The Yeast Glycerol 3-Phosphatases Gpp1p and Gpp2p Are Required for Glycerol Biosynthesis and Differentially Involved in the Cellular Responses to Osmotic, Anaerobic, and Oxidative Stress*

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We have characterized the strongly homologous GPP1RHR2 and GPP2HOR2 genes, encoding isoforms of glycerol 3-phosphatase. Mutants lacking both GPP1 and GPP2 are devoid of glycerol 3-phosphatase activity and produce only a small amount of glycerol, confirming the essential role for this enzyme in glycerol biosynthesis. Overproduction of Gpp1p and Gpp2p did not significantly enhance glycerol production, indicating that glycerol phosphatase is not rate-limiting for glycerol production. Previous studies have shown that expression of both GPP1 and GPP2 is induced under hyperosmotic stress and that induction partially depends on the HOG (high osmolarity glycerol) pathway. We here show that expression of GPP1 is strongly decreased in strains having low protein kinase A activity, although it is still responsive to osmotic stress. The gpp1Δ/gpp2Δ double mutant is hypersensitive to high osmolarity, whereas the single mutants remain unaffected, indicating GPP1 and GPP2 substitute well for each other. Transfer to anaerobic conditions does not affect expression of GPP2, whereas GPP1 is transiently induced, and mutants lacking GPP1 show poor anaerobic growth. All gpp mutants show increased levels of glycerol 3-phosphate, which is especially pronounced when gpp1Δ and gpp1Δ/gpp2Δ mutants are transferred to anaerobic conditions. The addition of acetaldehyde, a strong oxidizer of NADH, leads to decreased glycerol 3-phosphate levels and restored anaerobic growth of the gpp1Δ/gpp2Δ mutant, indicating that the anaerobic accumulation of NADH causes glycerol 3-phosphate to reach growth-inhibiting levels. We also found the gpp1Δ/gpp2Δ mutant is hypersensitive to the superoxide anion generator, paraquat. Consistent with a role for glycerol 3-phosphatase in protection against oxidative stress, expression of GPP2 is induced in the presence of paraquat. This induction was only marginally affected by the general stress-response transcriptional factors Msn2p/4p or protein kinase A activity. We conclude that glycerol metabolism plays multiple roles in yeast adaptation to altered growth conditions, explaining the complex regulation of glycerol biosynthesis genes.

Glycerol production in the yeast Saccharomyces cerevisiae is known to have fundamental physiological functions in two distinct adaptive responses: osmoregulation and anaerobic redox control (1). At high external osmolarity, yeast cells compensate for the loss of water by increased glycerol production (2). This adaptive response is regulated, at least in part, by an osmosensing and signaling system called the HOG (high osmolarity glycerol) MAPK pathway (3). Two putative osmosensors encoded by SLN1 (1) and SHO1 (2), which communicate with a MAPK module involving the MAPK Hog1p and its activator Pbs2p, control the HOG pathway (3).

Cells that are transferred from aerobic to anaerobic conditions are faced with the problem of catabolizing substrate molecules into a balanced blend of oxidized and reduced end-products, to maintain intracellular redox balance. Fermentation of glucose to ethanol is a redox inverting process in the sense that the NADH produced in glycolysis is reoxidized by conversion of pyruvate to ethanol and CO2. However, the assimilation of sugar to biomass generates excess NADH, which is re-oxidized by diversion of part of the sugar substrate to the more reduced end product, glycerol (4–6). Glycerol production is essential for S. cerevisiae to dispose of surplus reducing power under anaerobic conditions, because mutants with a blocked glycerol production abruptly stop glucose catabolism when shifted to anoxic conditions (7, 8).

Glycerol is produced by reduction of the glycolytic intermediate dihydroxyacetone phosphate to glycerol 3-phosphate (G3P) followed by a dephosphorylation of G3P to glycerol. The first step is catalyzed by NAD-dependent glycerol 3-phosphate dehydrogenase (GPD), which is encoded by the two isogenes GPD1 and GPD2. GPD1 encodes the major isofrom in aerobically growing cells. The expression of this isogene, which is required for growth at high osmolarity (9), is induced by hyperosmotic stress and is a target gene for the HO pathway (10). The second isogene, GPD2, is required for glycerol production in the absence of oxygen, and its expression is stimulated

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1 The abbreviations used are: HOG pathway, high osmolarity glycerol pathway; PAG, polyacrylamide gel electrophoresis; MAPK, mitogen activated protein kinase; PKA, protein kinase A; Gpd1p, -2p, NAD-dependent glycerol dehydrogenase (GPD), which is encoded by the two isogenes GPD1 and GPD2. GPD1 encodes the major isofrom in aerobically growing cells. The expression of this isogene, which is required for growth at high osmolarity (9), is induced by hyperosmotic stress and is a target gene for the HO pathway (10). The second isogene, GPD2, is required for glycerol production in the absence of oxygen, and its expression is stimulated

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under these conditions (8). Hence, the two GPD genes have defined physiological roles in the adaptation of S. cerevisiae to high osmolarity and anoxic conditions. We previously reported the purification and characterization of two isoforms of a glycerol 3-phosphatase (11). In this work we have studied the expression of the corresponding genes, GPP1 (RHR2) and GPP2 (HOR2) and the phenotype of the deletion mutants. We report that the GPP isoforms are differentially expressed under conditions of osmotic and anaerobic stress and that the expression of GPP1 is under strong influence of protein kinase A (PKA) activity. We also show that the two isoforms have interchangeable functions under osmotic stress but that deletion of GPP1 results in an anaerobic growth defect. This study also reveals a role for glycerol metabolism in oxidative stress protection, because the gpp1Δ/gpp2Δ deletion mutant is hypersensitive not only to osmotic stress and anoxic conditions but also to the oxidant parquat.

**EXPERIMENTAL PROCEDURES**

*Strains, Media, and Yeast Genetic Methods—*S. cerevisiae strains and genotypes are listed in Table I. Cells were routinely grown at 30 °C in synthetic yeast nitrogen base (YNB) medium (Difco) supplemented with ergosterol (10 mg/liter) and Tween 80 (v/v) from an overnight YNB culture. Media used for anaerobic incubation of single mutants. Tetrads gave rise to viable double mutants on aerobic plates.

**TABLE I**

| Strain | Genotype | Source |
|--------|----------|--------|
| W303–1A* | MATa | (61) |
| YA101* | MATa gdp1::kanMX4 | This study |
| YA102* | MATa gpd2::HIS3 | This study |
| YA103* | MATa gpd1::kanMX4 gpd2::HIS3 | This study |
| YA104* | MATa gpd1::kanMX4 gpd2::HIS3 gpd2::URA3 | This study |
| YA105* | MATa gpd1::kanMX4 gpd2::TRP1 | This study |
| YA106* | MATa gpd1::kanMX4 gpd2::URA3 | This study |
| YA107* | MATa gpd2::HIS3 gpd1::TRP1 | This study |
| YA108* | MATa gpd2::HIS3 gpd2::URA3 | This study |
| YA109* | MATa gpd1::LEU2 gpd2::URA3 | This study |
| YA110* | MATa msn2::3::HIS3 msn4::1::TRP1 | (62) |
| YA111* | MATa gpd1::TRP1 | (6) |
| YA112* | MATa gpd2::URA3 | (8) |
| YA113* | MATa gpd1::TRP1 gpd2::URA3 | (8) |
| YA114* | MATa gpd1::URA3 | S. Hohmann |
| SP1 | MATa leu2 his3 trp1 ura3 ade8 can1 | (64) |
| S7–7A | TRP1 Mata leu2 his3 trp1 ura3 ade8 can1 tpk2::HIS3 tpk3::TRP1 | (35) |
| S18–1DA | Mata leu2 his3 trp1 ura3 ade8 can1 tpk1::kanMX4 tpk2::HIS3 tpk3::TRP1 | (35) |

* These strains harbor the following additional mutations: leu2–3/112 ura3–1 trp1–1 his3–11/15 ade2–1 can1–100 GAL SUC2 null.

**Cloning and Characterization of S. cerevisiae GPP1 and GPP2**

The GPP1-specific 5′-TGT GAT CAA AGG CAT TGAT GAC G-3′ oligonucleotide (20) was used as the probe. The 2815-bp XbaI/SalI fragment covering the 753-bp GPP1 ORF and 1199 bp upstream of the translational start was cloned into the pRS316 vector. For multicopy expression, NotI/SalI (GPP1) or NotI/ClaI (GPP2) fragments were transferred from the centromeric pRS316 to the 2μ vector pRS326.

Deletion of the GPP1 gene was accomplished by the long flanking homology PCR-targeting technique (21, 22). In the first step, a set of primers (5′-TGGTGAAGTTCCCTTTTTCTT-3′ and 5′-CAAAAGCCATTGCGATTTCT-3′) was used to amplify 263 bp of genomic DNA from S. cerevisiae W303, upstream from the third codon in the GPP1 ORF. A second set (5′-CTCGTAAGGATCGCTTTG-3′ and 5′-CTCTCAACTTCTCTGATTGT-3′) was used to amplify a 358-bp fragment from the ninth codon in the GPP1 ORF upstream the stop codon. The 5′-end of the primers adjacent to the insertion site carried 25 nucleotide extensions homologous to the 5′ and 3′ regions of the hisG MX6 or kanMX4 disruption cassette of plasmid pFA6a-hisGMX6 and pFA6α-kanMX4 (21). In the second PCR reaction, pFA6a-hisGMX6 and pFA6α-kanMX4 were used as templates and the 5′ and 3′ homologous regions of the first PCR reaction were fused to the disruption cassette by serving as primers together with the upstream forward and downstream reverse primers of the flanking regions, thus producing the ORF targeting cassette. This cassette was transformed into a haploid S. cerevisiae W303 strain, and independent transformants were selected for verification of GPP1 replacement. Using a set of primers (forward: 5′-CAAGCGAGGAATC-3′ and reverse 5′-CTATATGGACTTGG-3′) hybridizing upstream and downstream, respectively, of the disruption cassette chromosomal DNA was amplified. The length of the PCR products was verified by agarose-gel electrophoresis. The GPP2 ORF was disrupted in a similar way using a set of primers (5′-GACGACTGTAATGTTG-3′ and 5′-GTTGGATAATGTTG-3′) to amplify a 346-bp fragment upstream from the fourth codon in the ORF. The second set (5′-GGACGATGTTGAAAATG-3′ and 5′-GGCGATGTTG-3′) was used to amplify a 287-bp fragment from the seventh codon in the GPP2 ORF downstream the stop codon. Correct integration of the disruption modules into the GPP1 and GPP2 alleles was verified by PCR.

The gpp1Δ/gpp2Δ double mutant was constructed by crossing of the single mutants. Tetrad gave rise to viable double mutants on aerobic incubation in complete medium. GPD1 and GPD2 were overexpressed from their own promoters in the YEplac112 (23) multicopy plasmid, transformed into the gpd1Δ/ gpd2Δ mutant.

**Northern Blotting—**Total RNA was isolated by standard procedures at indicated time points from cultures treated as described in the figure legends. RNA samples were screened on ethidium bromide agarose gels and quantified by spectrophotometry, using the Beckman DU65 (Beckman Instruments) nuclear acid program. Samples containing 15 μg of total RNA were denatured and run on low formaldehyde (2.5% v/v) agarose gels at 10 V/cm for 75 min, tested for quality at 254 nm on thin-layer chromatography plates, and blotted to positively charged nylon membranes (Roche) by capillary transfer, using 10× SSC as transfer buffer. Blotted filters were cross-linked by 1-min exposure to low wavelength UV and baked at 80 °C for 2 h. Prehybridization was
performed for 3–4 h at 55 °C in 5× SSC, 10 mM sodium phosphate (pH 6.5), 10 × Denhardt’s solution, 2% SDS, and 100 μg/ml herring sperm DNA.

Hybridization was performed at 55 °C for 18–20 h using the same solution supplemented with 10% polyethylene glycol 4000 and having labeled oligonucleotides added at 5 ng/ml. The filters were washed twice in 1× SSC/1% SDS, for 20 min at room temperature and once for 15 min at the hybridization temperature. Membranes were stripped for rehybridization by shaking in sterile water at 80 °C for 10 min. To quantify transcript levels, signal intensity was quantified with a Molecular Dynamics PhosphorImager and normalized to that for ACT1 or IPP1 mRNA. For most experiments two or more independent RNA blot analyses were performed for each growth condition and transcript examined.

The oligonucleotides used were 5′-labeled with 25 μCi of [γ-32P]ATP (Amersham Pharmacia Biotech) and 5′ units of polynucleotide kinase (Roche Molecular Biochemicals) per 50 ng of probe, left at 37 °C for 30 min. Unincorporated ATP was displaced using a Sephadex G-50 (Amersham Pharmacia Biotech) column. Sequences of oligonucleotides used were: 5′-TGTGGTCAAAGGCATTGCGATGG-3′ to probe for GPP1 mRNA, and 5′-TCTGCTGATGATCAGTACCTA-3′ to assay for GPP2 mRNA. Oligonucleotides used to probe for ACT1 and IPP1 were 5′-ATGGTCTTATACCAAACTGTGGAGG-3′ and 5′-GCTGTGCTGTAAGTTGGT-3′, respectively. The specificities of the oligonucleotides used for probing GPP1 and GPP2 mRNA were controlled using the gpp1Δ and gpp2Δ strains.

Analysis of Glycerol and Glycerol 3-Phosphate—Sampled cell suspensions of 1 ml were analyzed for total (intra- plus extracellular) glycerol by boiling for 10 min followed by centrifugation and enzymatic determination of the glycerol content of the supernatant using a commercial glycerol analysis kit (Roche Molecular Biochemicals). For glycerol extraction, cells were cleared by centrifugation, and the glycerol 3-phosphate was removed by repeated extraction with 4 ml of water-saturated diethylether. Residual diethylether was removed by percolation with water-saturated nitrogen gas for 10 min (24). The extract was cleared by centrifugation, and the glycerol 3-phosphate content was determined by the enzymatic method described by Bergmeyer (25) using a Cobas FARA analyzer.

Preparation of Cell-free Extracts—Cells were washed twice in TRED buffer (10 mM triethanolamine, 1 mM EDTA, pH 7.5) and resuspended in the same buffer plus 1 mM dithiothreitol and a protease inhibitor mix (Complete, Roche) added in amounts as recommended by the supplier. Extracts were prepared by disruption of cells using acid-washed glass beads and subsequent centrifugation (14,000 × g for 15 min). The obtained supernatants were desalted by gel filtration according to procedures described previously (26).

Protein Determination—For enzyme assays protein concentration was determined by the method of Bradford (Bio-Rad), whereas metabolite levels were related to cell protein using the Lowry method for protein determination. Bovine serum albumin was used as standard for both methods.

Enzyme Assay—Glycerol 3-phosphatase was assayed as described previously (11). Briefly, cell-free extracts were incubated in 20 mM Tricine-HCl (pH 6.5), 5 mM MgCl2, and 10 mM Nl-glycerol 3-phosphate in a total volume of 1.0 ml. After starting the reaction, samples of 90 μl were withdrawn at different time points and the reaction was stopped by adding 10 μl of 50% HClO4. Inorganic phosphate was analyzed according to a previous study (27), and the reaction rate was calculated from the slope of a linear plot of released phosphate versus time. All glassware used was immersed overnight in 1 M HCl and rinsed thoroughly in distilled water, to eliminate phosphate contamination.

Western Blot Analysis—Proteins were separated by SDS-PAGE in 10% acrylamide gels at 125 V for 1.5 h using a Mini-PROTEAN II electrophoresis system (Bio-Rad) and transferred to Hybond membranes (Amersham Pharmacia Biotech, UK), according to the manufacturers’ protocols. The membranes were incubated for 1 h at room temperature in 20 mM Tris, pH 7.6, 0.8% NaCl, 0.1% Tween 20 (buffer A) supplemented with 5% blocking reagent (Amersham Pharmacia Biotech, UK) and then for 1 h with rabbit anti-Gpp antibody diluted 1:5000 in buffer A) supplemented with 5% blocking reagent (Amersham Pharmacia Biotech, UK) and then for 1 h with rabbit anti-Gpp antibody diluted 1:5000 in buffer A. The Gpp antibodies were kindly provided by Dr. Dunn-Coleman, Genencor International (Palo Alto, CA). Membranes were washed for 15 min followed by 2×5 min in buffer A. The membranes were probed for 1 h with a 1:15,000 dilution of anti-rabbit peroxidase-linked antibodies (Amersham Pharmacia Biotech, UK) in buffer A. Blots were washed as before, and antibody detection was performed using the ECL labeling system (Amersham Pharmacia Biotech, UK).

RESULTS

Loss of GPP Genes Results in Osmosensitivity and Poor Growth under Anaerobic Conditions—Because glycerol production is strictly required by S. cerevisiae for osmoregulation and anaerobic redox adjustments (8), we examined the importance of the glycerol 3-phosphate genes GPP1 and GPP2 for growth at high osmolarity and anaerobiosis. The single gpp1Δ and gpp2Δ mutants showed no obvious growth defects on aerobic incubation in YNB medium, whereas the gpp1Δ/gpp2Δ double mutant grew somewhat more slowly under these conditions (Fig. 1A). The growth of the single gpp1Δ and gpp2Δ mutants also appeared indistinguishable from that of the wild type under NaCl stress, whereas the gpp1Δ/gpp2Δ double mutant was strongly inhibited by increased salinity (Fig. 1, A and B), or osmotically comparable concentrations of sorbitol (data not shown).
Under anaerobic conditions (Fig. 2, A and B) cells lacking \textit{GPP1} displayed a much prolonged lag phase and a decreased exponential growth rate, whereas \textit{gpp2} mutants grew like the wild type strain. Growth of the \textit{gpp1}\textsubscript{Δ}\textit{gpp2} mutant was severely inhibited by transfer to anaerobiosis and only on prolonged incubation growth occurred in liquid medium (Fig. 2A). The mechanism underlying this slow adaptation to anaerobiosis is unexplored, although an involvement of the recently described dihydroxy acetone pathway (20) for glycerol production is possible.

\textbf{Glycerol Production Is Blocked in a gpp1/gpp2 Double Mutant but Little Affected in \textit{gpp} Single Mutants—To clarify the role of the \textit{GPP} genes in glycerol biosynthesis, we analyzed glycerol production of wild type and mutant cells following transfer to increased osmolarity or anaerobiosis (Fig. 3). As expected from the growth phenotype, the two single mutants showed stimulated glycerol production much like the wild type, whereas the double mutant did not produce any significant amounts of glycerol under any conditions. Unexpectedly, the \textit{gpp1}Δ mutant produced glycerol like the wild type under anaerobic conditions, despite the growth defect of this mutant under these conditions (see above).

\textbf{Expression of Both \textit{GPP} Genes Is Affected by Osmotic Shock Whereas Expression of \textit{GPP1} Is Also Influenced by Anaerobic Conditions and \textit{PKA} Activity—We then analyzed the extent to which the observed glycerol response is associated with changes in \textit{GPP} gene expression. Northern analysis (Fig. 1C) confirmed previous reports of osmotic induction of the \textit{GPP} gene expression (11, 28, 29), demonstrating a weak response of the \textit{GPP1} gene, whereas \textit{GPP2} expression was strongly induced in a salt concentration-dependent fashion. After shift to anaerobic conditions, the mRNA level of \textit{GPP1} showed a transient increase (Fig. 2C), whereas that of \textit{GPP2} remained unaltered (not shown). The induction profiles of \textit{GPP1} or \textit{GPP2} in mutants carrying only one of the isogenes were similar to those observed for wild type cells (not shown), indicating that loss of one isoform does not markedly affect expression of the other.

The \textit{PKA} pathway plays a role in integrating growth control with environmental stress (30, 31), and works antagonistically with the HOG pathway on a variety of osmoreponsive genes (32). Although it was previously reported that the osmotic induction of \textit{GPP2} is \textit{PKA}-independent (28, 29, 33), we noted that the basal \textit{GPP1} mRNA level is strongly dependent on \textit{PKA} activity. This \textit{PKA} effect is in contrast to a previous report (28) but in agreement with other findings (34). In a \textit{tpk1}\textsuperscript{Δ} \textit{tpk2} strain having a low \textit{PKA} activity (35), \textit{GPP1} expression was severely decreased but still responsive to osmotic stress (Fig. 4). Attenuated \textit{PKA} activity also did not affect the anaerobic response of \textit{GPP1} gene expression (data not shown). Apparently, the \textit{PKA} pathway exerts an activating or derepressing effect on \textit{GPP1} expression as was previously noted for that of yeast ribosomal protein genes (36) and the AP-1 factorencoding \textit{GCN4} gene (37).

\textbf{Determination of the specific enzyme activity of Gpp1p and Gpp2p following transfer of cells to increased osmolarity or anaerobic conditions (Fig. 5), indicates that the \textit{Gpp1p} isozyme contributes between 70–90% of the glycerol 3-phosphatase activity. Because Gpp2p activity does not increase after shift to anaerobic conditions, this enzyme is responsible for only about 10% of the phosphatase activity in anaerobically grown cells. Moreover, the absence of activity in the \textit{gpp1}\textsubscript{Δ}\textit{gpp2} mutant

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure2}
\caption{A, growth of the wild type (W303-1A) strain (○), the \textit{gpp1}\textsubscript{Δ}, \textit{gpp2} Δ, and \textit{gpp1}\textsubscript{Δ}gpp2 Δ mutants in liquid YNB medium under anaerobic conditions. B, growth of the wild type strain, the \textit{gpp1}\textsubscript{Δ}, \textit{gpp2} Δ, and \textit{gpp1}\textsubscript{Δ}gpp2 Δ mutants when spotted in serial 10-fold dilutions onto YNB medium incubated in an anaerobic jar. Plates were scored after 2 days of incubation. C, \textit{GPP1} mRNA levels in wild type cells incubated in YNB medium and then shifted to anaerobic conditions. Cells were shifted to new conditions at time point zero. ACT1 mRNA served as internal control. The graph represent mRNA levels for \textit{GPP1} relative to those of \textit{ACT1}, the highest relative mRNA level being set to 1.}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure3}
\caption{Kinetics of total glycerol production by wild type (W303-1A), the \textit{gpp1} Δ, \textit{gpp2} Δ, and \textit{gpp1}\textsubscript{Δ}gpp2 Δ strains after shift to YNB medium containing 0 M (○), 0.5 M (●), or 1.0 M (□) NaCl, or YNB medium incubated under anaerobic conditions (□). Cells were grown to \textit{A}_{\text{540}} = 0.8–1.0, collected by centrifugation, and suspended at half of the original cell density with fresh YNB medium containing an appropriate concentration of NaCl.}
\end{figure}
Despite the more (Fig. 6) or immunoblot analysis (Fig. 6) cells showed a 50- to 100-fold increased expression of either of their native promoters in wild type cells. The transformed incubated in YNB medium containing 0 M (gpp1 wild type (W303-1A), B than 10-fold increase in phosphatase activity, there was no observed dephosphorylation of G3P. Taken together, measurements of transcript levels and specific enzyme activity suggest that a major control on the cellular glycerol 3-phosphatase activity is exerted at the mRNA level.

During anaerobic growth or under osmotic stress, the G3P level of the gpp2Δ mutant became 4- to 6-fold higher than in the wild type strain. Deletion of GPP1 caused, on the other hand, an immediate 4-fold increase of the G3P level, and this level increased another 2-fold after shift to anaerobic conditions. In the gpp1Δgpp2Δ mutant, the G3P pool was about 40-fold increased in aerobically grown cells irrespective of salinity, whereas anaerobic incubation provoked a progressive further accumulation to a level about 100-fold higher than that of the wild type. These results show that the size of the G3P pool is sensitively dependent on the glycerol 3-phosphatase activity.

We also noted that G3P accumulation in the double mutant was much higher after transfer to anoxic conditions than after shift to 0.5 M NaCl, whereas both conditions gave rise to a similar increase of glycerol production in wild type cells (cf. Fig. 3). As a possible explanation for this effect we could rule out aerobic reoxidation of G3P via the GUT2-encoded mitochondrial G3P dehydrogenase (38, 39), because the salt-induced accumulation of G3P in a gpp1Δgpp2Δgut2Δ triple mutant was similar to that of a gpp1Δgpp2Δ double mutant (Fig. 7A). We next considered the possibility that the anaerobically induced isosform of glycerol-3-phosphate dehydrogenase, Gpd2p, allows for stronger G3P accumulation than the osmostress-induced Gpd1p isozyme, by being less sensitive to product (G3P) inhibition. To test this idea we overexpressed GPD1 or GPD2 in gpd1Δgpd2Δ double mutants and assayed the glyceral-3-phosphatase dehydrogenase activity in extracts in the presence of 5 and 10 mM G3P. These measurements demonstrated, however, that both isoforms responded very similarly to increased product concentrations (data not shown). We finally examined whether anaerobic accumulation of NADH contributes to the massive anoxic expansion of the G3P pool in gpp1Δgpp2Δ mutants. To this end, the G3P pool was analyzed in double mutants following anaerobic incubation in the presence or absence of 10 mM acetaldehyde (Fig. 7B). S. cerevisiae efficiently reduces added acetaldehyde to ethanol (8), leading to regeneration of NAD+ from NADH. After addition of acetaldehyde, the G3P level decreased to about 50% and growth resumed. Following incubation, G3P levels increased again and growth gradually declined. These effects were clearly due to consumption of added acetaldehyde, because repeated addition released growth inhibition (data not shown). Hence, we conclude that an increased NADH/NAD+ ratio constitutes a strong driving force for the anaerobic accumulation of G3P in gpp1Δgpp2Δ mutants.

Interplay between Gpd and Gpp Isozymes for Tolerance to Osmotic and Anaerobic Stress—To examine a possible interplay between the Gpd and Gpp isozymes in the glycerol production pathway, we constructed the four possible gpd1Δgpp2Δ double mutants and analyzed their growth behavior at high salinity and anaerobiosis (Fig. 8). Growth was limited at high

**Fig. 4.** GPP1 mRNA levels in *S. cerevisiae* wild type strain SP1 (TPK1 TPK2 TPK3), S7-7A (TPK1 tpk2Δ tpk3Δ), and S18-1DA (tpk1Δ tpk2Δ tpk3Δ) cells shifted to fresh YNB medium containing 0 or 1 M NaCl. Experiments were initiated with cells that were grown to A600 = 0.8–1.0 and diluted to half the original cell density with fresh YNB medium containing an appropriate concentration of NaCl. Cells were sampled for RNA extraction at the indicated times. IPP1 mRNA served as internal control. The graphs show quantified GPP1 mRNA levels relative to those of IPP1 for the different strains, and the highest level for each set of experiments was adjusted to 1.0.

**Fig. 5.** Changes in glycerol 3-phosphatase-specific activity of wild type (W303-1A), gpp1Δ, gpp2Δ, and gpp1Δgpp2Δ strains incubated in YNB medium containing 0 M (○), 0.5 M (□), and 1.0 M (●) NaCl, or in YNB medium incubated under anaerobic conditions (△). Experiments were initiated with cells that were grown to A600 = 0.8–1.0 and diluted to half the original cell density with fresh YNB medium containing an appropriate concentration of NaCl. Samples of 50 ml were taken at the indicated time points and analyzed for glycerol 3-phosphatase specific activity.

Confirms that the enzyme assay specifically detects glycerol 3-phosphatase and that this activity is responsible for the observed dephosphorylation of G3P. Taken together, measurements of transcript levels and specific enzyme activity suggest that a major control on the cellular glycerol 3-phosphatase activity is exerted at the mRNA level.

**Overexpression of GPP1 or GPP2 Does Not Increase Glycerol Production**—To examine the role of glycerol 3-phosphatase in controlling the metabolic flux to glycerol, we introduced multicopy plasmids carrying either GPP1 or GPP2 under the control of their native promoters in wild type cells. The transformed cells showed a 50- to 100-fold increased expression of either isoform as demonstrated by enzyme activity measurements (Fig. 6A) or immunoblot analysis (Fig. 6C). Despite the more than 10-fold increase in phosphatase activity, there was no significant change in the glycerol production by cells cultured in basal medium (Fig. 6B).

**Intracellular G3P Levels Are Strongly Affected by Phosphatase Activity**—To discover whether loss of GPP genes leads to intracellular accumulation of G3P, we determined the G3P pool in wild type and mutant cells following exposure to environmental changes. This analysis pointed to a minor influence of GPP2 on the G3P level under nonstressed conditions (Fig. 7A).
salinity only in cells lacking GPD1, whereas under anaerobic conditions growth limitation was seen only for cells lacking GPD2. The anaerobic plate test used was not sufficiently sensitive to detect growth changes due to the absence of GPP1 (cf. Fig. 2). Nevertheless, the result supports the notion that flux in the glycerol pathway is primarily controlled at the level of glycerol 3-phosphate dehydrogenase.

Diminished GPP1 Expression in Cells Cultured on a Nonfermentable Carbon Source—Cultured on the nonfermentable carbon source, ethanol, *S. cerevisiae* increased GUT2 gene expression (38, 39) leading to respiratory oxidation of produced G3P via the G3P shuttle (40). We therefore examined the fate of the GPP1 and GPP2 transcript levels in cells cultured on ethanol as the sole carbon source. Although the GPP2 mRNA level appeared to be insensitive to the carbon source, the GPP1 mRNA level dropped to about one-third of that observed in cells cultured on glucose (Fig. 9A). Following exposure to increased extracellular osmolarity, both GPP1 and GPP2 mRNA levels increased, demonstrating that the osmotic response operates under these conditions. Overexpression of GPP1 or GPP2 did not increase the osmotolerance of ethanol-grown cells (Fig. 9B).
Cloning and Characterization of S. cerevisiae GPP1 and GPP2

Loss of GPP Genes Results in Sensitivity to Oxidative Stress—Because glycerol production appears to be crucial for redox regulation, and glycerol has been suggested as a radical scavenger (41), we explored a possible role of the GPP genes under conditions of oxidative stress. A plate test (Fig. 10A) revealed hypersensitivity of the gpp1Δ/gpp2Δ mutant to the superoxide ion-generating agent paraquat, whereas the single mutants grew like wild type. Because a gpd1Δ/gpp2Δ mutant also showed increased sensitivity, glycerol metabolism per se seems required for tolerance to paraquat. The difference in sensitivity between the two double mutants might be due to the accumulation of G3P in the gpp1Δ/gpp2Δ mutant, which may increase vulnerability to oxidative conditions. However, this accumulation does not generate increased sensitivity by affecting the electron flow to the respiratory chain via Gut2p, because a gpp1Δ/gpp2Δ/gut2Δ triple mutant is equally sensitive to paraquat as a gpp1Δ/gpp2Δ mutant (data not shown).

Consistent with the sensitivity of the gpp1Δ/gpp2Δ double mutant to oxidative stress, exposure to paraquat elicited increased expression of GPP2 (Fig. 10B). Expression of GPP1 was not markedly affected (data not shown). The stress-response element (STRE)-binding transcription factors Msn2p and Msn4p have been reported to mediate, among others, induction of gene expression by oxidative stress (42), and their nuclear localization is controlled by PKA (43). Although the promoter of GPP2 contains a single STRE element (at position −272 nt), mutants lacking the genes MSN2 and MSN4 or having attenuated PKA activity showed a similar degree of induction, although with slightly delayed kinetics (Fig. 10B).

DISCUSSION

We here report cloning and characterization of GPP1 and GPP2, two yeast isoenzymes for glycerol 3-phosphatase. Mutants deleted for both genes fail to produce glycerol, exhibit strong osmosensitivity, and arrest growth when shifted to anaerobic conditions, demonstrating that glycerol 3-phosphatase is essential for glycerol production and confirming the vital importance of the glycerol pathway for tolerance to osmotic and anaerobic stress.

GPP1 and GPP2 Have Redundant Roles in Osmoadaptation

Whereas GPP1 Is Specifically Required for Adaptation to Anaerobic Conditions—The observation that expression of both GPP1 and GPP2 is stimulated by osmotic stress suggested involvement of both genes in the osmoregulatory glycerol response. This notion was verified by the finding that neither the gpp1Δ nor the gpp2Δ single mutants exhibited osmosensitivity (Fig. 1A). Thus, the strongly homologous GPP isoenzymes appear to be functionally exchangeable when expressed at sufficiently high level. The osmostress-induced expression of number of genes is reported to depend on the HOG pathway and/or the PKA pathway (28, 32, 44, 46, 47). Previous studies have shown that the osmotic response of GPP1 expression is also largely PKA-independent, whereas the basal expression of this gene is strongly dependent on PKA activity. Hence, PKA activity influences cellular glycerol 3-phosphatase activity by affecting GPP1 expression but does not significantly contribute to altering GPP gene expression in response to changed medium osmolality.

Although the GPP isoenzymes have redundant roles in osmoadaptation, the anaerobic growth defect of the gpp1Δ mutant (Fig. 2) shows that full adaptation to anaerobic conditions is dependent on the GPP1 gene. This is consistent with the observations that the weakly expressed GPP2 gene does not respond to anaerobic conditions and is responsible for only a small fraction of the total glycerol 3-phosphatase activity. Following a shift to anaerobic conditions, the gpp1Δ/gpp2Δ mutant increases its G3P level to about 17 mM (calculated from data of Fig. 7A), approximately a 15-fold increase as compared with wild type levels. Considering that Gpp2p has a Km value for G3P at 3.9 mM (11), the elevated G3P concentration will substantially enhance the catalytic activity of the Gpp2p isoenzyme, which might explain the surprising finding that the gpp1Δ mutant maintains wild type glycerol production (Fig. 3A), despite its low phosphatase content. The observed anaerobic growth defect might therefore be associated with the accumulation of G3P rather than result from poor NADH reoxidation. In fact, the massive NADH-driven anaerobic buildup of G3P in the gpp1Δ/gpp2Δ mutant seems to be the prime reason for the anaerobic growth arrest of this mutant. This notion is supported by the observation that acetaldehyde addition decreases the G3P accumulation of the double mutant and alleviates its growth inhibition (Fig. 7B), whereas addition of acetoin, which, similar to acetaldehyde, prevents excessive NADH accumulation (7), causes no reduction of the G3P pool, and does not relieve the anaerobic growth arrest (data not shown). Hence, accumulation of G3P rather than NADH seems responsible for the anaerobic growth inhibition of the double mutant.

Glycerol 3-Phosphatase Is Not Rate-limiting in the Glycerol Metabolism—In wild type cells the G3P phosphatase did not appear to limit the flux to glycerol under basal growth conditions, because overproduction of either of the GPP isozymes did not significantly enhance glycerol production. However, because several pathways compete for G3P the cell has to coordinate the flux from G3P to glycerol with that to glycerolipids (49, 50) and the G3P shuttle (Fig. 11). Although the flux to glycerolipids is probably low (cf. ref. 51), the G3P shuttle effi-
Fig. 10. A, tolerance to oxidative stress conditions of wild type (W303-1A) cells and the gpp1Δ, gpp2Δ, gpp1Δgpp2Δ, gpd1Δgpd2Δ mutants spotted in serial 10-fold dilutions onto YPD medium containing 0.57 mg/ml paraquat. Plates were scored after 5 days of incubation. B, GPP2 mRNA levels following exposure to paraquat in the W303-1A strain and its iso-
genic msn2Δmsn4Δ mutant and the SP1 (TPK1 TPK2 TPK3) strain and its iso-
genic S7-7A (TPK1 tpk2Δ tpk3Δ) and S18-
1DA (tpk1Δ tpk2Δ tpk3Δ) mutants. Ex-
periments were initiated with cells that were grown to A_{490} = 0.8–1.0 and diluted to half the original cell density with fresh YNB medium containing paraquat to give a final concentration of 0.16 mg/ml. IPP1 mRNA served as internal control. The graphs show quantified GPP2 mRNA lev-
els relative to those of IPP1 for the different strains, and the highest level for each set of experiments was adjusted to 1.0.

Fig. 11. Scheme of the pathways involved in glycerol metabo-
lism in S. cerevisiae.

ciently competes for G3P in cells grown on nonfermentable carbon sources. Cells cultured on ethanol induce Gut2p activity due to derepression of GUT2 (38, 39), and do not produce significant amounts of glycerol (40). Thus, the derepressed cells direct the reducing equivalents to the respiratory chain rather than having them sacrificed on glycerol, which appears to be the rationale behind the diminished GPP1 expression in cells cultured on ethanol. The cAMP-PKA pathway may contribute to this control, because cells cultured on ethanol have low cAMP levels (52), and diminished PKA activity decreases GPP1 expression (Fig. 4). A requirement for low GPP1 expression in cells growing on nonfermentable substrates may explain why GPP1 is regulated oppositely to most of the PKA target genes studied, which being involved in stationary phase functions or stress responses, become transcriptionally activated by de-
creased PKA activity (30, 31). However, when exposed to hyperosmotic stress, cells cultured on ethanol increase expression of both GPP1 and GPP2 and this induction appears fully suf-
ficient for an appropriate osmoregulatory glycerol response, because overexpressing either the GPP1 or GPP2 gene does not further enhance osmotolerance. It thus appears that glycerol-
producing wild type cells, despite the complex metabolic re-
quirements, maintain the phosphatase activity at levels giving this reaction little rate control in the glycerol pathway.

A Role for the Glycerol Metabolism in Oxidative Stress Pro-
tection—Wong and colleagues (53) observed increased sensitiv-
ity to reactive oxygen species of a S. cerevisiae mutant, having defective glycerol production (9). They also noted that sensitivity to oxidative stress was reversed in mutants engineered to produce mannitol, a polyol that is reported to protect phospho-
ribulokinase against inactivation by hydroxyl radicals in iso-
lated thylakoids (54). Consistent with these findings we ob-
served that mutants with completely blocked glycerol production display increased sensitivity to the superoxide-pro-
ducing agent paraquat. Furthermore, we noted that oxidative conditions generated by either paraquat (Fig. 10B) or 0.2 mM H$_2$O$_2$ (data not shown) stimulated GPP2 gene expression. Al-
though glycerol has been shown to serve as an efficient hy-
droxyl radical scavenger in vitro (41), other protective mecha-
nisms are equally plausible. It was recently pointed out that glycerol metabolism might serve to generate NADPH (20, 55), an important electron donor for several defense systems against oxidative stress (56). A transhydrogenase function con-
verting NADH to NADPH (cf. Fig. 11) was suggested to result from recycling of glycerol to dihydroxyacetone phosphate via two recently identified enzymes, a putative NADPH-dependent glycerol dehydrogenase and a dihydroxyacetone kinase en-
coded by the isogenes DAK1 and DAK2 (20). Using a two-
dimensional PAGE gel approach Godon et al. (55) examined changes in protein expression during the adaptive response of S. cerevisiae to H$_2$O$_2$ stress and noted, among other changes, an increased relative amount of isozymes for each step in the suggested glycerol cycle. In S. cerevisiae the transcription fac-
tors Yap1p (57, 58) and Skn7p (59, 60) are specifically involved in the oxidative stress response. A recent two-dimensional PAGE analysis detected, however, only DAK1 expression was Yap1p- and Skn7p-dependent among the genes encoding gly-
cerol-metabolizing enzymes. Furthermore, we observed that GPP2 induction in response oxidative stress was largely inde-
pendent of the transcriptional factors Msn2p and Msn4p and PKA activity (Fig. 10B), suggesting control by as yet unidenti-
fied factors.

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The Yeast Glycerol 3-Phosphatases Gpp1p and Gpp2p Are Required for Glycerol Biosynthesis and Differentially Involved in the Cellular Responses to Osmotic, Anaerobic, and Oxidative Stress

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