NADP⁺-dependent Glutamate Dehydrogenase Activity is Impaired in Mutants of *Saccharomyces cerevisiae* That Lack Aconitase

By ALICIA GONZÁLEZ,* LAURA RODRÍGUEZ, HIRAM OLIVERA
AND MARIO SOBERÓN

*Centro de Investigación sobre Fijación de Nitrógeno, Universidad Nacional Autónoma de México,
Apartado Postal 565-A CP 62280 Cuernavaca, Morelos, México*

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A mutant of *Saccharomyces cerevisiae* lacking aconitase did not grow on minimal medium (MM) and had five- to tenfold less NADP⁺-dependent glutamate dehydrogenase (GDH) activity than the wild-type, although its glutamine synthetase (GS) activity was still inducible. When this mutant was incubated with glutamate as the sole nitrogen source, the 2-oxoglutarate content rose, and the NADP⁺-dependent GDH activity increased. Furthermore, carbon-limited cultures showed a direct relation between NADP⁺-dependent GDH activity and the intracellular 2-oxoglutarate content. We propose that the low NADP⁺-dependent GDH activity found in the mutant was due to the lack of 2-oxoglutarate or some other intermediate of the tricarboxylic acid cycle.

INTRODUCTION

In yeasts and filamentous fungi such as *Saccharomyces cerevisiae*, *Neurospora crassa* and *Aspergillus nidulans*, the interconversion of ammonia and glutamate is catalysed by two types of glutamic dehydrogenase (GDH) (Holzer & Schneider, 1957; Pateman & Kinghorn, 1975; Sanwal & Lata, 1961). One of them uses NADP⁺ as a cofactor, and has been assigned a biosynthetic role, whereas the other one uses NAD⁺ and plays a catabolic role.

The NADP⁺-dependent GDH catalyses the synthesis of glutamate from ammonium and 2-oxoglutarate. The first substrate of this reaction comes from the metabolism of nitrogenous compounds, or NH₄⁺ in the medium, and the second from carbon metabolism. Since ammonium assimilation constitutes the metabolic process in which carbon and nitrogen metabolism meet, it could be regulated by both the nitrogen and the carbon source. There have been reports on the metabolic regulation of NADP⁺-dependent GDH by the nitrogen source in some eukaryotic micro-organisms including *S. cerevisiae* (Hernández *et al.*, 1983; Roon & Even, 1973); in all cases, it has been found that the activity of this enzyme is maximal when ammonia is the sole nitrogen source. In one case, it has been shown that the nitrogen source exerts its effect at the level of *de novo* enzyme synthesis (Hernández *et al.*, 1983). Although the effect of carbon deprivation on NADP⁺-dependent GDH has been studied (Hemmings, 1978; Mazon, 1978; Mazon & Hemmings, 1979), the regulation of its activity by the carbon source has not been analysed in detail (Kapoor & Grover, 1970).

We have studied the regulation of the NADP⁺-dependent GDH in mutants of *S. cerevisiae* which are blocked in the tricarboxylic acid cycle and are thus unable to synthesize 2-oxoglutarate, which is one of the substrates of the enzyme.

METHODS

Organisms. The wild-type strain S288C was obtained from the Cold Spring Harbor Laboratory, NY, USA.

Media. The minimal medium (MM) contained salts, trace elements, vitamins, dextrose, and unless otherwise

Abbreviations: MM, minimal medium; GDH, glutamate dehydrogenase; GS, glutamine synthetase.

stated, 40 mM-(NH₄)₂SO₄ as a nitrogen source; it was prepared following the formula of Difco Yeast Nitrogen Base. Yeast extract/peptone/dextrose (YPD) plates contained 2% (w/v) glucose, 1% (w/v) yeast extract (Difco), 2% (w/v) peptone (Difco) and 2% (w/v) agar (Difco). The cultures were incubated at 30 °C and agitated in a New Brunswick water bath at 200 r.p.m. Cells were grown on YPD until they reached stationary phase; they were then washed and resuspended in MM without nitrogen. These cells were used to inoculate cultures to an OD₆₅₀ of 0.05.

Inocula and maintenance of continuous cultures. These cultures were inoculated and maintained as described by Senior (1975). A New Brunswick Chemostat model C30 was used for these experiments.

Mutagenesis and mutant selection. Strain S288C was mutagenized by treatment with ethyl methane sulphonate (EMS) using the method reported by Fink (1970). After mutagenesis cells were allowed a period of growth on MM plus 0.05% glutamate (added as glutamic acid). Glutamate-requiring strains were selected on MM plus 50 mM-glycine, by using *N*-glycosyl-polyfungin, as described by Polaina & Conde (1981). Cells were spread on agar plates containing MM plus 0.05% glutamate. Colonies which failed to grow on MM plus ammonium sulphate, but which did grow on the same medium supplemented with glutamate, were purified by successive streakings and characterized further.

Extraction of intracellular metabolites. For the estimation of 2-oxoglutarate, extracts were prepared as described by Kang *et al.* (1982). Ammonium pools were estimated by the method of Tempest *et al.* (1970) and Tachiki *et al.* (1981).

Determination of ammonium and 2-oxoglutarate. The concentration of these metabolites in extracts was determined with beef GDH by following NADH oxidation at 340 nm (Dubois *et al.*, 1974).

Determination of amino acid pools. Cells were harvested by centrifugation and immediately suspended in 80% (v/v) ethanol and heated in boiling water for 10 min. Cells were disrupted by grinding with a Braun cell disruptor and debris was removed by filtration with 0.22 µm Millipore filters. The filtrates containing the free amino acids were lyophilized and amino acid pools were determined as described previously (González *et al.*, 1983).

Enzyme assays. Soluble extracts for enzyme assays were prepared by grinding whole cells, suspended in their corresponding extraction buffer, with glass beads in a Braun cell disruptor. Glutamine synthetase (EC 6.3.1.2), NADP⁺-dependent GDH (EC 1.4.1.4) and aconitase (EC 4.2.1.3) were assayed by the methods of Ferguson & Sims (1974), Doherty (1970) and Anfinsen (1955), respectively.

Protein determination. Protein was determined by the Lowry method with bovine serum albumin as a standard.

Chemicals. All amino acids, beef GDH and bovine serum albumin were obtained from Sigma. *N*-Glycosyl-polyfungin was a kind gift from Dr Norman J. Pieniazek (Department of Genetics, University of Warsaw, Poland).

RESULTS AND DISCUSSION

Isolation and characterization of mutants

Glutamate auxotrophs were obtained as described in Methods. These mutants were unable to grow on MM unless glutamate or glutamine was added; they were also characterized by a negligible growth on media containing glycerol as the main carbon and energy source, and were unable to synthesize 2-oxoglutarate. The phenotype displayed by these mutants was similar to that reported for the *glt1* mutant (Ogur *et al.*, 1964), which has an alteration in aconitase synthesis. One of the mutants, CN11, had very low aconitase activity (Table 1).

Spontaneous revertants to glutamate independency were obtained with a frequency of 10⁻⁸; all had simultaneously recovered the capacity to grow on glycerol. Furthermore, the aconitase activity found in one of the revertants (CN13) was similar to that found in the wild-type strain S288C (Table 1). The main pathway for the synthesis of 2-oxoglutarate is the tricarboxylic acid cycle; a mutant lacking aconitase should thus be impaired in the synthesis of this compound. Strain CN11 had a very low 2-oxoglutarate content as compared to the wild-type, when these strains were incubated on MM (Fig. 1*a*).

The catabolism of glutamate results in the production of 2-oxoglutarate, and the cell content of this compound rose in the mutant and the wild-type when these strains were grown in the presence of ammonium plus glutamate (Fig. 1*b*), although the wild-type still contained more 2-oxoglutarate, presumably because this intermediate was also being synthesized via the tricarboxylic acid cycle. The intracellular content of ammonia was also determined in extracts obtained from the mutant and the wild-type strains incubated on ammonia. In these conditions, the intracellular amounts of this compound are higher in the mutant strain CN11 than in the wild-type (Fig. 1*c*). This result can be explained as an impairment in the synthesis of 2-oxoglutarate, which would result in a decreased ammonium assimilation and in the accumulation of the latter compound.

Fig. 1. Accumulation of 2-oxoglutarate (a, b) and ammonium (c, d) in the wild-type strain S288C (●) and in the mutant strain CN11 (○) of *S. cerevisiae*. Cells were incubated with either 40 mM-(NH₄)₂SO₄ (a, c) or 40 mM-(NH₄)₂SO₄ plus 0.05% glutamate (b, d) as the nitrogen source.

Table 1. *Aconitase activity in S. cerevisiae strains*

Cells were grown for 12 h on MM with (NH₄)₂SO₄ or 0.05% glutamate as the nitrogen source. ND, Not detected.

Strain	Nitrogen source...	Aconitase activity [units (mg protein) ⁻¹]	
		(NH ₄) ₂ SO ₄	Glutamate
S288C (wild-type)		62.6	31.2
CN11 (glutamate auxotroph)		ND	2.3
CN13 (revertant)		47.6	17.1

When the wild-type and the mutant strains were grown on MM plus glutamate, both strains accumulated similar ammonium pools (Fig. 1d).

The sizes of the glutamate pools in the mutant strain CN11 and in the wild-type strain are shown in Fig. 2. The mutant had a very low content of this amino acid when it was incubated on MM (Fig. 2a); this content rose when the mutant was grown on MM plus glutamate (Fig. 2b).

Effect of glutamate auxotrophy on NADP⁺-dependent GDH and GS activity

Ammonium assimilation can be accomplished via two enzymes: NADP⁺-dependent GDH and the glutamine synthetase (GS). Since 2-oxoglutarate and glutamate are substrates of the NADP⁺-dependent GDH and GS respectively, we examined whether the lack of these substrates had an effect on the activity of these enzymes.

Activities of NADP⁺-dependent GDH were measured in the wild-type strain and in the mutant CN11 after different periods of incubation on MM. In the wild-type strain the NADP⁺-dependent GDH activity increased during the incubation, and reached a maximal value after 6 h; in the mutant CN11 the NADP⁺-dependent GDH activity was five- to tenfold lower than that present in the wild-type strain (Fig. 3a). When the wild-type strain was grown on MM plus glutamate the activity of the NADP⁺-dependent GDH was lower than that found when this strain was grown on ammonium (Fig. 3a, b). These results agree with those of Roon & Even (1973), and with their proposition that glutamate could act as a repressor of NADP⁺-dependent GDH. As shown in Fig. 2(b), this amino acid is accumulated in these conditions. When the

Fig. 2. (a, b), Accumulation of glutamate in the wild-type strain S288C (●) and in the mutant strain CN11 (○) of *S. cerevisiae*. Cells were incubated with either 40 mM- $(\text{NH}_4)_2\text{SO}_4$ (a) or 40 mM- $(\text{NH}_4)_2\text{SO}_4$ plus 0.05% glutamate (b) as the nitrogen source. (c) Growth curve of the wild-type strain S288C. Cells incubated with either 40 mM- $(\text{NH}_4)_2\text{SO}_4$, 40 mM- $(\text{NH}_4)_2\text{SO}_4$ plus 0.05% glutamate or 0.05% glutamate alone all gave similar growth curves (■). (d) Growth curves of the mutant strain CN11. Cells were incubated with either 40 mM- $(\text{NH}_4)_2\text{SO}_4$ (Δ), 40 mM- $(\text{NH}_4)_2\text{SO}_4$ plus 0.05% glutamate (▲) or 0.05% glutamate (□).

Fig. 3. Specific activity of NADP⁺-dependent GDH in the wild-type strain S288C (●) and in the mutant strain CN11 (○) of *S. cerevisiae*. Cells were incubated with 40 mM- $(\text{NH}_4)_2\text{SO}_4$ (a), 40 mM- $(\text{NH}_4)_2\text{SO}_4$ plus 0.05% glutamate (b) or 0.05% glutamate (c) as the nitrogen source.

mutant strain CN11 was grown on MM plus glutamate, its NADP⁺-dependent GDH activity was similar to that found in CN11 grown on unsupplemented MM (Fig. 3b). When the two strains were grown on glutamate as the sole nitrogen source, the NADP⁺-dependent GDH activity in the mutant was higher than that present in the wild-type strain grown in these conditions, and higher than in the mutant grown on MM (Fig. 3c).

These results suggest that the lack of NADP⁺-dependent GDH induction found in the mutant CN11 incubated on ammonium is due to the lack of 2-oxoglutarate, or some other intermediate of the tricarboxylic acid cycle. When the mutant CN11 was grown on glutamate as the sole nitrogen source, the cell content of 2-oxoglutarate was greatly increased, as compared to that of cells grown on MM or on MM plus glutamate (Table 2; Fig. 1a, b), thus allowing the induction of NADP⁺-dependent GDH. The extent of induction was not as great as that in the wild-type

Table 2. Intracellular pools of 2-oxoglutarate and glutamate in *S. cerevisiae* CN11

Cells were grown on 0.05% glutamate as the nitrogen source. ND, Not detected.

Time (h)	2-Oxoglutarate [nmol (mg protein) ⁻¹]	Glutamate [μmol (mg protein) ⁻¹]
0	ND	0.40
6	45.0	1.80
12	8.5	0.24

Table 3. GS activity in *S. cerevisiae* strains S288C and CN11

Cells were incubated on MM with (NH₄)₂SO₄ as the nitrogen source. One unit of GS activity represents 1 μmol γ-glutamyl hydroxamate produced min⁻¹ at 30 °C. ND, Not determined.

Time (h)	Strain...	GS activity [units (mg protein) ⁻¹]	
		S288C	CN11
3		0.015	0.0046
6		0.014	0.010
9		0.010	0.020
12		ND	0.024

strain grown on MM, due to the presence of glutamate (Table 2), which could act as a repressor allowing only a partial induction. It has been previously reported (Dubois *et al.*, 1974) that mutants lacking aconitase have a normal NADP⁺-dependent GDH activity; however, our results clearly demonstrate that this is not the case in our mutant strain. We do not know the reason for this discrepancy. The lack of induction of NADP⁺-dependent GDH observed when the mutant CN11 was incubated on MM could be attributed to a decrease in protein synthesis, and not to a particular effect of 2-oxoglutarate, since this strain does not grow in these conditions.

In order to determine whether this was the case, we measured the activity of GS in strain CN11 incubated in MM, a condition in which one of the substrates of GS is also absent. Evidence has been presented indicating that glutamine regulates nitrogen catabolism in fungi by repressing various enzymes such as GS in *N. crassa* (Vaca & Mora, 1977) and in *S. cerevisiae* (Legrain *et al.*, 1982). As can be seen from the results in Table 3, strain CN11 had similar GS activity to that of the wild-type strain grown on the same medium. This shows that in the absence of glutamate or glutamine, there was no nitrogen catabolite repression, and that GS can be induced even in the absence of growth.

NADP⁺-dependent GDH activity in carbon-limited cultures

Cells from the wild-type strain were grown on MM containing 3 mM-glucose. NADP⁺-dependent activity, and 2-oxoglutarate and glutamate pools were assayed upon the attainment of a carbon-limited steady state. NADP⁺-dependent GDH activity increased as the intracellular content of 2-oxoglutarate rose (Fig. 4). This indicates that 2-oxoglutarate, or some other intermediate of the tricarboxylic acid cycle, is a positive modulator of NADP⁺-dependent GDH induction. The increase in NADP⁺-dependent GDH activity stopped when the specific growth rate was around 0.15 h⁻¹; this can be explained as being due to the glutamate pool reaching a maximum and exerting a repressive effect. Messenguy *et al.* (1980) have demonstrated that the intracellular compartmentalization of amino acids is affected by certain growth conditions: when protein synthesis is reduced, amino acids are mainly localized in the vacuole, whereas during balanced growth these compounds are mainly cytoplasmic. This could explain why, at low growth rates, glutamate is accumulated even though NADP⁺-dependent GDH activity rises: as the specific growth rate increases protein synthesis increases, and amino acids, including glutamate, move from the vacuole to the cytoplasm.

From the results presented above we conclude that due to the lack of 2-oxoglutarate, induction of NADP⁺-dependent GDH is prevented in a mutant lacking aconitase. Since this type of

Fig. 4. Effect of dilution rate on specific activity of NADP⁺-dependent GDH (●), and intracellular content of glutamate (□) and 2-oxoglutarate (△) in glucose-limited chemostat cultures of *S. cerevisiae* S288C. Several steady states were examined at each dilution rate and the activities are the means of triplicate samples.

mutant could have low contents of some other intermediates of the tricarboxylic acid cycle, we do not exclude the possibility that some other carbon skeleton, and not 2-oxoglutarate itself, could be the effector.

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