analysis were taken from batch cultures and immediately transferred to liquid nitrogen prior to storage at −20 °C. Total RNA was extracted using RNeasy Mini kit (Qiagen) using cell cultures that had reached an OD₆₀₀ of value of 1. Samples containing 15 μg of total RNA were separated on a low formamide (2.5%, v/v) denaturing agarose gels at 75 V/cm. Separated RNA was blotted by capillary transfer to positively charged nylon membranes (Hybond, Amersham Biosciences) using 10 × SSC as transfer buffer. Cross-linking of the RNA was achieved using low wavelength UV light and subsequent baking of the membrane at 80 °C for 2 h. Pre-hybridization was accomplished at 55 °C for 3–4 h in 5 × SSC, 10 mM sodium phosphate (pH 6.5), 1 × Denhardt’s solution, 2% SDS and 10 mg/ml carrier DNA. Hybridization was achieved at 55 °C for 18–20 h in the above mentioned pre-hybridization buffer supplemented with 10% PEG 4000. Oligonucleotides were used for transcript detection, and 5 ng/ml hybridization solution was labeled with [32P]ATP (Amersham Biosciences) using 5 units of polynucleotide kinase (MBI) for every 50-ng probe used, and the labeling reaction was conducted at 37 °C for
30 min. Excess ATP nucleotides was removed via centrifugation through a Sephadex G50 mini column.

Post-hybridization washes were performed in 1× SSC plus 1% SDS for 2×15 min at room temperature followed by a 15-min wash at hybridization temperature (55°C). Membranes were scanned and quantified using a Molecular Dynamics Phosphorimager (FX, Bio-Rad) using the software Quantity One. Sequence of the oligonucleotides used were: GPD1: 5'-TGTACTATTGGAGCGAAAACTTCT-3', GPD2: 5'-GGTCCTCATGACAGTGTTTGTGCT-3', IDP3 5'-GTC CCT CAC TGG GAG AAA CCT ATA ATT ATA-3', and for the control IPP: 5'-TGTCCTGTAGGTAGTGATTAGTT-3'.

Preparation of Cell-free Extracts—Cell samples were centrifuged
(4000 × g for 5 min) and the pellet resuspended in protein extraction buffer (50 mM Tris-Cl, 10 mM imidazole, 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 10 mM Na3F, 0.2 mM Na3VO4 (pH 7.5) and supplemented with a protease inhibitor mix (Complete™, Roche Applied Science). The cells were homogenized at 6.0 m/s in a FastPrep FP120 homogenizer (Bio101, Savant) for 20 s. Cell debris was removed by a 15-min centrifugation at 14,000 × g. Glycerol-3-phosphate dehydrogenase activity and immunoblot analysis of Gpd1p levels in wild-type, gpd1Δ, gpd2Δ, cox18Δ, gpd1Δcox18Δ, and gpd2Δcox18Δ strains grown in minimal glucose medium plus amino acids are shown.

**FIG. 5.** Strains lacking COX18 have increased levels of Gpd1p. Glycerol-3-phosphate dehydrogenase activity assay was assayed by addition of 200 μg of protein extract to 20 mM imidazole (pH 7.0), 1 mM MgCl2, 1 mM dithiothreitol, and 0.1 mM NADH. The reaction was started by the addition of dihydroxyacetone.
phosphate to 0.7 mM concentration in a final volume of 1 ml. The depletion of NADH was followed at 340 nm using a Beckman DU7400 spectrophotometer.

Western Blot Analysis—To prepare cell extracts for SDS-PAGE and Western analysis cell samples were taken from exponentially growing cultures, transferred to liquid nitrogen and stored at −20 °C prior to analysis. Cell-free extracts were prepared as described above, and protein concentration was determined using the method of Bradford (Bio-Rad). A total of 100 μg of protein extracts was loaded per lane for SDS-PAGE on 10% acrylamide gels (Bio-Rad). Western blot was performed using standard methods (29). For detection of Gpd1p, rabbit anti-Gpd1p antibodies (diluted 1:2000) and horseradish peroxidase-linked anti-rabbit secondary antibody (Sigma) were used. Signal detection was achieved using the ECL-plus system (Amersham Biosciences), and analysis was performed using the Luminescent Image Analyser LAS-1000 plus (Fujifilm).

Fluorescence Microscopy—Mid-log cells were observed using a Leica DMRXA microscope and green fluorescent protein was visualized using a GFP filter. MitoTracker (Molecular Probes) was added to the cell samples 10 min prior to observation using FITC filter.

RESULTS

Aerobic Growth and Glycerol Production—To evaluate the roles of Gpd1p and Gpd2p under aerobic redox imbalance in S. cerevisiae, we first examined the growth phenotype and glycerol production of a series of W303 strains made prototrophic for growth factor requirements, i.e. wild-type, gpd1Δ, gpd2Δ, cox18Δ, gpd1Δcox18Δ, and gpd2Δcox18Δ. The strains were cultured in YNB glucose medium under aerobic conditions in high performance bioreactors. Respiratory insufficiency was achieved by deletion of the COX18 gene, encoding a protein involved in the assembly of the cytochrome c oxidase complex (33, 34). Cells lacking COX18 consumed glucose as the wild-type cells but were unable to use the ethanol that was produced during the glucose-consuming phase, while the respiratory competent wild-type cells continued to grow on ethanol upon depletion of glucose (Fig. 1, A and B). During aerobic growth on glucose the cox18Δ cells showed the same generation time as the wild-type cells as did the gpd1Δ and gpd2Δ mutants (Fig. 2). The gpd1Δcox18Δ mutant exhibited a decreased growth rate and the gpd2Δcox18Δ strain, which could be precultured on YNB glucose medium supplemented with amino acids, did not grow at all in the minimal YNB glucose medium. Since cox18Δ strains are unable to oxidize NADH by respiration, these strains experience physiological conditions similar to anaerobic bios. In agreement with this notion the cox18Δ mutant produced 5 times as much glycerol as wild-type cells, a production similar to that of wild-type cells cultured under anaerobic conditions (Figs. 1A and 3). Deletion of GPD1 in the cox18Δ background resulted in a slightly reduced aerobic growth rate and under these conditions the strain generated ~80% of the glycerol produced by the cox18Δ single mutant (Fig. 3). These findings suggest a minor role for GPD1 in the aerobic redox-adjusting glycerol production of cox18Δ mutants. The observation that cox18Δ strains lacking GPD2 experience a complete growth arrest indicates that GPD2 is given a more important role for relieving the redox constraints in respiratory deficient mutants than reported for cells that are deprived of respiration due to anaerobic conditions (17, 18, 35).

Growth and Glycerol Production under Anaerobic Conditions—We then wanted to analyze whether aerobic non-respiring S. cerevisiae strains differed from strains grown under anaerobic conditions in their requirement for NAD+–regenerating glycerol production. Under anaerobic conditions the wild-type, gpd1Δ and cox18Δ strains exhibited close to identical heat production (data not shown), growth rate (Fig. 2), and glycerol production (Fig. 3). The gpd1Δcox18Δ mutant showed improved growth and glycerol production under anaerobic versus aerobic conditions, displaying anaerobic growth characteristics similar to wild-type cells. This suggests that the slight contribution given by GPD1 to aerobic redox driven glycerol production is fully substituted by an increased contribution from GPD2. It is also clear that GPD2 is more important in respiratory-deficient mutants than in wild-type cells even under anaerobic conditions. Deletion of GPD2 in wild-type background results in an about 50% decrease in anaerobic glycerol production (Fig. 3), and growth rate (Fig. 2), as is previously reported (17, 18, 35), whereas removal of GPD2 in the cox18Δ background is completely growth inhibiting under aerobic as well as anaerobic conditions.

Growth and Glycerol Production in the Presence of Acetoin—We next examined whether the severe growth defect exhibited by the gpd2Δcox18Δ cells was caused by the predicted redox imbalance. To this end growth of the cells was followed in an aerobic bioreactor before and after the addition acetoin (1 g/liter). This supplementation offers an alternative route for NAD+ re-
The predicted targeting sequence of the Gpd2p N-terminal 17 amino acids targets proteins to mitochondria. Auxotrophic wild-type (A) and gpd1Δcox18Δ (B) cells were transformed with a centromeric plasmid (pRS426) containing the GPD2 5′-promoter region and first 51 bp fused with the GFP sequence (NT-GFP). Cells were cultured aerobically in glucose minimal medium and collected at exponential phase for microscopy. The same field was photographed by bright field microscopy (BF, left) and by fluorescence microscopy to detect the NT-GFP (GFP, center), and to detect mitochondria after staining with Mitotracker (MT, Molecular Probes; right).

Role of Gpd1p and Gpd2p in Yeast Redox Control

Generation by reduction of acetoin to 2,3-butanediol (18, 36). Following acetoin addition growth of the gpd2Δcox18Δ strain resumed (Fig. 4), and a similar effect was seen in closed YNB glucose shaker cultures following the addition of acetaldehyde (not shown), another effective oxidizer of NADH. We then examined the effect of acetoin addition on growth and glycerol production for all strains when cultured in the bioreactor under aerobic conditions. For these experiments acetoin was added prior to inoculation. Although growth of the wild-type, gpd1Δ, cox18Δ, and gpd1Δcox18Δ strains was not significantly affected by the addition of acetoin (Fig. 2), their glycerol production decreased by about 40%, supporting the view that the acetoin addition relieves the requirement for redox-driven glycerol production. As previously reported anaerobic growth of the gpd2Δ strain is improved by acetoin addition (37), causing an about 2-fold increase in growth rate (Fig. 2). In fact, after acetoin addition the anaerobic growth rate of the gpd2Δcox18Δ strain becomes similar to that of the gpd2Δ strain. These strains have a rather low glycerol production (Fig. 3), suggesting that the Gpd1p activity do not compete well for NADH with that of the acetoin reducing 2,3-butanediol dehydrogenase.

**GPD Expression**—To further evaluate the roles of GPD1 and GPD2 for the redox-driven glycerol production, we analyzed the GPD activity (Fig. 5) in extract from exponentially growing cells cultured in shake flasks under aerobic conditions. Since the gpd2Δcox18Δ mutant does not grow unless provided with redox-adjusting supplements, this strain was cultured in a medium supplemented with amino acids. The used enzyme assay primarily assesses Gpd1p activity (18), as is also clear from the low activity observed in mutants lacking GPD1, despite the glycerol production by such cells (Figs. 3 and 5). Interestingly, the cox18Δ mutant exhibits a strongly increased Gpd activity, which is maintained by the cells even after deletion of GPD2. Hence, it appears that the gpd2Δcox18Δ mutant, despite a Gpd1p activity that is 2–3 times higher than in wild-type cells, cannot drive glycerol production to an extent sustaining redox balance and growth. These observations were further confirmed by Western blot analysis using a Gpd1p specific antibody, showing 2–3-fold higher Gpd1p levels in the cox18Δ mutants (Fig. 5). Obviously, GPD1 cannot compensate for lack of GPD2 despite an increased level of Gpd1p.

Lacking a correspondingly representative assay for Gpd2p enzyme activity as well as antibodies specific for this protein, we used Northern blot analysis to assess GPD2 expression in relevant strains. This analysis demonstrated that GPD2 expression under aerobic conditions is slightly induced in cox18Δ and gpd1Δ mutants but more strongly (3-fold) in gpd2Δcox18Δ mutants (Fig. 6A). However, the addition of acetoin to a cox18Δ mutant gives rise to a rapid decrease and apparent disappearance of GPD2 mRNA (Fig. 6B), illustrating a direct or indirect sensitivity of GPD2 expression to changes in the cellular NADH/NAD+ ratio. A shift from aerobic to anaerobic growth conditions of a wild-type strain gives rise to a 2-fold increase in GPD2 steady state mRNA levels (17). Since this induction appears sufficient to meet the cell requirements for redox-induced glycerol production, the stronger increase in steady-state GPD2 mRNA observed in the anaerobically grown gpd1Δ,
cox18Δ, and gpd1Δcox18Δ mutants (Fig. 6A) may result from deranged regulation rather than as a response to meet requirements for increased glycerol production.

Overexpression of GPD1 Rescues Growth of the gpd2Δcox18Δ Strain—To further examine whether the ability of Gpd1p to substitute for Gpd2p is related to the level of expression of the GPD1 gene, GPD1 was expressed from a multicopy plasmid in a gpd2Δcox18Δ strain. As is clear from Fig. 7 enhanced expression of GPD1 permits growth of the gpd2Δcox18Δ mutant similar to what is seen for wild-type cells carrying an empty vector (not shown) and when overexpressing GPD2 in the mutant background. The results demonstrate that massive overexpression of GPD1 generates conditions that relieve the redox constraints of the gpd2Δcox18Δ mutant.

Growth in the Presence of Amino Acids—Since NADH/NAD⁺ balance seems critical for growth of respiratory compromised cells, suggested that a mitochondrial localization of Gpd2p might explain its redox relieving function in such strains. Global analysis of cellular distribution of GFP-tagged proteins (38) did not support such localization, showing a cytosolic as well as a nuclear localization of Gpd2. However, immuno-fluorescence studies (ygac.med.yale.edu/triples) have indicated a cytosolic as well as mitochondrial distribution of the enzyme (39), and a recent study of the proteome of highly purified S. cerevisiae mitochondria (40) identified Gpd2 as member of the mitochondrial set of proteins. Furthermore, the PSORT II (41) and MIPS MitoProt II (42) programs for prediction of protein localization give Gpd2 a very high probability of being a mitochondrial protein. The MitoProt II program identifies a cleavable 17 amino acid classical targeting sequence, enriched for basic, hydrophobic, and hydroxylated amino acids in the N-terminal end of the protein. The calculated MitoProt score of the presequence is 0.993, indicating strong targeting properties (www.mips.biochem.mpg.de/cgi-bin/proj/medgen/mitofilter). Since the cytosolic localization of Gpd2p might impede detection of a partial localization to mitochondria (38), we fused a fragment of GPD2 encoding the authentic promotor and the first 17 amino acids to GFP (NT-GFP). The expression of NT-GFP in wild-type and gpd1Δcox18Δ cells revealed distinct mitochondrial localization in both strains (Fig. 9). These results support a mitochondrial distribution of Gpd2p and strongly indicate that the first 17 amino acids contain information that is sufficient for mitochondrial targeting of the protein.

Fig. 10. The peroxisomal distribution of Gpd1p is more pronounced in respiratory deficient cells. Auxotrophic wild-type (A), and cox18Δ (B) cells were transformed with a centromeric plasmid (pRS426) containing functional GFP-tagged GPD1 sequence expressed from the GPD1 promoter. Cells were cultured aerobically in glucose minimal medium and collected at exponential phase for microscopy. The same field of cells was photographed by bright field microscopy (BF, left) and by fluorescence microscopy to detect the GFP-tagged Gpd1p (GFP, right).

Fig. 11. Expression of IDP3 is increased in cox18Δ mutants. Northern blot analysis of IDP3 mRNA in wild-type and cox18Δ cells during exponential growth in aerobic batch cultures is shown. IPP mRNA was used as internal control. IDP3 transcript levels normalized to control mRNA are given as relative values.

Gpd2p Is Distributed between Cytosol and Mitochondria—The observation that maintenance of mitochondrial NADH/ NAD⁺ balance seems critical for growth of respiratory compromised cells, suggested that a mitochondrial localization of Gpd2p might explain its redox relieving function in such strains. Global analysis of cellular distribution of GFP-tagged proteins (38) did not support such localization, showing a cytosolic as well as a nuclear localization of Gpd2p. However, immuno-fluorescence studies (ygac.med.yale.edu/triples) have indicated a cytosolic as well as mitochondrial distribution of the enzyme (39), and a recent study of the proteome of highly purified S. cerevisiae mitochondria (40) identified Gpd2 as member of the mitochondrial set of proteins. Furthermore, the PSORT II (41) and MIPS MitoProt II (42) programs for prediction of protein localization gives Gpd2p a very high probability of being a mitochondrial protein. The MitoProt II program identifies a cleavable 17 amino acid classical targeting sequence, enriched for basic, hydrophobic, and hydroxylated amino acids in the N-terminal end of the protein. The calculated MitoProt score of the presequence is 0.993, indicating strong targeting properties (www.mips.biochem.mpg.de/cgi-bin/proj/medgen/mitofilter). Since the cytosolic localization of Gpd2p might impede detection of a partial localization to mitochondria (38), we fused a fragment of GPD2 encoding the authentic promotor and the first 17 amino acids to GFP (NT-GFP). The expression of NT-GFP in wild-type and gpd1Δcox18Δ cells revealed distinct mitochondrial localization in both strains (Fig. 9). These results support a mitochondrial distribution of Gpd2p and strongly indicate that the first 17 amino acids contain information that is sufficient for mitochondrial targeting of the protein.

Gpd1p Shows an Increased Peroxisomal Distribution in cox18Δ Cells—Global analysis of protein localization (38) recently reported a cytosolic as well as a peroxisomal localization Gpd1p (yeastgfp.ucsf.edu/). This is consistent with the fact that Gpd1p carries a peroxisomal targeting signal of PTS2 type at amino acid position 7 (RLNLTSGLH). To examine the distribution of Gpd1p in cytosol and peroxisomes in wild-type and cox18Δ cells we introduced into the strains a low copy plasmid carrying GFP-tagged GPD1 gene expressed from its own promotor. This plasmid complements the salt sensitivity of mutants lacking the GPD1 gene, indicating the construct is functional (data not shown). Fluorescence microscopy showed that the dual distribution of fluorescence between cytosol and peroxisomes in wild-type cells was clearly shifted toward a more distinct distribution to the peroxisomes in cox18Δ cells (Fig. 10).

DISCUSSION

Metabolic control analysis of glycerol production in S. cerevisiae (43) has demonstrated that most of the flux control is
exerted at the reaction catalyzed by the \textit{GPD1/2} encoded glycerol-3-phosphate dehydrogenase. Here we confirm a key role for \textit{GPD2} in redox-mediated glycerol production and demonstrate that \textit{GPD1} needs strong overexpression to be able to substitute for \textit{GPD2} in a respiratory-deficient mutant, despite the fact that the corresponding two iso-enzymes have very similar kinetic properties (17). But why are cells with a compromised respiratory chain more dependent on \textit{GPD2} for redox adjustments than cells that cannot respire due to lack of oxygen? Butow and co-workers (27) have demonstrated that yeast with compromised mitochondria induces peroxisome biogenesis and increases peroxisome abundance. This response contributes to provide metabolic intermediates from anaplerotic pathways to fuel the blocked tricarboxylic acid cycle cycle with oxaloacetate and acetyl-CoA (27, 44). Since aerobic expression of \textit{GPD1} is not increased in respiratory compromised cells (Refs. 27 and 45 and Fig. 6), the enhanced peroxisome biogenesis might lead to an increased retention of the Gpd1p pool in the peroxisomes, leaving less activity in the cytosol to substitute for Gpd2p in the \textit{cox18} mutant. Our observations on the distribution of Gpd1p-GFP fluorescence in wild-type and \textit{cox18}Δ cells (Fig. 10), clearly support this view. However, wild-type cells do not appear to increase their peroxisome abundance on transfer to respiratory inhibiting anoxic conditions (46). This finding presumably explains why respiratory incompetent mutants become more strongly dependent on Gpd2p for redox adjustment than anaerobically grown wild-type cells, because in the mutant cells a higher proportion of the backup Gpd1p enzyme would be trapped in the peroxisome. The surprising observation that \textit{cox18}Δ cells maintain a higher Gpd1p level than wild-type cells (Fig. 5), despite similar expression of the \textit{GPD1} gene in the two strains (Fig. 6A), might also be related to the cellular distribution of Gpd1p. The peroxisome may provide a stabilizing environment for the enzyme and increase its half-life.

The physiological role for Gpd1p in the peroxisome seems linked to the function of this organelle in fatty acid degradation. By analogy with the suggested role for Gpd in peroxisomes of mammalian cells and \textit{Candida tropicalis} (47–49), \textit{S. cerevisiae} Gpd1p may contribute to maintain a suitable level of NAD⁺ for \(\beta\)-oxidation of fatty acids, either by local NADH production (which would require presence of the yeast glycerol-3-phosphatase Gpp1p or Gpp2p) or by being part of glycerol 3-phosphate shuttle for transfer of reducing equivalents from the peroxisome to the cytosol. Such a shuttle may provide an alternative to the previously suggested malate/oxaloacetate cycle (50) to ensure continued reoxidation of NADH produced by the intraperoxisomal \(\beta\)-oxidation.

Our observation that the addition of lysine and/or glutamic acid/glutamine could restore growth of the \textit{gpd2Δcox18}Δ mutant, suggested that the growth inhibiting redox imbalance of this mutant has its origin in the mitochondrial matrix. Glutamate derives from the tricarboxylic acid cycle intermediate \(\alpha\)-ketoglutarate, and is a precursor of other amino acids. The synthesis of the \(\alpha\)-ketoglutarate is considered a major source of mitochondrial NADH production (13). \(\alpha\)-Ketoglutarate is also an intermediate in lysine biosynthesis and at least one of the NADH generating enzymes of the lysine pathway, homo-isocitrate dehydrogenase (Lys12p), has a distinctly mitochondrial localization (38, 39). At first sight it seemed surprising that addition of glutamate did not improve growth of the respiratory defective strain as efficient as did addition of lysine. However, anaplerotic production of acetyl-CoA (27) will decrease requirement for mitochondrial production of this precursor for tricarboxylic acid cycle intermediates, thereby decreasing mitochondrial NADH production from the pyruvate dehydrogenase complex. It is also reported that yeast treated with the respiratory inhibitor antimycin, shows an increased expression of \textit{IDP3} (27), encoding a peroxisomal isoform of NADP-dependent isocitrate dehydrogenase. Since enhanced expression of \textit{IDP3} in the respiratory compromised \textit{cox18}Δ mutants might reduce the need for NADH producing mitochondrial synthesis of \(\alpha\)-ketoglutarate, we analyzed for \textit{IDP3} expression in wild-type and mutant strains (Fig. 11). The 3-fold up-regulated expression of
IDP2 in cox18Δ mutants gives credence to a scenario in which peroxisomal production of α-ketoglutarate partly substitutes for the mitochondrial synthesis. If the growth-inhibiting problem for the gpd2Δcox18Δ mutant is NADH accumulation in the mitochondrial matrix, why does addition of acetoin or overexpression of GPD1 restore growth of the mutant? Both of these rescuing activities are likely to take place in the cytosol; acetoin is reduced to 2,3-butanediol by the cytosolic NAD+-linked 2,3-butanediol dehydrogenase, Bdh1p (36, 38), and Gpd1p is a cytosolic/peroxisomal enzyme. Based on experimental evidence (51), the reversible ethanol-acetaldehyde shuttle is the most likely pathway for resolving the redox-problem of non-respiring mitochondria. This shuttle is driven by the NADH/NAD+ ratio gradient over the inner mitochondrial membrane (3). Hence, extensive localization of Gpd1p to the peroxisome might prohibit the generation of a sufficient gradient to drive the shuttle, whereas overexpression of GPD1 might provide for adequate amounts of cytosolic Gpd1p to compensate for absence of Gpd2p. Similarly, cytosolic reduction of acetoin might produce the driving redox conditions. This brings us to the role of Gpd2p, which appears to have acquired a cellular distribution that is optimal for adjustment of the cellular NAD+/NADH ratio in non-respiring cells; a cytosolic (38) as well as mitochondrial localization (40). In agreement with its mitochondrial distribution, the enzyme possesses an amino-terminal sequence of 17 amino acids that has the capacity to target proteins into mitochondria (Fig. 9). In this respect Gpd2p is reminiscent of yeast fumarase, which is encoded by a single gene (FUM1) that produces a major cytosolic and a minor mitochondrial form (52). The FUM1 translation product contains a cleavable targeting signal and both the cytosolic and mitochondrial product appears to be processed by mitochondria, whereupon the mature cytosolic form returns to the cytosol via release from the mitochondria (53). NADH oxidation in the mitochondrial matrix catalyzed by Gpd2p, would require transport of its second substrate, dihydroxyacetone phosphate, from the cytosol to the matrix, and no such transporter has so far been identified. It would also require export of glyceraldehyde 3-phosphate to the cytosol, since Gpp1p and Gpp2p appear to be primarily located in the cytosol (38) and absent from mitochondria (40). We therefore prefer a model in which mitochondrial Gpd2p is targeted to the intermembrane space rather than to the matrix. Such a distribution would allow for free exchange of substrates and products with the cytosol through the permeable outer membrane (cf. Ref. 4). In this scenario a Gpd2p-driven G3P production in the intermembrane region would generate a NAD+/NADH gradient across the inner mitochondrial membrane that provides a driving force for the ethanol-acetaldehyde shuttle to export reducing equivalents from the matrix to the cytosol (Fig. 12). Hence, despite substantial reconfiguration of cellular metabolism to outsource NADH production from mitochondria, maintenance of mitochondrial redox balance in cells that cannot respirate appears to require export of reducing equivalents. The differential cellular distribution of Gpd1p and Gpd2p provides an interesting system for further studies of compartmental redox adjustments.

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REFERENCES
1. de Koning, W., and van Dam, K. (1992) Anal. Biochem. 204, 118–123
2. von Jagow, G., and Klingenberg, M. (1970) Eur. J. Biochem. 12, 583–592
3. Bakker, B. M., Overkamp, K. M., van Maris, A. J., Kötter, P., Luttik, M. A., van Dijken, J. P., and Pronk, J. T. (2000) J. Bacteriol. 182, 4551–4555
4. Roos, B. M., Bakker, B. M., Kötter, P., van Tuuli, A., de Vries, S., van Dijken, J. P., and Pronk, J. T. (1998) J. Bacteriol. 170, 4529–4534
5. Luttik, M. A. H., Bakker, K. M., Kötter, P., de Vries, S., van Dijken, J. P., and Pronk, J. T. (1998) J. Biol. Chem. 273, 24529–24534
6. Larsson, C., Pählman, L. L., Persall, R., Rigosu, M., Adler, L., and Gustafsson, L. (1998) Yeast 14, 347–357
7. Marres, C. A., de Vries, S., and Grivel, L. A. (1991) Eur. J. Biochem. 195, 557–562
8. de Vries, S., van Wiltenburg, R., Grivel, L. A., and Marres, C. A. (1992) Eur. J. Biochem. 203, 587–592
9. Nakamura, K. (1998) J. Biol. Chem. 273, 429–432
10. Albers, E., Liden, G., Larsson, and Gustafsson, L. (1998) Rev. Devel. Microbiol. 2, 253–279
11. Nissen, T. L., Schulze, U., Nielsen, J., and Villadsen, J. (1997) Microbiology 143, 205–218
12. Oura, E. (1973) Process Biochem. 12, 19–35
13. van Dijken, J. P., and Scheffers, W. A. (1986) Microbes Microb. Rev. 20, 199–225
14. Pählman, A. K., Granath, K., Ansell, R., Hohmann, S., and Adler, L. (2001) J. Biol. Chem. 276, 3555–3563
15. Åhlman, A. K., Granath, K., Hohmann, S., Thevelein, J., and Adler, L. (1997) EMBO J. 16, 2179–2187
16. Björkqvist, S., Ansell, R., Liden, L., and Adler, G. (1997) Appl. Environ. Microbiol. 63, 128–132
17. Brown, A. D. (1978) Adv. Microbial. Physiol. 17, 181–242
18. André, L., Hemming, A., and Adler, L. (1991) FEBS Lett. 286, 13–17
19. Larsson, K., Eriksson, P., Ansell, R., and Adler, L. (1993) Mol. Microbiol. 10, 1011–1111
20. Albertyn, J., Hohmann, S., Thevelein, J. M., and Prior, B. A. (1994) Mol. Cell. Biol. 14, 4135–4144
21. Gustafsson, E., Albertyn, J., Alexander, M., and Davenport, K. (1998) Microbial. Mol. Biol. Rev. 62, 1264–1300
22. Hohmann, S. (1992) Microbial. Mol. Biol. Rev. 66, 300–372
23. Costable, R., Valadi, H., Gustafsson, L., Niklasson, C., and Franzen, C. J. (2000) Yeast 16, 1483–1495
24. Ansell, R., and Adler, L. (1999) FEBS Lett. 461, 175–177
25. Epstein, C. B., Waddell, J. A., Hale IV, D. E., Dave, V., Thornton, J., Macatee, T. L., Garner, H. R., and Rutov, R. A. (2001) Mol. Biol. Cell, 297–308
26. Pronk, J. T. (2002) Appl. Environ. Microbiol. 68, 2095–2100
27. Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K. (1995) Current Protocols in Molecular Biology, John Wiley & Sons, New York
28. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Labaratory Press, Cold Spring Harbor, New York
29. Larsen, C., Pålman, A. K., and Adler, L. (1991) J. Cell Comp. Physiol. 161, 143, 779–786
30. Large, L. J., and O’Hare, P. J. (1992) FEBS Lett. 301, 372–378
31. Skoneczny, M., and Rytka, J. (1996) Biochimie 78, 767–779
32. Small, W. C., and McAlister-Henn, L. (1998) J. Bacteriol. 180, 133–137
33. Roos, B. M., Bakker, B. M., Kötter, P., van Tuuli, A., de Vries, S., van Dijken, J. P., and Pronk, J. T. (1998) J. Bacteriol. 180, 133–137
34. van Roermund, C. W., Waterham, H. R., Ijlst, L., and Wanders, R. J. (2003) Mol. Cell. Biol. 23, 35786–35885
35. van Dijken, J. P., and Pronk, J. T. (2000) J. Bacteriol. 182, 4730–4737
36. Elbers, E., Blachinsky, E., Karney, S., and Pines, O. (2001) J. Biol. Chem. 276, 46111–46117
37. Sass, E., Karney, S., and Pines, O. (2003) J. Biol. Chem. 278, 45109–45116
38. Thomas, B. J., and Rothstein, R. (1989) Cell 56, 619–630
Distinct Intracellular Localization of Gpd1p and Gpd2p, the Two Yeast Isoforms of NAD$^+$-dependent Glycerol-3-phosphate Dehydrogenase, Explains Their Different Contributions to Redox-driven Glycerol Production

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