NADP-Glutamate Dehydrogenase Isoenzymes of *Saccharomyces cerevisiae*

PURIFICATION, KINETIC PROPERTIES, AND PHYSIOLOGICAL ROLES*

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In the yeast *Saccharomyces cerevisiae*, two NADP∗-dependent glutamate dehydrogenases (NADP-GDHs) encoded by *GDH1* and *GDH3* catalyze the synthesis of glutamate from ammonium and α-ketoglutarate. The *GDH2*-encoded NAD∗-dependent glutamate dehydrogenase degrades glutamate producing ammonium and α-ketoglutarate. Until very recently, it was considered that only one biosynthetic NADP-GDH was present in *S. cerevisiae*. This fact hindered understanding the physiological role of each isoenzyme and the mechanisms involved in α-ketoglutarate channeling for glutamate biosynthesis. In this study, we purified and characterized the *GDH1*- and *GDH3*-encoded NADP-GDHs; they showed different allosteric properties and rates of α-ketoglutarate utilization. Analysis of the relative levels of these proteins revealed that the expression of *GDH1* and *GDH3* is differentially regulated and depends on the nature of the carbon source. Moreover, the physiological study of mutants lacking or overexpressing *GDH1* or *GDH3* suggested that these genes play nonredundant physiological roles. Our results indicate that the coordinated regulation of *GDH1*, *GDH3*, and *GDH2*-encoded enzymes results in glutamate biosynthesis and balanced utilization of α-ketoglutarate under fermentative and respiratory conditions. The possible relevance of the duplicated NADP-GDH pathway in the adaptation to facultative metabolism is discussed.

Like most free living microorganisms, the yeast *Saccharomyces cerevisiae* possesses amino acid biosynthetic pathways that allow the cell to use ammonium as sole nitrogen source. Ammonium utilization occurs exclusively via its incorporation into glutamate and glutamine (1), a process that can be achieved by two metabolic routes. One of them is constituted by the concerted action of glutamine synthetase and the *Glt1*-dependent glutamate synthase. The other route is mediated by the NADP∗-dependent glutamate dehydrogenase (NADP-GDH)† (EC 1.4.1.4), a broadly distributed enzyme that catalyzes the reductive amination of α-ketoglutarate to form glutamate (4, 5). In *S. cerevisiae*, two genes (*GDH1* and *GDH3*) have been described whose products constitute NADP-GDH isoenzymes (6). Glutamate catabolism is achieved through a reaction catalyzed by a different but related enzyme, the *GDH2*-encoded NAD∗-dependent glutamate dehydrogenase (NAD-GDH) (EC 1.4.1.2), which determines glutamate degradation to ammonium and α-ketoglutarate (4, 7, 8).

*S. cerevisiae* is the first microorganism described in which the NADP-GDH activity is encoded by two genes (6); the physiological significance of this apparent redundancy is not clear. When this yeast is grown on glucose and ammonium as carbon and nitrogen sources, Gdh1p is the primary pathway for glutamate biosynthesis (6, 9, 10). It has also been shown that *GDH1* expression is regulated by the *HAP* system (11), which is known to control expression of genes involved in carbon metabolism and respiratory function (12). Null *gdh3Δ* mutants show no evident growth phenotype on glucose, and *GDH3*-dependent activity is negligible on this carbon source. Nevertheless, a biosynthetic role was established for *GDH3* in a double *gdh1Δ glt1Δ* mutant that grows on ammonium sulfate as sole nitrogen source by means of Gdh3p (6). Moreover, global analysis of transcription suggests that *GDH3* expression is influenced by the general nitrogen control system (13).

*S. cerevisiae* is able to grow using a variety of carbon sources under fermentative and respiratory conditions. This fact has stimulated discussion as to which specific mechanism allows α-ketoglutarate utilization for glutamate biosynthesis without impairing the integrity of the tricarboxylic acid cycle as an energy-providing system. In this regard, it has been shown that *Klebsiella aerogenes* strains overexpressing their *gdhA* gene coding for the biosynthetic NADP-GDH display an auxotrophy that is interpreted as a limitation for α-ketoglutarate and succinyl-coenzyme A (14). Accordingly, α-ketoglutarate modulates NADP-GDH activity so that fluctuations in the intracellular levels of tricarboxylic acid cycle intermediates would regulate glutamate biosynthesis. Indeed, it has been shown that the signal that coordinates regulates carbon and nitrogen metabolism in *Escherichia coli* depends on the intracellular levels of α-ketoglutarate and glutamine (15). Interestingly, the presence of Gdh3p has been found to be increased during diauxic transition in *S. cerevisiae* (16), suggesting a particular role of this enzyme in respiratory metabolism.

To understand the function of the duplicated NADP-GDH pathway present in *S. cerevisiae*, we purified both isoenzymes and studied their biochemical properties. Our results revealed...
that Gdh1p and Gdh3p have different allosteric properties and rates of α-ketoglutarate utilization. The construction of chimerical plasmids harboring combinations of the GDH1 and GDH3 promoter and coding regions allowed us to determine that expression of these two genes is differentially modulated by the carbon source. Finally, physiological analysis of mutants lacking or overexpressing GDH1 or GDH3 showed that expression of both genes is required to achieve wild-type growth on ethanol. Our results indicate that existence of different NADP-GDH isoenzymes allows the functioning of a regulatory system in which the relative abundance of each isoform modulates the rate at which α-ketoglutarate is channeled to glutamate biosynthesis.

EXPERIMENTAL PROCEDURES

Strains

Table I describes the characteristics of the strains used in the present work. All strains constructed for this study were LEU2 derivatives of CLA1 (ura3 leu2) and thus suited for URA3 selection. To obtain a gdh3Δ mutant, CLA1 was transformed with the BglII-linearized plasmid pLV6 (6) harboring a 760-bp GDH3 fragment and the yeast LEU2 gene, generating strain CLA12 (GDH1 gdh3Δ::LEU2 ura3). A gdh1Δ gdh3Δ ura3 mutant was obtained from CLA12, using the PCR-based gene replacement protocol described by Wach et al. (17), with kanMX4 as a marker. Two deoxyoligonucleotides were designed based on the GDH1 nucleotide sequence and that of the multiple cloning site present in the pFA6a vector (17). The deoxyoligonucleotide D1 (5’-CAG AAT TTC AAC AAG CTG AGT GTC TTG ACC AAT GTG A-3’) was used to create a 522-bp fragment. The deoxyoligonucleotide D2 (5’-AAC ACC GAT TGC CTT ACG AAG AAG TTG TCT CCT CTT TGG AAG C-3’) was used to create a 2596-bp fragment by PCR amplification in a Stratagene Robocycler 40 with the following program: one denaturing cycle for 3-min at 94 °C, followed by 26 cycles of 30-s denaturation at 94 °C, 1-min annealing at 50 °C, and 1-min extension at 72 °C. The 522-bp PCR product obtained was gel-purified and used to transform strain CLA12, generating strain CLA14. A CLA1 LEU2 derivative was obtained by transforming this strain with plasmid YIp351, generating strain CLA11. To obtain a gdh1Δ gdh3Δ ura3 mutant, the CLA11 strain was transformed with a 762-bp PCR product, then generating CLA13.

Yeast was transformed by the method described by Ito et al. (18). Transformants were selected for either leucine prototrophy on minimal medium (MM), or G418 resistance (200 mg/liter) (Life Technologies, Inc.) as a marker.

Growth Conditions

Strains were routinely grown on MM containing salts, trace elements, and vitamins following the formula of yeast nitrogen base (Difco). Filter-sterilized glucose (2%, w/v) or ethanol (2%, w/v) was used as a carbon source, and 40 mM ammonium sulfate was used as a nitrogen source. Supplements needed to satisfy auxotrophic requirements were added at 0.1 mg/ml. Cells were incubated at 30 °C with shaking (250 rpm).

Construction of Low Copy Number and High Copy Number Plasmids Bearing GDH1 or GDH3 Genes

All standard molecular biology techniques were followed as previously described (19). GDH1 or GDH3 were PCR-amplified together with their 5’ promoter sequence and cloned into either the pRS316 (CEN6 ARSH4 URA3) low copy number or pRS426 (2µ or URA3) high copy number yeast shuttle vectors (20, 21). For GDH1, the 2596-bp region between +952 from the start codon and +295 from the stop codon was considered to comprise the full GDH1 promoter and coding sequences (11). For GDH3, a 2646-bp fragment was PCR-amplified, containing the putative regulatory region (−1213 from the start codon) plus the full coding sequence and +48 from the end codon, as reported in the nucleotide sequence of chromosome I from S. cerevisiae (22). Deoxyoligonucleotides used for this purpose were S1 (5’-CGC GGG ATC TAG TAG TTC AGC GAC AGA AG-3’), S2 (5’-CCG GGC GAT CCC GAG TAA GGT CAT CAA TAA G-3’), S3 (5’-CCG GGC CTC CCG CTA TAT GAT CTT C-3’), and S4 (5’-CCG GGC CTC ACT ACA TAC ACA GAT AG-3’), generating plasmids pLM1 (GDH1 CEN URA3), pLM11 (GDH1 2µ URA3), pLM2 (GDH3 CEN URA3), and pLM22 (GDH3 2µ URA3). DNA sequencing was carried out, using the T3/T7 priming sites of pRS316 and pRS426, at the Unidad de Biologia Molec-
Yeast NADP-GLUTAMATE DEHYDROGENASE ISOENZYMES

\[ \text{Reaction: } \text{GluA+H}^+ + \text{NADP}^+ \rightarrow \text{GluB} + \text{NADPH} + \text{H}^+ \]

**Fig. 2. Comparative kinetic analysis of two NADP-GDH isoenzymes.** Plots show the dependence of the relative rate of the reductive amination reaction on the concentration of \( \alpha \)-ketoglutarate (A), NADPH (B), and ammonium (C). Reactions were carried out in 100 mM Tris buffer (pH 7.2) at 25 °C (see “Experimental Procedures”). ■ Gdh1p enzyme; ● Gdh3p enzyme. Insets represent double reciprocal plots.

- **Construction of GDH1 and GDH3 Chimerical Fusion Plasmids**
  - Fusions containing either the GDH1 promoter and the GDH3 coding sequence or the GDH3 promoter and the GDH1 coding sequence were generated by overlapping PCR amplification. For this purpose, primers S1 and S5 (5'-GAA ATT CTG GCT GCT GGC TTT TTG CAT TCC TTC TTT G-3') were used to obtain a 980-bp product corresponding to the GDH1 promoter and the first 17 bp of the GDH3 coding sequence (in boldface type), and overlapped with the 1632-bp product of primers S3 and S4 (5'-GAC AAG CGA ACC AGA GTT TC-3') were used to obtain a 1244-bp product corresponding to the GDH3 5' cognate sequence, together with the first 17 bp of the GDH1 coding sequence (in boldface type), and overlapped with the 1832-bp product of primers S3 and S10 (5'-GCT AGA GGC AGA ATT TCA AC-3') were used to obtain a 1244-bp product corresponding to the GDH3 5' cognate sequence, together with the first 17 bp of the GDH1 coding sequence (in boldface type), and overlapped with the 1832-bp product of primers S3 and S10 (5'-GCT AGA GGC AGA ATT TCA AC-3') which included the complete GDH1 coding sequence. Similarly, primers S2 and S9 (5'-GAA ATT CTG GCT GCT GGC TTT TTG CAT TCC TTC TTT G-3') were used to obtain a 1244-bp product corresponding to the GDH3 5' cognate sequence, together with the first 17 bp of the GDH1 coding sequence (in boldface type), and overlapped with the 1832-bp product of primers S3 and S10 (5'-GCT AGA GGC AGA ATT TCA AC-3') which included the complete GDH1 coding sequence. The whole procedure led to the generation of the following plasmids: pLAM3 (5'-GDH3-GDH1 CEN URA3), pLAM3 (5'-GDH3-GDH1 CEN URA3), pLAM4 (5'-GDH3-GDH1 CEN URA3), and pLAM44 (5'-GDH1-GDH3 2α URA3). Constructs were verified by DNA sequencing as described above.

- **NADP-GDH Purification**
  - NADP-GDH activity was purified from ethanol-grown cultures of CLA 14-11 (gdh1 Δ gdh3Δ pLAM11 (GDH1 2μ URA3)), CLA 14-22 (gdh1 Δ gdh3Δ pLAM22 (GDH3 2μ URA3)), and the CLA4 wild-type strain. Strains were grown in 10 liters of MM supplemented with ethanol and ammonium sulfate, in a fermentor at the Unidad de Escalamiento, Instituto de Investigaciones Biomedicas, UNAM. Cultures were incubated at 30 °C and 200 rpm and aerated with 7 liters of oxygen/min. Cells were harvested at an optical density of 0.8–1.0 at 600 nm and stored at −70 °C until used. NADP-GDH was purified by a modified version of the method of Doherty (23). All steps were carried out at 5 °C.

**Fig. 2, A**

**Fig. 2, B**

**Fig. 2, C**

- **Step 1: Whole Cell Soluble Protein Extract**—Cells were thawed and resuspended in 1 ml of buffer A (100 mM Tris (pH 7.5), 1 mM EDTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 50 μg of N\textsuperscript{−}-p-tosyl-l-lysine chloromethyl ketone (TLCK)/ml)/g of cells. Crude extracts were obtained after mechanical disruption of cells with a Bead-Beater (8 cycles of 1 min). After centrifugation at 30,000 × g for 30 min, protein extracts were resuspended in buffer A and diluted to 25 mg/ml.

**Step 2: Ammonium Sulfate Fractionation**—Proteins that precipitated between 40 and 65% saturation of ammonium sulfate were resuspended in buffer A. Mixtures were dialyzed twice against 4 liters of buffer B (20 mM Tris (pH 7.5), 1 mM EDTA).

**Step 3: DEAE Bio-Gel A Chromatography**—Dialyzed fractions were applied to a DEAE Bio-Gel A column (23 by 2.8 cm) equilibrated with buffer C (20 mM Tris (pH 7.5), 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 50 mg of TLCK/liter). After sample application, the column was washed with 5 column volumes of buffer B. NADP-GDH was subsequently eluted with a linear NaCl gradient of 10 column volumes (0–0.5 M). Fractions with NADP-GDH activity were pooled and dialyzed against 4 liters of buffer B.

**Step 4: Affinity Chromatography**—A Reactive Red-agarose column (13 by 1.2 cm) was equilibrated with buffer A. After application of the sample from the previous step, the column was washed with 10 volumes of buffer A. NADP-GDH was eluted with buffer A containing 0.1 mM NADPH. Fractions with NADP-GDH activity were pooled, dialyzed against buffer B, concentrated by ultrafiltration to ~1 mg/ml with an Amicon YM30 membrane, and stored at −70 °C until used.

- **Preparation of Anti-NADP-GDH Antibodies**
  - Antibodies were raised in rabbits injected with purified yeast GDH1-encoded NADP-GDH and partially purified by ammonium sulfate precipitation according to the method of Gonzalez-Halphen et al. (26).

- **Electrophoresis and Immunoblotting**
  - SDS-polycrylamide gel electrophoresis (PAGE) and native PAGE were performed with 10 and 6% slab gels, respectively. Proteins on polyacrylamide gels were visualized with Coomasie Blue. Immunoblot analysis of SDS-electrophoresed crude extract or pure NADP-GDH was carried out as described by Towbin et al. (27). Immunoblot signaling was optimized by analyzing a number of combinations of antigen and antibody concentrations in the linear range of detectability. Scanned blots were subjected to densitometric analysis using the program ImageQuant 4.2 (Molecular Dynamics, Inc., Sunnyvale, CA). Data were
reaction, respectively. For experiments in which pH was varied, 25 mM pH 7.2 or 8.0 for the reductive amination or oxidative deamination.

150 mM NaCl, and 1 mM dithiothreitol. The column was calibrated with the same as those shown in Fig. 1

°C, wild-type NADP-GDH; /c141°, Gdh1p enzyme; /c141/Œ, Gdh3p enzyme; f, wild-type NADP-GDH; v, Gdh1p plus Gdh3p (3:1 mixture).

normalized to the immunoblot signals of the corresponding purified protein.

Molecular Mass Determination

Native molecular mass was determined on a Sephacryl S-300 gel filtration column (2.6 by 90 cm) equilibrated with 50 mM Tris (pH 7.5), 150 mM NaCl, and 1 mM dithiothreitol. The column was calibrated with molecular mass standards (29–700 kDa) from Sigma. Purified NADP-GDH was diluted in the same buffer, loaded into the column, and eluted at a rate of 6 ml/h. Molecular mass was determined from a plot of the log molecular mass against elution volume per void volume.

The apparent molecular masses of denatured subunits were determined by SDS-PAGE with molecular mass standards (29–205 kDa) from Sigma.

Amino-terminal Sequencing

The isolation of polypeptides for amino-terminal sequencing was carried out as described previously (28). Edman degradation was carried on an Applied Biosystems Sequencer at the Laboratoire de Microsequençage des Protéines (Institut Pasteur, Paris, France).

Enzyme Kinetics and Analysis of Kinetic Data

NADP-GDH activity was assayed for the reductive amination reaction at different concentrations of α-ketoglutarate, NADPH, or ammonium chloride and at saturating concentrations of the remaining substrates (8 mM α-ketoglutarate, 200 μM NADPH, and 50 mM ammonium chloride). For the oxidative deamination reaction, different concentrations of glutamate or NADP+ and saturating concentration of the remaining substrate (100 mM glutamate and 300 μM NADP+) were used. The progress of the reaction was always kept below 5% conversion of the initial substrate. Measurements were made at 25 °C in 100 mM Tris at pH 7.2 or 8.0 for the reductive amination or oxidative deamination reaction, respectively. For experiments in which pH was varied, 25 mM acetic acid, 25 mM MES, 50 mM Tris was used as buffer. This buffer

minimizes the change of ionic strength with pH (29). Kinetic data were analyzed by nonlinear regression using the program Origin 4.1 (MicroCal Software, Inc.).

Extraction and Determination of Intracellular α-Ketoglutarate

Protein-free cell extracts were prepared as described by Kang et al. (30). The intracellular concentration of α-ketoglutarate relative to protein concentration was determined with beef glutamate dehydrogenase (Sigma) by following NADH oxidation (31).

Determination of Extracellular Glucose Concentration

Cells were filtered through 0.22-μm Millipore membranes. Extracellular glucose concentration was determined in the filtrate with the Glucose [HK] kit from Sigma.

RESULTS

NADP-GDH Purification from Mutant and Wild-type Strains—S. cerevisiae is the first microorganism in which the existence of two NADP-GDH isoenzymes has been reported (6). Although yeast NADP-GDH has been previously purified and characterized (32), the properties described could be ascribed to either or both isoenzymes. Therefore, we purified the Gdh1p and Gdh3p enzymes to electrophoretic homogeneity to study their individual biochemical properties. Gdh1p was 36-fold purified from the CLA14-11 mutant strain harboring plasmid pLAM11, whereas Gdh3p was 49-fold purified from strain CLA14-22 bearing plasmid pLAM22. Additionally, NADP-GDH was 252-fold purified from the wild-type strain CLA4 grown on ethanol, a condition in which both isoenzymes are readily expressed (see below). Apparent molecular masses of the monomers were 51 and 46 kDa for Gdh1p and Gdh3p, respectively (Fig. 1A). The observed molecular mass of the latter was at variance with the expected value deduced from its amino acid sequence, which is 49.6 kDa. This suggested the existence of a post-translational modification of Gdh3p, which remains to be identified. Amino-terminal sequencing was not possible, because both Gdh1p and Gdh3p purified polypeptides were blocked.

The active oligomeric structures of the purified samples obtained from the wild-type and mutant strains were hexameric, as revealed by gel filtration experiments (data not shown). This is in agreement with results obtained for all members of the small glutamate dehydrogenase subfamily, which show an α5 50-kDa oligomeric structure (33) and whose three-dimensional crystal structure has been reported (34, 35). Native PAGE
Yeast NADP-Glutamate Dehydrogenase Isoenzymes

Clairenl
The time at which glucose was exhausted from the medium.

Rival in all experiments (130–131). Both isoenzymes showed hyperbolic behavior at increasing concentrations of the two remaining substrates (Fig. 2).

The abundance of each isoenzyme relative to the total NADP-GDH of the wild-type strain was calculated from the normalized densitometric analysis of immunoblot signals obtained for electrophoresed protein extracts of strain CLA4. Black bars, Gdh1p; white bars, Gdh3p.

Figure 5. Relative levels of Gdh1p and Gdh3p change depending on growth phase. A, yeast were cultured by extended growth in YPD-rich medium. Aliquots were withdrawn at different times, and protein extracts were assayed for NADP-GDH activity. Extracellular glucose concentration was determined in parallel; a dark arrow indicates the time at which glucose was exhausted from the medium. , CLA7 (gdh3Δ); , CLA6 (gdh1A); , CLA4 (wild type). B, the abundance of each isoenzyme relative to the total NADP-GDH of the wild-type strain was calculated from the normalized densitometric analysis of immunoblot signals obtained for electrophoresed protein extracts of strain CLA4. Black bars, Gdh1p; white bars, Gdh3p.

Analysis of the protein purified from the wild-type strain showed a smeared pattern (Fig. 1B, lane 3), compared with the sharp bands observed when a mixture of equivalent amounts of purified homomeric proteins was electrophoresed (Fig. 1B, lane 4). Hence, the enzyme purified from the wild-type strain was most probably a natural mixture of several isoforms built up by the oligomerization of the two different monomers encoded by GDH1 and GDH3. In SDS-PAGE electrophoresis, the NADP-GDH purified from the wild-type strain grown on ethanol showed two bands corresponding to Gdh1p and Gdh3p monomers (Fig. 1A). Densitometric analysis of Coomassie-stained gels revealed that 73% of the total NADP-GDH was composed of Gdh1p; the remaining 27% corresponded to Gdh3p.

Kinetic Analysis of NADP-GDH Isoenzymes—Enzymological properties were separately determined for the Gdh1p and Gdh3p homomeric NADP-GDHs. Activities were measured in Tris-MES-acetic acid buffer at pH values ranging from 4.5 to 9.5 (data not shown); maximum activity was obtained at pH 6.8 for both enzymes. We examined the dependence of NADP-GDH activity on α-ketoglutarate, NADPH, or ammonium, using saturating concentrations of the two remaining substrates (Fig. 2). Both isoenzymes showed hyperbolic behavior at increasing NADPH and ammonium concentrations but sigmoidal responses to increasing α-ketoglutarate concentrations (Fig. 2A).

NADP-GDH isoenzymes showed \( V_{\text{max}} \) values that were similar in all experiments (130–135 units mg\(^{-1}\)). All substrates caused inhibition of enzyme activity above a given threshold concentration (data not shown). NADPH began to inhibit the activity of both enzymes at a concentration of 300 \( \mu \text{M} \) (10% inhibition); with 100 \( \text{mM} \) ammonium chloride, we observed a similar effect. A 5% inhibition of the maximal activity was observed with 10 \( \text{mM} \) α-ketoglutarate for the Gdh1p enzyme, whereas a 25 \( \text{mM} \) substrate concentration was needed to generate the same inhibition of the Gdh3p enzyme.

For the Gdh1p enzyme assayed in both directions of the NADP-GDH reaction, the \( K_v \) values for NADPH, ammonium, NADP\(^+\), and glutamate were 11.3 \( \mu \text{M} \), 5.96 \( \mu \text{M} \), 14.1 \( \mu \text{M} \), and 9.79 \( \mu \text{M} \), respectively. Values of 33.1 \( \mu \text{M} \), 5.00 \( \mu \text{M} \), 10.5 \( \mu \text{M} \), and 6.36 \( \mu \text{M} \), respectively, were obtained for the Gdh3p isoenzyme. Phosphate competitive inhibition on NADPH binding has been previously described for yeast NADP-GDH (36). We confirmed that with respect to NADPH concentration, phosphate competitively inhibited both isoenzymes at various concentrations (0–250 \( \text{mM} \) sodium phosphate) (data not shown). However, Gdh3p was more sensitive to this effect, with a \( K_i \) value for phosphate of 9.3 \( \mu \text{M} \), compared with 72.5 \( \mu \text{M} \) for the Gdh1p enzyme.

Differences were also found between the two isoenzymes in their kinetics for α-ketoglutarate. At pH 7.2, substrate concentrations at which rates were equal to half the \( V_{\text{max}} \) (\( S_{0.5} \)) were 0.29 and 1.27 \( \mu \text{M} \) for the Gdh1p and Gdh3p enzyme, respectively. Hill coefficients (\( n_H \)) in the same experiments were 1.3 and 1.5 for the Gdh1p and Gdh3p enzyme, respectively. In this regard, hexameric glutamate dehydrogenases from other organisms are known to be allosteric enzymes activated by different molecules (AMP, ADP, GTP, ATP, NADP\(^+\), succinate, aspartate, and asparagine) (33, 37–39). The effect of these compounds was assayed for the yeast NADP-GDH isoenzymes, but none of them behaved as an allosteric effector (data not shown). However, sigmoidal kinetics could most likely reflect a phenomenon of cooperativity, since \( n_H \) values strictly depended on the pH at which the kinetics for α-ketoglutarate was assayed (Fig. 3A). The \( n_H \) plot for the Gdh3p isoenzyme against pH showed an inflection point at pH 6.2. Near optimum pH, Gdh3p exhibited a higher \( S_{0.5} \) value compared with its homologue; this difference was higher at low pH (Fig. 3B). Conversely, the Gdh1p isoenzyme showed no considerable changes in sigmoidicity and had higher affinity for α-ketoglutarate in terms of \( S_{0.5} \). Thus, the overall data indicate that the NADP-GDH isoenzymes differ in their allosteric properties and rates at which they use α-ketoglutarate.

We determined α-ketoglutarate kinetics for the NADP-GDH purified from the wild-type strain and compared them with those of the homomeric Gdh1p and Gdh3p isoenzymes. Since the maximum kinetic differences between the two isoenzymes were observed at pH 5.8, we analyzed the behavior of the wild-type enzyme at this pH. The wild-type enzyme exhibited kinetic parameters (\( S_{0.5}, 0.90 \mu\text{M}; n_H, 1.6 \)) similar to those of a preparation containing 75% Gdh1p and 25% Gdh3p homomeric isoenzymes (Fig. 3C). This indicates that kinetics toward α-ketoglutarate depend on the relative abundance of the GDH1- and GDH3-encoded monomers, whether or not these proteins associate in heteromeric structures.

Relative Levels of Gdh1p and Gdh3p Are Carbon-dependent—To compare the relative levels of the two NADP-GDHs under different conditions, extracts were prepared from the wild-type or the pertinent null mutant strains grown on glucose or ethanol as carbon sources. The specific activities and immunochemically detected levels of Gdh1p were similar in extracts obtained from the gdh3Δ strain grown on glucose or ethanol (Fig. 4, lane 3). For Gdh3p, low levels of NADP-GDH activity were observed, and no signal in immunoblots could be detected when glucose was the carbon source. However, when extracts were prepared from ethanol-grown cells, Gdh3p enzymatic activity increased 20-fold, and an immunoblot signal was clearly observed (Fig. 4, lane 2). Normalized densitometric

![Figure 5](http://www.jbc.org/)

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**Fig. 5. Relative levels of Gdh1p and Gdh3p change depending on growth phase.** A, yeast were cultured by extended growth in YPD-rich medium. Aliquots were withdrawn at different times, and protein extracts were assayed for NADP-GDH activity. Extracellular glucose concentration was determined in parallel; a dark arrow indicates the time at which glucose was exhausted from the medium. , CLA7 (gdh3Δ); , CLA6 (gdh1A); , CLA4 (wild type). B, the abundance of each isoenzyme relative to the total NADP-GDH of the wild-type strain was calculated from the normalized densitometric analysis of immunoblot signals obtained for electrophoresed protein extracts of strain CLA4. Black bars, Gdh1p; white bars, Gdh3p.
when this gene was under the regulation of the GDH1 promoter sequence (5′GDH1-GDH3) than when expressed from its cognate promoter. Similar results were obtained using cells harboring high copy number plasmids. When NADP-GDH activity was monitored in extracts obtained from ethanol-grown cells, high levels were observed for either Gdh1p or Gdh3p, regardless of which promoter fostered expression. These results confirmed that GDH3 expression was repressed by glucose at the transcriptional level.

It is worth mentioning that in extracts prepared from glucose-grown cultures, Gdh1p activity was at least 5-fold higher than that of Gdh3p when the genes were expressed from either promoter; this effect was barely observed in ethanol (Table II). Considering that V_\text{max} values are similar for both isoenzymes, this differential level of expression could be attributed to a post-transcriptional level of regulation. In fact, it could be considered that the codon bias difference of these genes (0.75 and 0.19 for GDH1 and GDH3, respectively) may account for different translation rates of their transcripts.

NADP-GDH Isoenzymes Modulate \(\alpha\)-Ketoglutarate Utilization for Glutamate Biosynthesis—Gdh1p enzyme is the primary pathway for glutamate biosynthesis in glucose-grown cells (6). Double \(gdh1\Delta gdh3\Delta\) mutants lacking NADP-GDH activity are not full glutamate auxotrophs; this strain grows with a 2-fold higher doubling time than that observed in the wild-type strain. This growth is achieved through the action of the GLT1-encoded glutamate synthase, which constitutes an ancillary pathway for glutamate biosynthesis (42). Gdh3p expressed from a high copy number plasmid conferred only a partial recovery of the slow growth phenotype of a \(gdh1\Delta gdh3\Delta\) strain (Table III), as expected from the observed repression of the GDH3 gene by glucose. Conversely, Gdh1p expressed from a high copy number plasmid completely restored wild-type growth to a \(gdh1\Delta gdh3\Delta\) strain.

It is relevant that in cells grown on ethanol, single disruptions of either GDH1 or GDH3 resulted in a slower growth with respect to the wild-type strain. Furthermore, the \(gdh1\Delta gdh3\Delta\) analysis of immunoblots showed that 25% of the wild-type NADP-GDH from ethanol-grown yeasts corresponded to Gdh3p.

In light of the previous results, it was relevant to determine whether NADP-GDHs containing different Gdh1p/Gdh3p ratios could be found in long term yeast cultures. In YPD-rich medium, \textit{S. cerevisiae} grows by fermentation; diauxic shift occurs after glucose is exhausted from the medium and cells adapt to respiratory metabolism using the ethanol produced during glucose fermentation (40). In fermentative growth, with glucose as the only carbon source, NADP-GDH activity was solely due to Gdh1p (Fig. 5, A and B). However, as cells proceeded through postdiauxic growth, different Gdh1p/Gdh3p ratios were observed, and after 5 days of incubation, 70% of the total NADP-GDH activity in the wild-type strain corresponded to Gdh3p (Fig. 5B). Within this context, it is relevant that NADP-GDH proteolysis has been observed after glucose starvation (41); this could account for the specific inactivation of Gdh1p after glucose was exhausted from the medium. Taken together, these results indicate that the relative abundance of Gdh1p or Gdh3p depends on the carbon source.

**GDH3 Expression Is Transcriptionally Regulated by the Nature of the Carbon Source**—To determine whether GDH3 carbon-dependent regulation was exerted at the transcriptional level, several recombinant plasmids were constructed (see “Experimental Procedures”). NADP-GDH activities were determined for strains derived from the CLA14 mutant strain transformed with these plasmids (Table II). Cells bearing low copy number constructs showed differences in enzymatic activity, which could be mainly attributed to the different levels of expression allowed by the cognate 5′ promoter sequences of either GDH1 or GDH3. In glucose-grown cells, Gdh1p-dependent NADP-GDH activity was 27-fold higher when expressed from its own promoter as compared with that fostered by the 5′GDH3-GDH1 fusion. Likewise, Gdh3p-dependent activity was 20-fold higher when this gene was under the regulation of the GDH1 promoter.

| Strain | Glucose | Ethanol |
|-------|---------|---------|
| CLA14-0 (gdh1Δ gdh3Δ/pRS316 (CEN URA3)) | <0.005 | <0.005 |
| CLA14-1 (gdh1Δ gdh3Δ/pLM1 (GDH1 CEN URA3)) | 3.26 ± 0.920 | 1.38 ± 0.999 |
| CLA14-2 (gdh1Δ gdh3Δ/pAM2 (GDH1 CEN URA3)) | 0.03 ± 0.007 | 0.51 ± 0.042 |
| CLA14-3 (gdh1Δ gdh3Δ/pAM3 (5′GDH1-GDH3 CEN URA3)) | 0.12 ± 0.015 | 0.97 ± 0.076 |
| CLA14-4 (gdh1Δ gdh3Δ/pAM4 (5′GDH1-GDH3 CEN URA3)) | 0.60 ± 0.066 | 0.62 ± 0.031 |
| CLA14-00 (gdh1Δ gdh3Δ/pRS426 (2µ URA3)) | <0.005 | <0.005 |
| CLA14-11 (gdh1Δ gdh3Δ/pLM11 (GDH1 2µ URA3)) | 9.01 ± 0.85 | 10.6 ± 1.02 |
| CLA14-22 (gdh1Δ gdh3Δ/pLM22 (GDH3 2µ URA3)) | 0.17 ± 0.013 | 8.65 ± 0.77 |
| CLA14-33 (gdh1Δ gdh3Δ/pLM33 (5′GDH1-GDH3 2µ URA3)) | 0.86 ± 0.069 | 11.4 ± 0.94 |
| CLA14-44 (gdh1Δ gdh3Δ/pLM44 (5′GDH1-GDH3 2µ URA3)) | 2.41 ± 0.311 | 9.90 ± 0.86 |

* Values are presented as means from three independent experiments ± S.D.

* Values are shown relative to doubling time of the wild-type strain (2.3 and 9.5 h on glucose and ethanol, respectively) and are presented as means from three independent experiments (variation was always ±10%).

Values are given in \(\mu\text{mol min}^{-1} \text{mg of protein}\) and are presented as means from three independent experiments (variation was always ±10%).

### Table II

**Yeast NADP-Glutamate Dehydrogenase Isoenzymes**

| Strain | Glucose | Ethanol |
|-------|---------|---------|
| CLA11-00 (GDH1 GDH3/pRS426 (2µ URA3)) | 100 | 100 |
| CLA12-00 (gdh1Δ GDH3/pRS426 (2µ URA3)) | 63 | 55 |
| CLA13-00 (gdh1Δ gdh3Δ/pRS426 (2µ URA3)) | 105 | 67 |
| CLA14-00 (gdh1Δ gdh3Δ/pRS426 (2µ URA3)) | 58 | 45 |
| CLA14-11 (gdh1Δ gdh3Δ/pLM11 (GDH1 2µ URA3)) | 92 | 51 |
| CLA14-12 (gdh1Δ gdh3Δ/pLM12 (GDH3 2µ URA3)) | 75 | 82 |

### Table III

| Strain | Relative growth * | NADP-GDH Specific activity a | NADP-GDH Specific activity a |
|-------|------------------|-----------------------------|-----------------------------|
|       | Glucose | Ethanol | Glucose | Ethanol | Glucose | Ethanol |
| CLA11-00 (GDH1 GDH3/pRS426 (2µ URA3)) | 0.884 | 0.718 | 0.038 | 0.055 |
| CLA12-00 (gdh1Δ GDH3/pRS426 (2µ URA3)) | 0.019 | 0.459 | 0.032 | 0.063 |
| CLA13-00 (gdh1Δ gdh3Δ/pRS426 (2µ URA3)) | 1.34 | 0.940 | 0.043 | 0.156 |
| CLA14-00 (gdh1Δ gdh3Δ/pRS426 (2µ URA3)) | <0.005 | <0.005 | 0.033 | 0.041 |
| CLA14-11 (gdh1Δ gdh3Δ/pLM11 (GDH1 2µ URA3)) | 10.5 | 8.98 | 0.098 | 0.160 |
| CLA14-12 (gdh1Δ gdh3Δ/pLM12 (GDH3 2µ URA3)) | 0.294 | 7.87 | 0.035 | 0.123 |
double mutant strain overexpressing GDH1 from a plasmid grew considerably slower on ethanol than the one bearing the GDH3 high copy number construct. Thus, it can be concluded that wild-type growth on ethanol depends on both Gdh1p and Gdh3p and that overexpression of GDH1 could result in a deleterious effect.

Because of the differences in the rates of α-ketoglutarate in vitro utilization by the NADP-GDH isoenzymes, we explored if these differences could be observed in vivo. To this end, we measured α-ketoglutarate intracellular pools in cells lacking or overexpressing GDH1 or GDH3. We also determined the NAD-GDH-specific activities in the various strains, since this catalytic enzyme would be expected to increase α-ketoglutarate concentration. In yeast cells grown on glucose, the only evident phenotype was due to the lack of GDH1; either single (gdh1Δ) or double (gdh1Δ gdh3Δ) mutants exhibited a significant accumulation of α-ketoglutarate. A lack of GDH3 did not affect either the intracellular concentration of this intermediate or NAD-GDH activity (Fig. 6A, Table III). These results are in consonance with the growth phenotypes observed for the same strains on glucose. When GDH1 was overexpressed, NAD-GDH activity exhibited a 2-fold increase, suggesting that this activity increased as a result of glutamate accumulation (3, 43). As expected, GDH3 overexpression did not result in increased NAD-GDH activity (Table III).

When grown on ethanol, the single gdh1Δ mutant did not show a net increase in α-ketoglutarate concentration, whereas the gdh3Δ single mutant exhibited a 2-fold lower α-ketoglutarate pool size as compared with that of the wild-type strain. However, the gdh1Δ gdh3Δ strain had α-ketoglutarate levels similar to those found in the gdh1Δ mutant; this suggested that the α-ketoglutarate depletion observed in a gdh3Δ mutant was due to a Gdh1p-dependent consumption of this compound in the absence of the Gdh3p enzyme (Fig. 6B). These results are in agreement with the fact that Gdh1p enzyme has a higher rate of α-ketoglutarate utilization than the heteromeric enzyme that exists in ethanol-grown cells. Moreover, NAD-GDH specific activity was induced 3-fold in ethanol-grown cells lacking the Gdh3p enzyme (Table III), indicating that under this condition glutamate accumulated, resulting in induced GDH2 expression (43).

Overexpression of either GDH1 or GDH3 in ethanol-grown cells caused an increase in the specific activity of NAD-GDH. However, the effect on α-ketoglutarate concentration was contrasting. Cells overexpressing GDH1 showed a reduced α-ketoglutarate pool size, whereas GDH3 high copy number expres-
sion caused its accumulation. Thus, it can be concluded that GDH1 overexpression causes a drain of the intracellular α-ketoglutarate pool, suggesting that in vivo Gdh1p uses this compound at a higher rate than Gdh3p.

**DISCUSSION**

This study addresses the question of whether GDH1 and GDH3 play overlapping or distinct roles and whether these roles are involved in the inherent capacity of *S. cerevisiae* to grow under fermentative or respiratory conditions. The results presented in this paper indicate that the existence of different NADP-GDH isoforms results in glutamate biosynthesis and balanced α-ketoglutarate utilization. The main observations that support this assertion are the following: (a) NADP-GDHs showed differences in their allosteric properties and rates of α-ketoglutarate utilization; (b) the relative abundance of both isoenzymes depended on the nature of the carbon source; (c) a gdh3Δ mutant grew slowly on ethanol, although it had wild-type NADP-GDH activity levels (this mutant showed reduced α-ketoglutarate pools and high activity levels of the catabolic NAD-GDH, indicating an abnormal high glutamate production rate); and (d) GDH1 overexpression from a plasmid did not suppress slow growth or the reduced α-ketoglutarate pool phenotypes of a gdh1Δ gdh3Δ strain; in contrast, overexpression of GDH3 resulted in faster growth and α-ketoglutarate accumulation.

It has been recently shown that the regulated expression of yeast tricarboxylic acid cycle genes is governed by two transcriptional complexes that function alternatively, depending on the integrity of the respiratory function (44). The HAP system regulates the expression of genes that lead to the synthesis of α-ketoglutarate during respiratory metabolism (12), whereas expression of these genes is controlled by the RTG system when respiratory function is dampened or lost. This model considers that glutamate plays a central role by repressing respiratory function is dampened or lost. This model considers that glutamate plays a central role by repressing

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