JYI

RESEARCH

Extramitochondrial localization of glutamate dehydrogenase isozymes in *Saccharomyces cerevisiae*

Max C. Petersen[1] and Pamela J. Trotter, Ph.D.[1, 2]*

ABSTRACT

The metabolic process of glutamate synthesis in *Saccharomyces cerevisiae* is governed by a functional model that assumes extramitochondrial localization of glutamate dehydrogenase (GDH). In *S. cerevisiae*, GDH exists as three isozymes: Gdh1p and Gdh3p, which are NADP-dependent, and Gdh2p, which is NAD-dependent. Previous work has suggested nuclear and/or cytosolic localization, but definitive data are lacking. We attempted to determine the subcellular localization of the three GDH isozymes by enzymatic activity. Crude mitochondrial and extramitochondrial fractions were isolated from wild-type and mutant (*gdh3Δ*) cells by subcellular fractionation. Spectrophotometric measurement of NADP-GDH and NAD-GDH activity suggested extramitochondrial localization for Gdh1p and Gdh2p. Additionally, NADP-GDH activity was lower in *gdh3Δ* extramitochondrial fractions as compared to wild-type. However, this trend was not observed in mitochondrial fractions, suggesting extramitochondrial localization for Gdh3p. Since mammalian GDH is primarily mitochondrial, our findings have implications for the use of *S. cerevisiae* in modeling glutamate metabolism.

INTRODUCTION

The primary *in vivo* pathway for glutamate biosynthesis in the budding yeast *Saccharomyces cerevisiae* uses glutamate dehydrogenase (GDH) to catalyze the assimilation of ammonia into the amino group of glutamate (Magasanik, 2003) (Figure 1). This reversible reaction is catalyzed by three isozymes: Gdh1p, Gdh2p, and Gdh3p. NADP-dependent GDHs (Gdh1p & Gdh3p) catalyze the synthesis of glutamate, while NAD-dependent GDH (Gdh2p) catalyzes its reductive deamination (DeLuna et al., 2001). Either Gdh1p or Gdh3p can be the dominant source of NADP-GDH activity, depending on the carbon source. Gdh1p predominates during fermentation (e.g. glucose as carbon source), while Gdh3p predominates during respiratory metabolism (e.g. ethanol as carbon source) (Fraenkel, 1982; DeLuna et al., 2001). GDH activity is also affected by growth phase. NADP-dependent GDH activity shows significant increases during early-exponential and mid-exponential phases, while NAD-dependent GDH activity slightly increases throughout the exponential phase (Thomulka & Moat, 1972).

[1]Guehler Biochemistry Laboratory, Department of Chemistry, Augustana College, 639 38th Street, Rock Island, IL 61201, USA. [2]Advisor.

*To whom correspondence should be addressed. Email: pamtrotter@augustana.edu

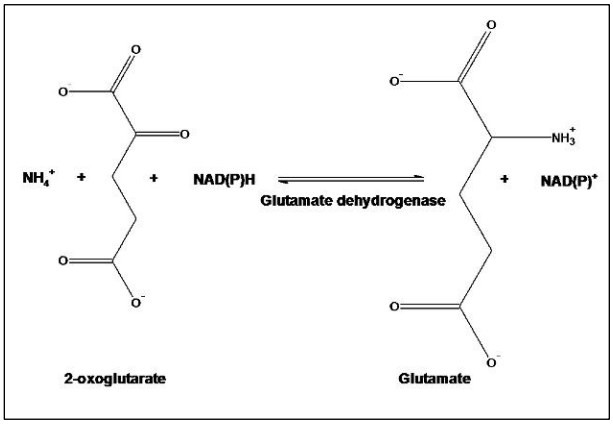


Figure 1. The conversion of 2-oxoglutarate to glutamate by glutamate dehydrogenase is reversible depending on reducing agent and concentrations of reagents.

In mammals, GDH is primarily mitochondrial (Mastorodemos et al., 2009). However, the prevailing model of *S. cerevisiae* glutamate biosynthesis assumes extramitochondrial localization of all three isozymes. Though this is consistent with much of the current literature, definitive localization studies are lacking. Perlman et al. (1970) localized both NADP-GDH and NAD-GDH to the cytosol; however, later work by Camardella et al. (1976) indicated nuclear localization of NADP-GDH. The sequencing of the *S. cerevisiae* genome led to the discovery of a second NADP-GDH isozyme, Gdh3p (Avendano et al., 1997). This multiplicity suggests that the localization studies performed prior to the sequencing of the *S. cerevisiae* genome, while likely valid, are incomplete and worthy of reexamination.

Global proteomic localization data are available for thousands of *S. cerevisiae* proteins (Huh et al., 2003; Sickmann et al., 2003). Huh et al. found Gdh1p to be cytoplasmic or nuclear and Gdh2p to be cytoplasmic, but list no data for Gdh3p. Sickmann et al. (2003) list Gdh2p and Gdh3p as mitochondrial. These studies are useful in a broad referential sense, but are contradictory in the case of one GDH protein, Gdh2p. This may be related to methodological differences. Huh et al. used GFP tag visualization, while Sickmann et al. used 2D PAGE and mass spectrometry. Taken together, available data leave the question of GDH localization unresolved. The extramitochondrial localizations of NADP-GDH and NAD-GDH found using enzymatic and proteomic methods (Perlman et al., 1970; Camardella et al., 1976; Huh et al., 2003) conflict somewhat with the mitochondrial localization of Gdh2p and Gdh3p found by Sickmann et al. (2003). Additionally, Gdh2p contains a putative bipartite nuclear localization sequence as analyzed by PSORT (Nakai & Horton, 1999; Dingwall et al., 1988), predicting a nuclear localization that was not observed in global proteomic analysis (Huh et al. 2003). These considerations highlight the importance of specific studies like the one presented here.

In this study, we utilized mutant strains to analyze each isozyme individually (Table 1). Our approach thereby avoided a major shortcoming of the work of Perlman et al. (1970) and Camardella et al. (1976), who analyzed Gdh1p and Gdh3p together as NADP-GDH. Previous indications of extra-mitochondrial localization of *S. cerevisiae* GDH (Perlman et al., 1970; Camardella et al., 1976; Huh et al., 2003) are particularly interesting in light of the discovery that mammalian GDH is primarily mitochondrial (Mastorodemos et al., 2009). Knowledge of the subcellular localization of budding yeast GDH will have implications for the use of *S. cerevisiae* as a model organism in metabolic studies, particularly those involving glutamate metabolism.

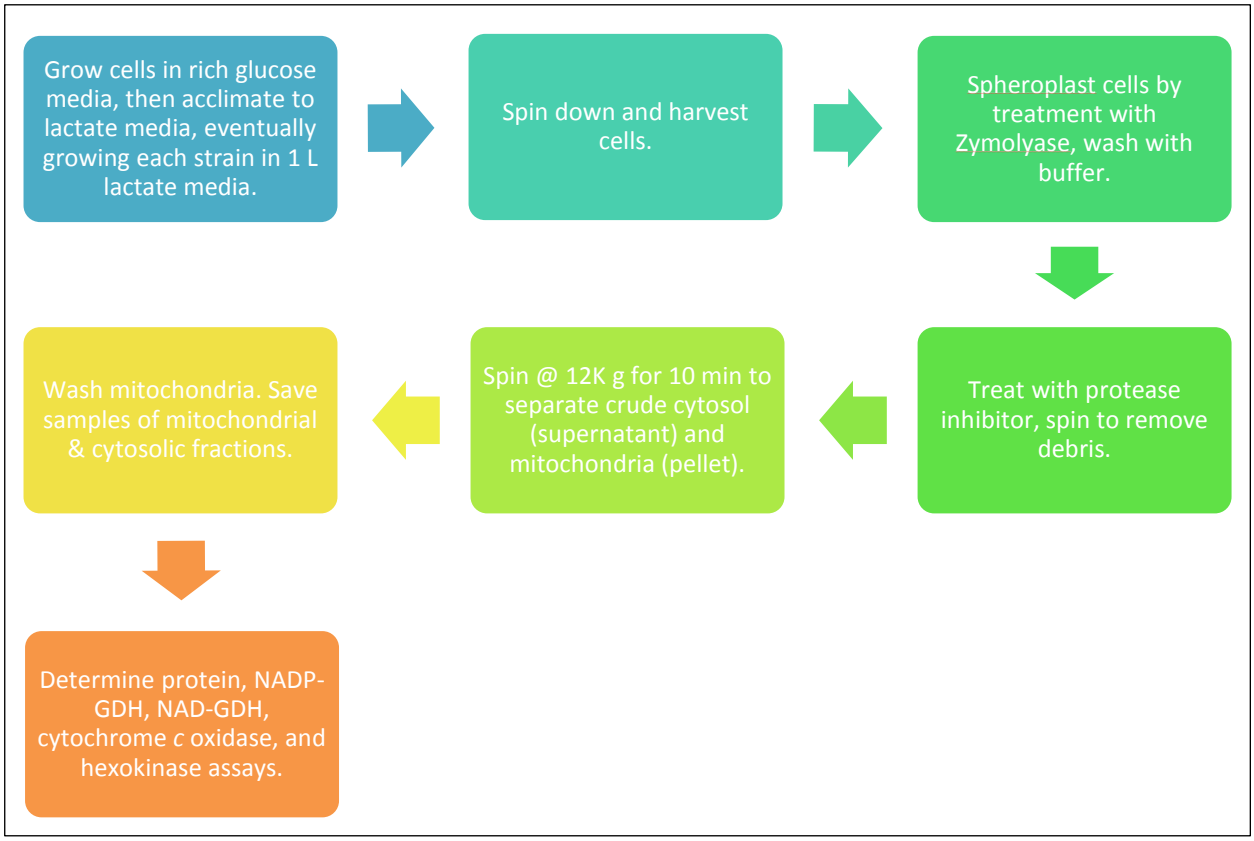


Figure 2. Schematic representation of experimental protocol.

METHODS AND MATERIALS

Yeast strains and growth conditions

Genotypes of strains used in this study are as follows: Wild-type (BY4742); *MATα his3 leu2 lys2 ura3*, and *gdh3Δ* (disrupted in open reading frame *YAL062w*); *MATα his3 leu2 lys2 ura3 gdh3Δ::G418*. All yeast strains were obtained from Research Genetics (Huntsville, AL).

Single yeast colonies were pre-grown in 20 mL rich glucose media (Yeast, Peptone, Dextrose (YPD)—1% yeast extract, 2% peptone, 2% glucose) overnight at 30° C. They were then acclimated to Daum, Böhni, and Schatz (DBS) lactate media [0.3% yeast extract, 0.1% glucose, 0.1% KH_2PO_4, 0.1% NH_4Cl, 0.05% $CaCl_2{\cdot}2H_2O$, 0.05% NaCl, 0.05% $MgSO_4$, 0.03% *v/v* 1% $FeCl_3$, 2.2% *v/v* 90% lactic acid (Daum et al., 1982) with standard concentrations (Sherman, 1991) of amino acids necessary to fulfill auxotrophic requirements] by incubating 50 µL YPD-grown cells in 10 mL DBS media overnight at 30° C. Cultures were finally transferred to 1 L DBS media and grown overnight at 30° C. Cells were harvested in mid-exponential phase, at OD_{600} between 0.8 and 1. The non-fermentable carbon source lactate was used because yeast utilize primarily fermentative metabolism in the presence of a fermentable carbon source (e.g. glucose), and therefore fail to accumulate significant quantities of mitochondria (Fraenkel, 1982).

Subcellular fractionation

Yeast strains grown as described above were harvested and separated into mitochondrial and extramitochondrial fractions using a modification of the method used by Daum et al. (1982). The procedure was followed to the point where crude mitochondria and cytosol were obtained (Figure 2). Aliquots of fractions were frozen immediately and stored at -20° C for up to several weeks before analysis.

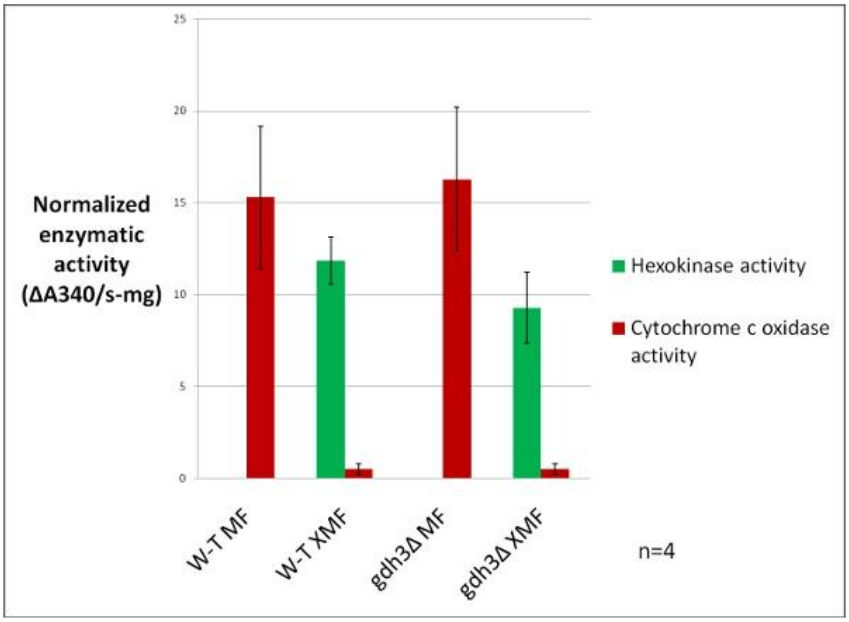


Figure 3. Hexokinase (a cytosolic enzyme) and cytochrome c oxidase (a mitochondrial enzyme) activity confirms identity of subcellular fractions. Cytochrome c oxidase is concentrated in mitochondrial fractions. Limited mitochondrial contamination of extramitochondrial fractions is present; however, in both strains, cytochrome c oxidase activity is increased at least 30x in mitochondrial fractions over extramitochondrial fractions. Hexokinase is found only in extramitochondrial fractions. Abbreviations: W-T: wild-type, MF: mitochondrial fraction, XMF: extramitochondrial fraction

Assays and analysis

NADP-GDH and NAD-GDH activity were measured by the oxidation of NADPH and NAD (Bergmeyer & Beutler, 1985). Briefly, we measured the decrease in absorbance at 340 nm of solutions containing mitochondrial or extra-mitochondrial fractions in the presence of excess ammonium and 2-oxoglutarate (Doherty, 1970). To ensure that oxidation of NAD(P)H was not due to enzymes other than GDH, absorbance curves were subtracted from curves of solutions containing no 2-oxoglutarate. Solutions with 1 M NH_4Cl, instead of fraction served as negative control, while purified bovine GDH served as positive control. Extinction coefficients were determined by spectrophotometric calibration using purified NADPH or NADH and the Beer-Lambert Law.

Cytochrome *c* oxidase, a mitochondrial marker enzyme, was measured by adding sample to a dithionite-reduced solution of cytochrome *c* and monitoring its oxidation spectrophotometrically (Mason et al., 1973). Hexokinase, a cytosolic marker enzyme, was measured by the reduction of NAD in a coupled reaction with an excess of glucose-6-phosphate dehydrogenase (Rose & Warms, 1967). Protein was measured by the bicinchoninic acid (BCA) assay (Smith et al., 1985). All assays were carried out on a Beckman-Coulter UV-Vis spectrophotometer.

Statistical analysis

All calculations were performed using Microsoft Excel. Statistical significance was calculated using Student's t-test, considering P value < 0.05 as statistically significant. All data shown is the average of data from at least three independent preparations.

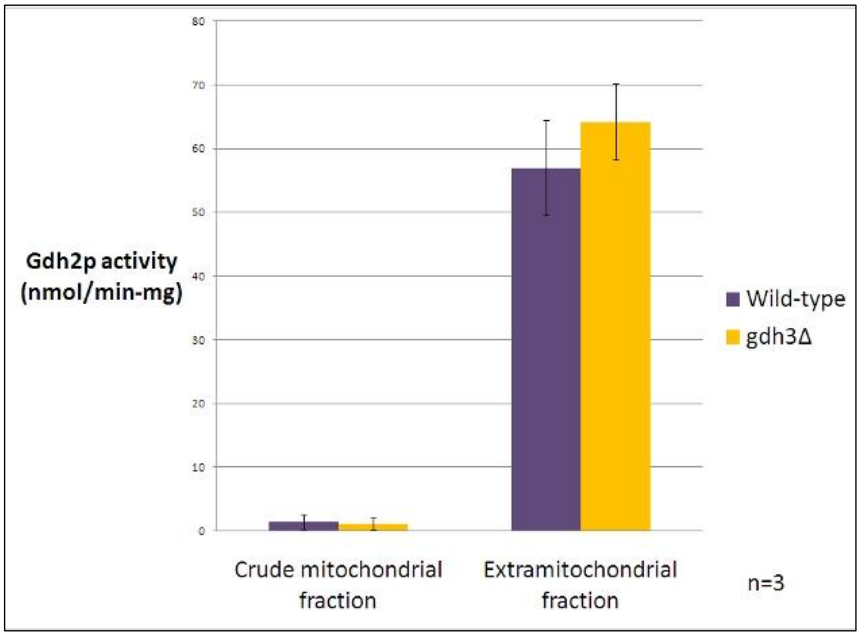


Figure 4. Gdh2p activity is extramitochondrial. NAD-GDH activity in both wild-type and gdh3Δ cells was assayed spectrophotometrically by the oxidation of NADH. Gdh2p activity is increased at least 40x in extramitochondrial versus mitochondrial fractions in both strains. The results were not statistically different at the 95% level (data not shown).

Isolation of mitochondrial and extra-mitochondrial fractions was confirmed by cytochrome *c* oxidase and hexokinase activities, respectively. Results are summarized in Figure 3. Mitochondrial fractions displayed more than 30x the cytochrome *c* oxidase activity of extra-mitochondrial fractions in both wild-type and *gdh3Δ* cells. Hexokinase activity was only detected in extramitochondrial fractions.

NAD-GDH activity and subcellular localization of Gdh2p

Mitochondrial and extramitochondrial fractions from wild-type and *gdh3Δ* strains, both of which express *GDH2*, were assayed for NAD-GDH activity. Gdh2p activity in extra-mitochondrial fractions was approximately 40x that of mitochondrial fractions, with similar activity in both wild-type and *gdh3Δ* fractions (Figure 4). Purity of fractions was assessed by marker enzyme analysis, using cytochrome *c* oxidase for mitochondrial fractions and hexokinase for extramitochondrial fractions (Figure 3).

RESULTS

Isolation of individual isozyme activities

The activity of Gdh2p—the only NAD-dependent GDH—was analyzed in both wild-type and *gdh3Δ* strains. We attempted to isolate the activities of the two NADP-dependent isozymes, Gdh1p and Gdh3p, through the use of mutant *gdh3Δ* and *gdh1Δ* strains (isolating Gdh1p and Gdh3p, respectively). The *gdh1Δ* strain failed to grow on the lactate media used to promote mitochondrial biogenesis, a finding consistent with the inability of this strain to grow on other non-fermentable carbon sources (DeLuna et al., 2001; Tang et al., 2011). Analysis of NADP-GDH activity in *gdh3Δ* fractions isolated Gdh1p activity.

Identification of subcellular fractions

NADP-GDH activity and subcellular localization of Gdh1p & Gdh3p

Mitochondrial and extra-mitochondrial fractions from wild-type and *gdh3Δ* strains were assayed for NADP-GDH activity (Figure 5). Mutants impaired in *GDH3* only express Gdh1p as an NADP-GDH, so *gdh3Δ* activity was

used to localize Gdh1p. Gdh1p activity in extramitochondrial fractions was approximately 12x that of mitochondrial fractions. Fractional purity was assessed using cytochrome *c* oxidase and hexokinase activities (Figure 3). Similar experiments using *gdh1Δ* cells to localize Gdh3p failed owing to the inability of the *gdh1Δ* strain to grow on non-fermentable carbon sources (DeLuna et al., 2001; Tang et al., 2011).

Comparison of the NADP-GDH activity data from the wild-type and *gdh3Δ* strains (Figure 5) reveals another observation: a statistically significant ($p < 0.05$) decrease in NADP-GDH activity from wild-type to *gdh3Δ* extramitochondrial fractions (Figure 6).

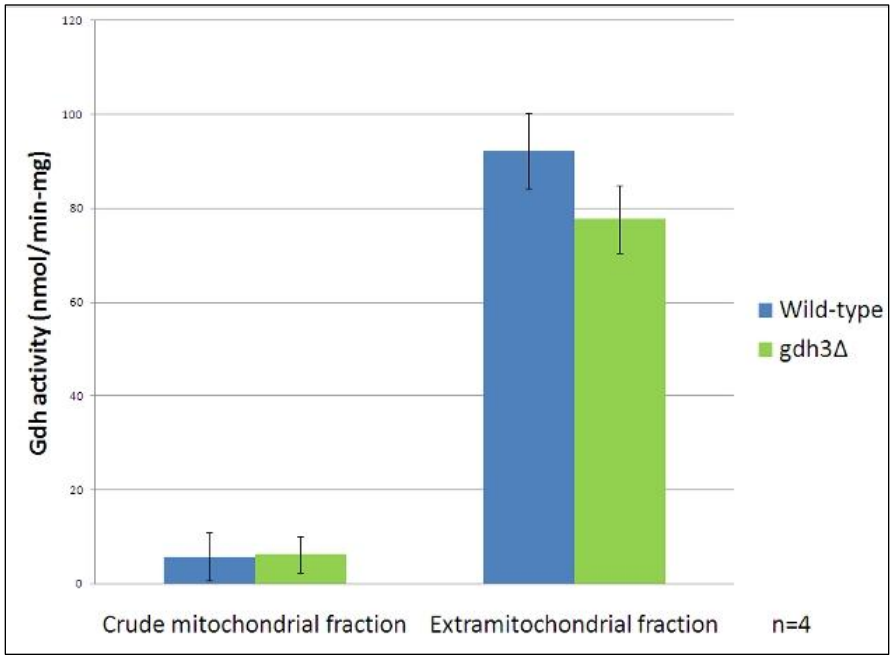
DISCUSSION AND CONCLUSION

The goal of this study was to localize the three *S. cerevisiae* glutamate dehydrogenase isozymes as either mitochondrial or extramitochondrial. Two studies done before the sequencing of the yeast genome (Perlman et al., 1970; Camardella et al., 1976) suggest extramitochondrial localization for both NADP-dependent and NAD-dependent GDH, and one global proteomic study (Huh et al., 2003) finds Gdh1p and Gdh2p to be extramitochondrial. In contrast, Sickmann et al. (2003) describe Gdh2p and Gdh3p as mitochondrial. Our data, obtained by subcellular fractionation and analysis of enzymatic activity, suggest that Gdh1p and Gdh2p are extramitochondrial.

Analysis of Gdh2p activity was carried out in wild-type and *gdh3Δ* strains, both of which carry an unmutated *GDH2* gene. Gdh2p activity was increased approximately 40x in the extramitochondrial fractions of both strains, suggesting extramitochondrial localization for Gdh2p.

Since *S. cerevisiae* has two NADP-dependent glutamate dehydrogenase isozymes, Gdh1p and Gdh3p, the mutant strain *gdh3Δ* was used to isolate Gdh1p activity. Gdh1p activity in extramitochondrial fractions was increased approximately 12x over mitochondrial fractions. Notably, the "crude mitochondrial fraction" is

Figure 5. Gdh1p activity is extramitochondrial. NADP-GDH activity in wild-type and gdh3Δ cells was assayed spectrophotometrically by the oxidation of NADPH. In gdh3Δ extramitochondrial fractions, Gdh1p showed significantly heightened activity (over 12x that of the mitochondrial fraction).



designated as such because the method used to isolate mitochondria (Daum et al. 1982) likely contaminates those fractions with small amounts of lysate debris and other extramitochondrial matter. Though marker enzyme analysis (Figure 3) revealed minimal cross-fractional contamination, we hypothesize that the limited amount of NADP-GDH activity observed in mitochondrial fractions owes to this factor. We cannot, however, formally exclude the possibility that a small amount of Gdh1p is mitochondrial.

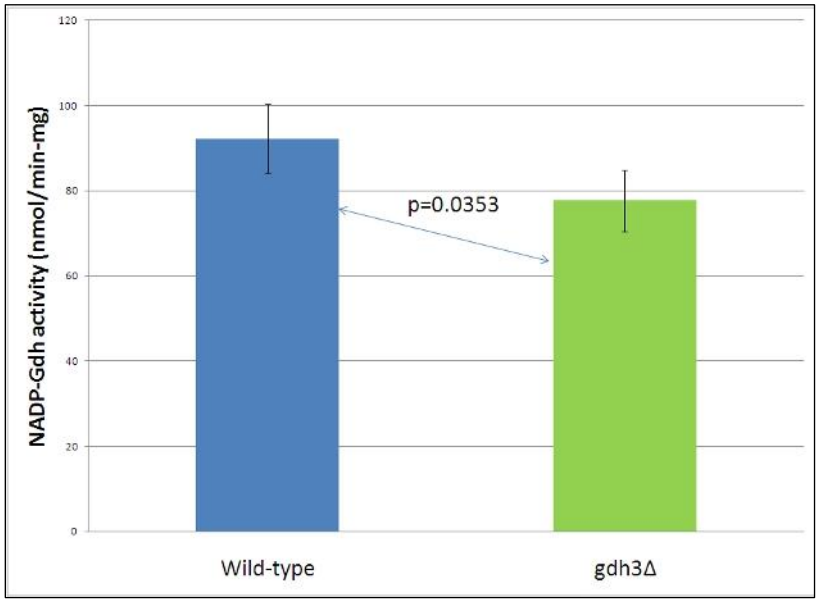


Figure 6. Yeast lacking Gdh3p show decreased extramitochondrial NADP-GDH activity. NADP-GDH activity in gdh3Δ cells is ≈84% of wild-type, and the data are statistically significant at the 95% confidence level (p=0.0353). The trend is nonexistent in the mitochondrial fractions, as seen in Fig. 5.

There are indications that Gdh3p is also extramitochondrial (Figure 6). It is possible that the significant decrease in NADP-GDH activity from wild-type to *gdh3Δ* extramitochondrial fractions (a trend not observed in mitochondrial fractions) owes to the lack of Gdh3p activity in *gdh3Δ* fractions (i.e. that Gdh3p is extramitochondrial), but it is also possible that other factors, such as differences in gene expression, caused the decrease in activity. Therefore, we refrain from drawing a firm conclusion with regard to Gdh3p subcellular localization, but rather note that definitive data are at this time lacking.

The literature generally supports these conclusions. One notable exception is the work of Sickmann et al. (2003), who found Gdh2p and Gdh3p in the mitochondria. Sickmann et al. used glycerol (a fermentable carbon source) and grew their cells to an OD_{600} of 1.5-2.0 (late exponential/linear growth phase), while our cells were grown in lactate (a non-fermentable carbon source) to an OD_{600} of 0.8 – 1.0 (mid-exponential phase). While the use of a fermentable carbon source by Sickmann et al. likely hindered mitochondrial biogenesis as compared to non-fermentable carbon sources (Fraenkel, 1982), it is unclear if this difference might contribute to the conflicting localization data. Additionally, the global tandem mass spectrometry experiments of Sickmann et al. represent a significant difference in approach from our GDH-specific assays, and it is possible that the small levels of both NAD-GDH and NADP-GDH activity in mitochondrial fractions (Figures 4 & 5) reflect a small amount of mitochondrial localization.

The study presented strongly suggests extramitochondrial localization for the three GDH isozymes. Future experiments may be designed with intent to further localize the isozymes to either a

specific organelle or the soluble phase of the cytoplasm. Reports of nuclear NADP-GDH activity, as well as the presence of a putative bipartite nuclear localization sequence in *GDH2* (Nakai & Horton, 1999; Dingwall et al., 1988), make the nucleus an appropriate organelle to study next.

A final interesting consideration arises when considering the findings of Camardella et al. (1976) in light of the more recent discovery of Gdh3p by Avendano et al. (1997). Camardella et al. (1976) characterized NADP-GDH both from isolated nuclei and the soluble phase of the cytosol. The NADP-GDH from these two sources yielded different K_m values, and the authors noted that this suggested the existence of two different NADP-GDH proteins—a prediction verified after the sequencing of the *S. cerevisiae* genome (Avendano et al., 1997). The cytosolic and nuclear proteins were present in an approximately 9:1 ratio, and cells were grown on glucose and harvested in early stationary phase growth (i.e. when the cells had begun to exhaust their supply of glucose and were beginning respiration using the ethanol produced by their fermentation). Considering the findings of Deluna et al. (2001) that Gdh1p predominates NADP-GDH activity during fermentation of glucose and gradually cedes to Gdh3p as more and more ethanol is utilized in respiration, it seems quite possible that the two NADP-GDH proteins characterized by Camardella et al. (1976) were in fact Gdh1p and Gdh3p, and that Gdh1p is cytosolic while Gdh3p is nuclear. Regardless, this speculation must be confirmed by experimentation, and such work will be a future direction of our research.

The major limitation of our study was the inability to isolate Gdh3p activity from the other NADP-dependent GDH, Gdh1p, and therefore our failure to localize this isozyme. This was due to the inability of the *gdh1Δ* strain to grow on non-fermentable carbon sources (Deluna et al., 2001; Tang et al., 2011), which we found necessary to stimulate enough mitochondrial biogenesis to obtain mitochondrial pellets of useful size. Additionally, our study might have benefited from direct detection (e.g. immunoblotting) of the GDH isozymes. However, our indirect measurement of GDH activity by the oxidation of NAD(P)H (Doherty, 1970) has been validated both by the widespread use of this assay in similar studies (Deluna et al., 2001) and our spectrophotometric analysis of solutions lacking 2-oxoglutarate to control for non-GDH sources of NAD(P)H oxidation in mitochondrial and extramitochondrial fractions.

Glutamate metabolism is a crucial process in biological arenas as diverse as nitrogen fixation, the urea cycle, and neural signaling (glutamate is the major excitatory neurotransmitter in the vertebrate central nervous system). *S. cerevisiae* is a good model organism owing to its ease of use and the sizable body of knowledge regarding its biology (Botstein et al., 1997). But without resolving the basic question of the subcellular localization of the GDH isozymes, much research into glutamate metabolism in *S. cerevisiae* suffers from a modicum of ambiguity. Equivocal suggestions of extramitochondrial localization of the three GDH isozymes are particularly undesirable in light of the finding that mammalian GDH is primarily mitochondrial (Mastorodemos et al. 2009). Our work, demonstrating via enzymatic activity that Gdh1p and Gdh2p are extramitochondrial, represents a step forward in addressing this interesting and elusive question.

ACKNOWLEDGEMENTS

We thank Patrick Crawford, Matthew Pautz, Alex Sieg, and Deidre Leist for helpful discussions and contributions. This work was supported by grants from the Merck Institute for Science Education (to Augustana College) and the National Institutes of Health R15-GM069372 (to P.J.T.).

REFERENCES

Avendano, A. et al. (1997) *GDH3* encodes a glutamate dehydrogenase isozyme, a previously unrecognized route for glutamate biosynthesis in *Saccharomyces cerevisiae*. Journal of Bacteriology 179, 5594-5597.

Bergmeyer, H.U., and Beutler, H-O. (1985) Methods of Enzymatic Analysis. 3rd ed. New York: VCH, 454-461.

Camardella, L. et al. (1976) Purification and properties of NADP-dependent glutamate dehydrogenase from yeast nuclear fractions. Biochimica et Biophysica Acta 429, 324-330.

Daum, G. et al. (1982) Import of Proteins into Mitochondria. Journal of Biological Chemistry 257, 13028-13033.

Deluna, A. et al. (2001) NADP-Glutamate dehydrogenase isozymes of *Saccharomyces cerevisiae:* purification, kinetic properties, and physiological roles. Journal of Biological Chemistry 276, 43775-43783.

Dingwall, C. et al. (1988) The nucleoplasmin nuclear localization sequence is larger and more complex than that of SV-40 large T antigen. Journal of Cell Biology 107, 841-849.

Doherty, D. (1970) L-Glutamate dehydrogenases (yeast). Methods in Enzymology 17, 850–856.

Fraenkel, D.G. (1982) Carbohydrate metabolism. In:*The Molecular Biology of the Yeast Saccharomyces cerevisiae: Metabolism and Gene Expression*. Editors: Strathern, J.N., Jones, E.W. and Broach, J.R., New York: Cold Spring Harbor Laboratory. pp. 1-37.

Huh, W-K. et al. (2003) Global analysis of protein localization in budding yeast. Nature 425, 686-691.

Magasanik, B. (2003) Ammonia Assimilation by *Saccharomyces cerevisiae*. Eukaryotic Cell 2, 827-829.

Mason, T.L. et al. (1973) Cytochrome *c* oxidase from baker's yeast. Journal of Biological Chemistry 248, 1346-1354.

Mastorodemos, V. et al. (2009) Human GLUD1 and GLUD2 localize to mitochondria and endoplasmic reticulum. Biochemistry and Cell Biology 87, 505-516.

Nakai, K., and Horton, P. (1999) PSORT: a program for detecting sorting signals in proteins and predicting their subcellular localization. Trends in Biochemical Sciences 24, 34-36.

Perlman, P.S., and Mahler, H.R. (1970) Intracellular localization of enzymes in yeast. Archives of Biochemistry and Biophysics 136, 245-259.

Rose, I.A., and Warms, J.V.B. (1967) Mitochondrial Hexokinase: Release, Rebinding, and Location.

Journal of Biological Chemistry 242, 1635-1645.

Sherman, F. (1991) Getting started with yeast. Methods in Enzymology 194, 3-21.Sickmann, A. et al. (2003) The proteome of Saccharomyces cerevisiae mitochondria. Proceedings of the National Academy of Sciences USA 100, 13207-13212.

Smith, P.K. et al. (1985) Measurement of protein using bicinchoninic acid. Analytical Biochemistry 150, 76-85.

Tang, Y. et al. (2011) ^{13}C-metabolic enrichment of glutamate in glutamate dehydrogenase mutants of Saccharomyces cerevisiae. Microbiological Research [Epub ahead of print].

Thomulka, K.W., and Moat, A.G. (1972) Inorganic Nitrogen Assimilation in Yeasts: Alteration in Enzyme Activities Associated with Changes in Cultural Conditions and Growth Phase. Journal of Bacteriology 109, 25-33.