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The glutamate synthase (GOGAT) of *Saccharomyces cerevisiae* plays an important role in central nitrogen metabolism

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Abstract

Central nitrogen metabolism contains two pathways for glutamate biosynthesis, glutaminases and glutamate synthase (GOGAT), using glutamine as the sole nitrogen source. GOGAT's importance for cellular metabolism is still unclear. For a further physiological characterisation of the GOGAT function in central nitrogen metabolism, a GOGAT-negative (*Δglt1*) mutant strain (VWk274 LEU$^+$) was studied in glutamine-limited continuous cultures. As reference, we did the same experiments with a wild-type strain (VWk43). Intracellular and extracellular metabolites were analysed during different steady states in both strains. The redox state of the cell was taken into account and the NAD(H) and NADP(H) concentrations were determined as well as the reduced and oxidised forms of glutathione (GSH and GSSG, respectively). The results of this study confirm an earlier suggestion, based on a metabolic network model, that GOGAT may be a link between the carbon catabolic reactions (energy production) and nitrogen anabolic reactions (biomass production) by working as a shuttle between cytosol and mitochondria.

Keywords: Glutamate synthase; Nitrogen metabolism; Continuous culture; *Saccharomyces cerevisiae*

1. Introduction

The yeast *Saccharomyces cerevisiae* is able to use a wide variety of compounds as a source of nitrogen. The yeast cell converts these molecules into ammonia, glutamate and glutamine, which function as amino donor in all other biosynthetic reactions [1,2]. All reactions that convert ammonia and glutamine into glutamate are referred to as the central nitrogen metabolism (CNM; Fig. 1).

CNM contains two pathways for glutamate biosynthesis (glutaminases and GOGAT) using glutamine as the sole source of nitrogen. GOGAT, which is NADH-dependent in *S. cerevisiae*, converts one molecule of glutamine and one molecule of α-ketoglutarate into two molecules of glutamate. The glutaminases (GDA) degrade glutamine to glutamate and ammonia [3,4]. The presence of several pathways has generated controversy about the significance of two routes for the biosynthesis of the same end product. Although the GDA-encoding genes have not yet been identified, these glutaminases obviously exist because mutants without the GOGAT enzyme grow well on glutamine [2]. Some authors have suggested that the role of the GOGAT pathway, with the concerted action of the glutamine synthetase (GS), is to assimilate ammonium and synthesise glutamate under ammonium limitation [5]. However, in other microorganisms NADPH-dependent glutamate dehydrogenase (NADPH-GDH) is used to incorporate ammonia during either nitrogen limitation or nitrogen excess [6]. This hypothesis suggests that NADPH-GDH is the main pathway for glutamate biosynthesis. Physiological studies have therefore been reported with both wild-type and mutant strains impaired in GOGAT or in NADPH-GDH activity. These show that GOGAT has different

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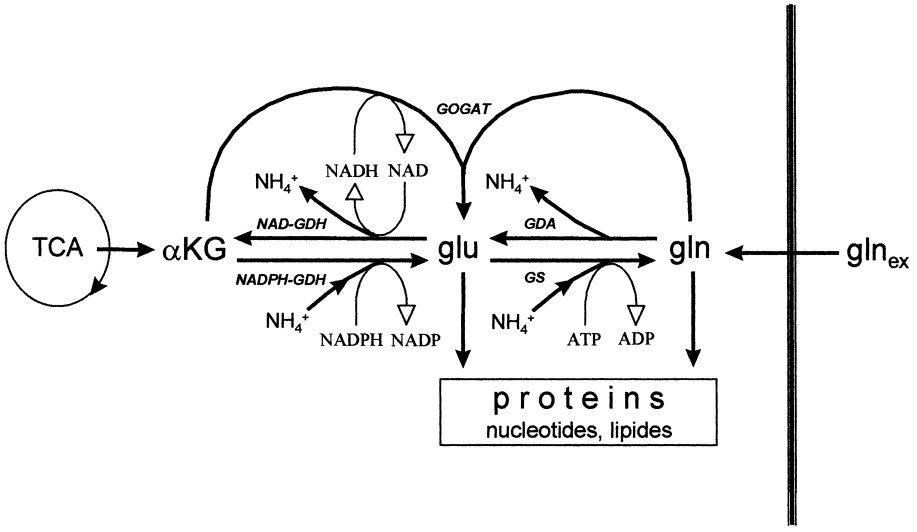

Fig. 1. Schematic presentation of the reactions involved in CNM.

roles in different microorganisms [7–12] but its function in *S. cerevisiae* is still unclear.

Mathematical models of central nitrogen metabolism have recently been constructed [13,14], based on previous data [15–17] from nitrogen-limited continuous cultures. In a mechanistic model [13] metabolic control was implemented as Michaelis–Menten enzyme kinetics. Changes in enzyme activities were modelled as first-order activation and inactivation rate constants. Modifications of gene expression, via the key transcription regulator Gln3p, were also modelled as first-order processes, representing a third level of regulation in the mechanistic model. The second model [14] was based on holistic principles of a cell as an optimally controlled system (cybernetic concept), focussing on homeostasis. Both models were successfully used to simulate and predict both the steady-state flux distribution in glutamine-limited chemostats as well as the (dynamic) responses to perturbations by pulses of ammonia and glutamine. According to the models, the GOGAT pathway plays a much more important physiological role in yeast than is generally assumed, in agreement with previous results [6,18–20]. Based on stability considerations, the role of GOGAT was predicted to be the rebalancing of the intracellular amino acid pools after disturbance of the homeostatic state by changes in the quantity or quality of the nitrogen source. It was suggested that GOGAT could be associated with the mitochondrial membrane, operating in a vectorial process, to fulfil this function. Based on these models and data evaluation it was concluded that it is worthwhile to study the function of the GOGAT pathway in *S. cerevisiae* more carefully.

Most studies of CNM use ammonia as the nitrogen source and there are few data of the CNM on growth with amino acids, such as glutamine. To evaluate the role of GOGAT in the CNM, a GOGAT-negative (*Δglt1*) mutant strain (VWk274 LEU^+) was used. This strain and the wild-type VWk43 (also called CEN.PK-113D) were grown in glutamine-limited continuous cultures, so nitrogen flux and growth rate were constant. Before the rebalancing hypothesis is experimentally verified using dynamic pulse experiments, the possible differences in the steady-state physiology of both strains is studied in this paper. Different intracellular and extracellular metabolites were analysed.

2. Materials and methods

2.1. Strains

A GOGAT-negative mutant was constructed in the diploid strain CEN.PK219. The gene was deleted by PCR targeting with short-flanking homology [21]. The DNA fragments for homologous integration were generated by PCR using a loxP-KanMX-loxP cassette with kanamycin resistance as the dominant marker [22]. After performing tetrad analyses of the resulting heterozygous deletion strain, the correct integration was verified by diagnostic PCR and subsequently the Kan^R gene was removed by expressing the *cre* recombinase (verified by PCR). In the resulting VWpk274 (*MATa*, *leu2-3,112*, *Δglt1* (41,6000)::*loxP*), the *leu2* mutations were removed by transforming with a wild-type *LEU2* gene derived from YDpLEU [23], yielding strain VWk274 LEU^+.

2.2. Growth conditions: continuous culture fermentations

S. cerevisiae VWk274 LEU^+ (*Δglt1*) and VWk43 (wild-type) strains were grown at 30°C in a BiofloIII fermenter (New Brunswick Scientific, Edison, NJ, USA), with a working volume of 2 l. The fermenter was connected to a computer controller unit with Advanced Fermentation Software (New Brunswick Scientific, Edison, NJ, USA). These strains were inoculated in a previously described

medium [24] in which NH_4Cl was replaced by 5 g l^{-1} glutamine. After batch growth, a continuous feed was connected at a dilution of 0.1 h^{-1}. This medium contained 3 g l^{-1} glutamine and 20 g l^{-1} glucose. The growth conditions were essentially as previously described [15]. The continuous culture was started when the respiration coefficient (RQ is the ratio of CO_2 evolution rate (CER (mmol l^{-1} h^{-1})) and oxygen uptake rate (OUR (mmol $l^{-1}h^{-1}$))) was below 0.9, which was usually the case after overnight growth.

During batch and glutamine-limited growth the pH was automatically controlled at 5 by adding 3 M KOH. The airflow was 1.5 l min^{-1} and the oxygen tension was kept above 50% saturation by controlling the stirrer speed. The steady state was assumed to be reached after five times the dilution time (50 h).

2.3. Sampling and sample preparation

Samples were taken when the steady state was reached and several repetitions of the sampling were carried out in the same steady state at different points in time for statistical analysis. Preparation of cell-free extracts and dry cell weight was performed as previously described [24]. Samples for determining intracellular metabolites and cofactors were taken aseptically from the fermenter, quenched immediately and extracted to assay metabolites with high turnover rates, as previously described [25]. An aliquot of the culture (5 ml) was dropped into 5 vols. of 60% methanol diluted with HEPES buffer (10 mM, pH 7.5) kept at −40°C. The mixture was centrifuged at low temperatures (the temperature of the suspension should be below −20°C after centrifugation). The supernatant was poured off and 3 ml of boiling 75% ethanol diluted with HEPES buffer (70 mM, pH 7.5) were added to the pellet and put in an 80°C water bath for 3 min. The ethanol was evaporated and the pellet resuspended in 1.5 ml of milliQ water. This solution was centrifuged at 5000 rpm for 10 min (4°C) to clean the supernatant from fines. The supernatant was then taken out and used for the metabolite assays.

2.4. Extracellular metabolite determination

Carbon compounds (acetaldehyde, acetic acid, ethanol, glycerol, pyruvic acid, succinate and trehalose) were measured by HPLC (Shimadzu, Kyoto, Japan). The temperature of the HPX-87H column was 60°C. An H_2SO_4 solution of pH 2.0 was used as eluent. To determine the glucose concentration in the feed and the supernatant a Cobas Mira S autoanalyser (Roche, Mannheim, Germany) with the glucose kit from IntruChemie, comprising hexokinase and glucose-6-phosphate dehydrogenase, was used. Free amino acids were measured by HPLC using the AccQ-tag system (Waters, Milford, MA, USA) equipped with a reversed-phase C^{18} column (temperature 37°C). Amino acids were derivatised with 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC). The separation was performed with a nonlinear gradient of 1–17% acetonitrile in a 130 mM sodium acetate buffer (AccQ.TAGTM eluent). Amino acid derivatives were detected by a fluorescence detector; excitation was performed at 245 nm and emission at 395 nm. Glutamate and glutamine were also measured with the L-glutamic acid determination kit (Boehringer Mannheim cat. No. 139 092, Mannheim, Germany).

2.5. Intracellular metabolite determination

Intracellular compounds and free amino acids were measured as described above. α-Ketoglutarate was measured enzymatically using glutamate dehydrogenase as previously described [26] (implemented on Cobas Mira S). Total glutathione and its oxidised form (GSSG) were determined as previously described [27] using a Cobas Fara autoanalyser (Roche, Mannheim, Germany). The method was almost the same in both cases, except that for GSSG, GSH was masked by vinylpyridine. The concentration of reduced glutathione (GSH) was calculated from these results. Redox cofactors were measured fluorometrically using the reaction catalysed by alcohol dehydrogenase for determining NAD^+, glucose-6-phosphate dehydrogenase for $NADP^+$, glycerol-3-phosphate dehydrogenase for NADH and glutamate dehydrogenase for NADPH [26].

3. Results

The results of the analysed metabolites are shown in Table 1. The residual concentrations of glutamine and glucose were almost zero, or undetectable, in the cultures of both strains. This could be qualified as 'double limitation'. Single nitrogen limitation does not exist in *S. cerevisiae* when it is grown with glucose as carbon and energy source in purely respiratory continuous cultures because this yeast consumes most of the available glucose, even if the energy and carbon requirements are already met [28].

The biomass yield of the *Δglt1* mutant was significantly lower than that of the wild-type (5.85 vs. 7.09 g l^{-1}). The residual concentrations of glutamine and glucose were almost zero in the cultures of both strains, so other by-products should be formed by the mutant.

Extracellular metabolites were analysed (Table 1). There were no significant differences between wild-type and *Δglt1* mutant in trehalose, acetic acid, glycerol, succinate or pyruvic acid concentration. Ethanol was either almost zero or undetectable. However, high concentrations of acetaldehyde (approx. 45 ± 13 mM) were determined in the steady-state cultures of the *Δglt1* mutant, whereas the wild-type did not exceed typical 'background' values for these fermentation conditions (approx. 5 mM). The concentrations of the same intracellular compounds were in

Table 1
Steady-state concentrations in glutamine-limited continuous cultures of wild-type and *Δglt1* mutant

		Wild-type VWk43		*Δglt1* mutant VWk274 LEU$^+$	
		Average	S.D.	Average	S.D.
Glutamine in feed	(mM)	19.96	0.0	18.48	1.2
Glucose in feed	(mM)	111.11	0.0	111.51	0.8
DCW	(g l^{-1})	7.05	0.0	5.85	0.3
Residual glutamine	(mM)	0.01	0.0	0.0	0.0
Residual glucose	(mM)	0.56	0.0	0.21	0.2
Extracellular metabolites					
Trehalose	(mM)	0.11	0.15	0.19	0.03
Pyruvate	(mM)	4.73	0.59	3.50	0.63
Succinate	(mM)	2.08	0.37	0.91	0.07
Glycerol	(mM)	1.11	0.34	0.85	0.31
Acetate	(mM)	0.48	0.68	0.45	0.06
Acetaldehyde	(mM)	4.43	0.55	45.12	12.86
Ethanol	(mM)	0.0	0.00	1.36	0.05
Intracellular amino acids[a], *ammonia and α-ketoglutarate*					
Glutamine	(μmol gX^{-1})	37.4	3.8	93.4	48.1
Glutamate	(μmol gX^{-1})	195.6	64.9	101.9	30.8
Ammonia	(μmol gX^{-1})	57.9	23.3	14.7	7.9
Aspartate/asparagine	(μmol gX^{-1})	23.6	18.2	10.8	2.6
Serine	(μmol gX^{-1})	13.0	0.7	7.1	3.0
Arginine	(μmol gX^{-1})	10.7	1.4	5.6	2.6
Alanine	(μmol gX^{-1})	25.4	1.1	9.8	4.6
Valine	(μmol gX^{-1})	11.1	2.0	5.9	2.2
α-Ketoglutarate	(μmol gX^{-1})	184.7	2.5	5.2	1.5
Redox compounds					
GSH	(μmol gX^{-1})	42.4	9.2	25.4	3.7
GSSG	(μmol gX^{-1})	1.7	0.9	5.5	1.9
GSH/GSSG	(μmol gX^{-1})	26.7	8.9	4.9	0.9
NAD^+	(μmol gX^{-1})	2.7	0.0	2.7	0.1
NADH	(μmol gX^{-1})	1.2	0.0	0.1	0.0
$NADP^+$	(μmol gX^{-1})	0.2	0.0	0.27	0.1
NADPH	(μmol gX^{-1})	0.3	0.0	0.1	0.1

Averages of $n = 3$ and $n = 4$ experiments for the wild-type and mutant, respectively.
[a]Only the amino acids with a free pool size greater than 10 μmol gX^{-1} have been included.

the same range for both strains, except for acetaldehyde, which had very low levels in the wild-type but reached up to 0.7 mmol gX^{-1} in the mutant (data not shown).

Significant differences in the steady-state levels of intracellular free ammonia and several amino acids were detected (Table 1). Glutamine in the mutant was approx. 2.5 times higher than in the wild-type (93.4 vs. 37.4 μmol gX^{-1}). On the other hand, glutamate and ammonia were lower in the mutant than in the wild-type, glutamate was twice as low (102 vs. 195 μmol gX^{-1}) and ammonia was approx. 3 times lower (14.7 vs. 58 μmol gX^{-1}). Another 13 amino acids were determined in all the steady-state samples. The intracellular concentration of these other amino acids was greater (approximately double) in the wild-type than in the mutant.

α-Ketoglutarate is essential for the interaction of carbon and nitrogen metabolism. Enormous differences were observed between the steady-state α-ketoglutarate levels of the two strains; the wild-type had a much higher level (184.7 ± 2.5 μmol gX^{-1}) than the mutant strain (5.2 ± 1.5 μmol gX^{-1}).

Glutathione has been described as storage of excess nitrogen. Concerning the nitrogen stored in glutathione (glutathione containing three nitrogen in reduced form (GSH) and six in oxidised form (GSSG)) the difference between wild-type and mutant is not striking (137.4 vs. 109.2 μmol gX^{-1}). However, the ratio of reduced vs. oxidised glutathione also indicates the redox state of the cell. For the wild-type GSH/GSSG = 26.7 (mol mol^{-1}) and only 4.9 for the mutant, indicating a significant difference in redox state.

The latter result could be supported by cofactor analysis. The levels of oxidised redox equivalents during the steady state for the wild-type and the mutant were similar (Table 1). On the other hand, the NADH and NADPH levels in all the samples of the mutant were very close to zero, but in the wild-type the NADH concentration was approx. 50% of the NAD^+ concentration.

4. Discussion

Earlier mathematical models [13,14] have indicated that the glutamate synthase (GOGAT) pathway plays a more important physiological role in yeast than is generally assumed. For a further physiological characterisation of the GOGAT function in the CNM, a GOGAT-negative (*Δglt1*) mutant strain (VWk274 LEU^+) has been studied in glutamine-limited continuous cultures. As reference, the same experiments were done with a wild-type strain (VWk43). A large number of intracellular and extracellular metabolites were analysed during several steady states in both strains. The redox state of the cell was taken into account and the NAD(H) and NADP(H) concentrations were determined as well as the reduced and oxidised forms of glutathione (GSH and GSSG, respectively).

Data from the intracellular amino acids showed that glutamine accumulated more in the mutant than in the wild-type, while the glutamate pool was higher in the wild-type than in the mutant. As expected, the GOGAT mutant converted glutamine into glutamate less efficiently. This also impaired the concentration of other amino acids, which was greater in the wild-type than in the mutant. However, the most striking differences were: (i) the biomass yield of the *Δglt1* mutant was significantly lower than that of the wild-type; (ii) the mutant produced high concentrations of acetaldehyde; (iii) the intracellular α-ketoglutarate concentration in the mutant was lower than in the wild-type; (iv) the reduced equivalents were undetectable in the mutant.

The incomplete metabolism of the nitrogen contained in glutamine for the *Δglt1* mutant is almost certainly directly related to the unbalanced redox state. The connection between the enzymes of the CNM and maintenance of the intracellular redox balance has previously been reported [29]. Overexpressing the GDH2 gene (coding for the NAD-GDH) restored the growth on glucose of a *S. cerevisiae* phosphoglucose isomerase mutant, because this CNM enzyme supplied $NADP^+$, which is needed as a cofactor in the oxidative reactions of the pentose phosphate pathway. However, the GOGAT enzyme uses NADH as cofactor and we should therefore have expected the $NADH/NAD^+$ ratio in the mutant to increase. It is known that biomass formation yields an excess of NADH for glucose-limited growth with ammonia as nitrogen source [30]. However, with glutamine as nitrogen source, significantly less NADH will be produced by biosynthesis both in the cytosol and mitochondrial matrix. Another unexpected result was the low level of α-ketoglutarate in the mutant while it is a substrate for the GOGAT reaction. Thus, we need to ask why the lack of this enzyme produced a shortage of cytosolic NADH and α-ketoglutarate. This is not easy to answer with our data, but we can postulate a possible hypothesis.

The structure analysis of the GOGAT enzyme has predicted two transmembrane domains (bp 1077–1093 and 1172–1188, data obtained from MIPS). Isolating the mitochondria of the wild-type strain showed that almost 100% of the activity was maintained [31]. This is the first experimental evidence that this protein may be associated with the mitochondrial membrane. Moreover, a possible connection between the GOGAT pathway and the maintenance of any of the intermediates of the Krebs cycle has previously been described [32]. From these observations, GOGAT's role in maintaining the redox balance could be its involvement in a redox shuttle between cytosol and mitochondria. GOGAT may use mitochondrial NADH instead of the cytosolic pool of this compound (the concentration of the local mitochondrial NADH pool in the mutant is probably not as low as the average measured in the cell extracts). The concerted action of GOGAT and NAD-GDH could then cycle glutamate while producing NADH in the cytosol, maintaining a correct redox balance. A similar cycling reaction (GS and GOGAT) in the CNM has also been reported for controlling the ATP/ADP ratio [33]. This shuttle mechanism is similar to other shuttles between cytosol and mitochondria, such as the glycerol 3-phosphate shuttle [34] and the glutamate-malate shuttle. Recently, a new ethanol-acetaldehyde redox shuttle has been reported [35] which transfers the redox equivalents from the mitochondria to the cytosol and which could take over the role of a GOGAT shuttle. But, bearing in mind our results, this alternative worked out insufficient, maybe because GOGAT is not just a redox shuttle and it is also related with the transport of mitochondrial compounds for biosynthetic reactions.

However, so far we have assumed that GOGAT utilises cytosolic α-ketoglutarate. Another possibility is that GOGAT withdraws α-ketoglutarate from the mitochondrial pool. This suggests that GOGAT is also related to the transport of α-ketoglutarate between cytosol and mitochondrion. This hypothesis also explains the low level of cytosolic α-ketoglutarate in the mutant. The glycerol 3-phosphate shuttle provides NADH that is produced in the cytoplasm for mitochondrial oxidation [34]. Concerted action of GOGAT and NAD-GDH could provide α-ketoglutarate and NADH from the mitochondria to the cytosol, working in a reverse way to the former system. It also provides ammonia for aminating reactions to the cells growing with glutamine as the sole nitrogen source.

Carbon catabolism was also impaired in the mutant, and the result was the large amount of acetaldehyde excreted. Our hypothesis suggests that α-ketoglutarate may accumulate in the mitochondria of the mutant, which would slow down the tricarboxylic acid cycle and limit the respiratory capacity of the cells. This saturation of the respiratory capacity, which is widely described as the Crabtree effect, produces an accumulation of glycolytic intermediates and a shift from the respiratory metabolism to the fermentative one. Ethanol production was not higher in the mutant than in the wild-type. The fact that more acetaldehyde was produced than ethanol may be due to

the lack of NADH in the mutant. In a previous study, the overexpression of the glycerol-3-phosphate dehydrogenase (GPD1) increased the production of glycerol, which enhanced the utilisation of NADH through the glycerol pathway, limited the formation of ethanol and caused a transient accumulation of acetaldehyde [36]. In the same study, acetate and succinate production increased in which way the cells generated some NADH. We did not detect differences in the acetate concentration between the wild-type and the mutant and succinate was a bit higher in the wild-type. A lower biomass concentration was also reported by GPD1-overexpressing strains, probably because acetaldehyde production increased during the growth phase [37]. However, our cultures were limited in glutamine and the yield was determined by the nitrogen content in the feed. As the residual glutamine concentrations of both strains in the growth medium were similar, the mutant used about 15% more energy than the wild-type did. The lower biomass yields of the GOGAT mutant are probably related to an imbalance of redox state, as shown before for *Hansenula polymorpha* [38].

In conclusion, the CNM is not just the central node between catabolism and anabolism of amino acids. It should also be considered the connection between carbon and nitrogen metabolisms, playing an important role in maintaining balanced growth. Our results point out that GOGAT (working as a shuttle between cytosol and mitochondria) may be a link between the carbon catabolic reactions (energy production) and nitrogen anabolic reactions (biomass production). There are several redox shuttles in yeast, recently reviewed [30], and the shuttle proposed in this study could be an additional one so far not recognised for glucose-limited growth with ammonia, but significant for nitrogen-limited growth with glutamine. Further analysis should be done to prove this hypothesis.

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