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Glutamine Synthetase/Glutamate Synthase Ammonium-Assimilating Pathway in *Schizosaccharomyces pombe*

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Abstract. Kinetic parameters of glutamine synthetase (GS) and glutamate synthase (glutamine-oxoglutarate aminotransferase) (GOGAT) activities, including initial velocity, pH, and temperature optima, as well as K_m values, were estimated in *Schizosaccharomyces pombe* crude cell-free extracts. Five glutamine auxotrophic mutants of *S. pombe* were isolated following MNNG treatment. These were designated *gln1-1,2,3,4,5*, and their growth could be repaired only by glutamine. Mutants *gln1-1,2,3,4,5* were found to lack GS activity, but retained wild-type levels of NADP-glutamate dehydrogenase (GDH), NAD-GDH, and GOGAT. One further glutamine auxotrophic mutant, *gln1-6*, was isolated and found to lack both GS and GOGAT but retained wild-type levels of NADP-GDH and NAD-GDH activities. Fortuitously, this isolate was found to harbor an unlinked second mutation (designated *gog1-1*), which resulted in complete loss of GOGAT activity but retained wild-type GS activity. The growth phenotype of mutant *gog1-1* (in the absence of the *gln1-6* mutation) was found to be indistinguishable from the wild type on various nitrogen sources, including ammonium as a sole nitrogen source. Double-mutant strains containing *gog1-1* and *gdh1-1* or *gdh2-1* (mutations that result specifically in the abolition of NADP-GDH activity) result in a complete lack of growth on ammonium as sole nitrogen source in contrast to *gdh* or *gog* mutants alone.

The assimilation of inorganic ammonium, the preferred nitrogen source for most bacteria, algae, yeast, and filamentous fungi, into glutamate (and glutamine) is catalyzed by NADP-specific glutamate dehydrogenase (NADP-GDH: EC 1.4.1.4.) or by a coupled glutamate synthase (GOGAT: EC 1.4.1.14 = glutamine-oxoglutarate aminotransferase) and glutamine synthetase (GS: EC 6.3.1.2.) reaction. The physiological role of these two glutamate-forming pathways varies substantially within the microbial world, but has been studied best in prokaryotic cells [13]. In enteric and nitrogen-fixing bacteria NADP-GDH reportedly is the major ammonium-assimilating pathway at high intracellular ammonium concentrations, whereas the GS/GOGAT system takes over when ammonium availability is low [5, 28, 20]. In cyanobacteria GS/GOGAT has been suggested as the only route of ammonium assimilation [21]. The relative importance of these pathways in fungi is not well understood. The GS/GOGAT pathway has been suggested to be unimportant in yeasts like *Saccharomyces cerevisiae* [4], *Candida boidinii* [10], and *C. utilis* [30]. However, in *C. albicans* GS/GOGAT proved to be a major ammonium assimilation pathway [13]. In fungi like *Aspergillus nidulans* [17] and *Neurospora crassa* [18] the major role in ammonium assimilation is played by NADP-GDH with GS/GOGAT utilized at low ammonium concentrations.

The purpose of our current work is to characterize the GS/GOGAT catalytic system in the fission yeast *Schizosaccharomyces pombe*, an organism amenable to biochemical, genetic, and molecular studies [15, 16]. Previous preliminary studies using chemostat cultures showed that NADP-GDH, GS, and GOGAT are present in *S. pombe* and that GS/GOGAT are plausibly responsible for assimilation of lower concentrations of intracellular ammonium [6, 14, 26]. This is

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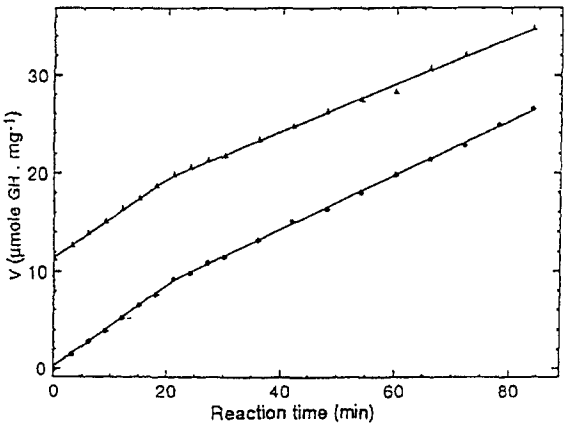

Fig. 1. Velocity of GS activity. ●, reaction mixture without added GH; ▲, 12 µmoles of GH per mg protein added to the reaction mixture at time 0.

Table 1. Kinetic parameters of *S. pombe* GS and GOGAT activities

Kinetic parameter	GS[a]	GOGAT[a]
Specific activity	0.50 ± 0.06	0.050 ± 0.004
Temperature optimum	40°C	40°C
pH optimum	5.80 and 6.35	6.35
K_m (mM Gln)	100	0.5
K_m (mM $NH_2OH.HCl$)	4.0	NA[b]
K_m (mM α-kg)	NA	0.07

[a] Cells were grown on MM with 5 mM $(NH_4)_2SO_4$ as sole N source.
[b] NA, not applicable.

further supported by certain novel biochemical, physiological, and genetic factors associated with the GS/GOGAT assimilatory pathway in *S. pombe* presented in the present report.

Materials and Methods

Strains and media. *Schizosaccharomyces pombe* wild-type strains 975 h^+ and 972 h^- [16] were used throughout this work. Cells were grown in complete (YE) or minimal media (MM) [11]. Glucose (3% wt/vol) and $(NH_4)_2SO_4$ (5 mM, or as otherwise specified in the text) were used as the carbon and nitrogen sources respectively. All other nitrogen sources were used at a concentration of 10 mM. Cells were grown in 200-ml cultures with 0.5-L conical flasks and agitated in a shaking (180 r.p.m.) orbital incubator at 30°C. Starting cultures contained 10^6 cells ml^{-1} at a pH value 4.5. Under these conditions, the generation time was 220 ± 10 min. Cells from mid-exponential phase (OD_{540} = 0.5, 1.5 × 10^7 cells ml^{-1}, pH value of culture 2.8) were harvested by centrifugation (3 min, 2300 *g*). To achieve nitrogen starvation conditions, cells were harvested as before, washed twice with fresh minimal medium lacking a nitrogen source, resuspended in this medium, and agitated as above for the desired time period. A specific nitrogen source was added in this medium when required, as specified in the text.

Preparation of cell-free extracts. Mid-exponentially growing cells were chilled in ice, harvested as before at 4°C, washed once with distilled H_2O, and cell pellets were extracted immediately as previously described [24].

Enzyme assays. NADP-GDH, NAD-GDH, and GOGAT activities were assayed essentially as described by Doherty [8] and Meers et al. [22], modified as previously described [24]. The specific activities of NADP-GDH, NAD-GDH, and GOGAT activities were estimated according to the function a · min^{-1} · mg(protein)$^{-1}$ · v.(6.22)$^{-1}$, where a = dOD and v = 3 ml (total reaction volume) [2, 3]. GS activity was measured by following glutamyltransferase activity essentially as described by Woolfolk and associates [29], modified as described previously [24]. Specific activity was estimated as µmole L-glutamic acid-γ-monohydroxamate min^{-1} mg-(protein)$^{-1}$. Protein estimations of cell-free extracts were performed routinely with the Lowry test [19]. All values were the mean of at least eight independent experiments with each cell-free extract assayed in triplicate. Standard errors were calculated by common numerical analysis software with a confidence coefficient of 99%.

Isolation of mutants. *S. pombe* cells were mutagenized by N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) treatment as described previously [24]. Minimal medium containing ammonium and 10 mM glutamine as the sole nitrogen sources was inoculated with a mutagenized cell suspension. The volume of this suspension was adjusted to give around 200–300 single cell colonies per plate. After 4 days' incubation, colonies were velvet replicated onto minimal medium agar (MMA) containing ammonium as the sole nitrogen source. Mutant isolates were purified by single cell colony isolation and used for further genetic and biochemical analysis.

Determination of generation times. Determination of generation times was carried out in liquid medium with sole nitrogen sources as stated in the Results by following changes in optical densities at 540 nm. For this purpose, 200-ml liquid batch cultures of wild-type and mutant strains were sampled (3 ml) every 60 min for a total period of 20 h.

Genetic analysis. Genetic crosses were performed according to Gutz and colleagues [11] and Kohli and coworkers [16]. Recombination frequencies were determined by free spore analysis [11, 16].

Results

GS activity. GS was measured following its γ-glutamyltransferase activity. Van Andel and Brown [26] have shown that the ratio of synthetase and transferase activities of glutamine synthetase was constant under a variety of different conditions. The GS specific activity was determined as 0.50 ± 0.06 µmole GH min^{-1} · mg^{-1} (Fig. 1, Table 1), and it was found to be specific for glutamine over a number of other amino acids tested (asparagine, glutamate, aspartate, proline, leucine, arginine, lysine, and alanine). The velocity of the activity demonstrated a hysteric phenomenon with two linear curves (0–17 min and 23–90 min, Fig. 1). Specific activity was estimated after 15 min during the period of the first linear phase. This activity had a linear response in respect to total protein concentration within a range of 0.1–0.8 mg

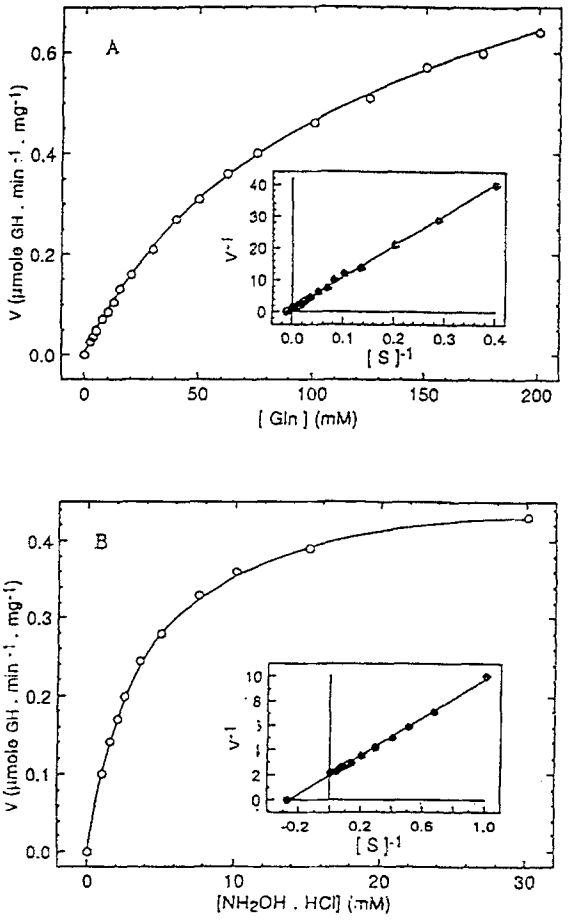

Fig. 2. The influence of glutamine (panel *a*) and hydroxylamine (panel *b*) concentration on GS activity.

and a temperature optimum at 40°C. Two pH optima were found at 5.8 and 6.35 (Table 1). These data may reflect possible different glutamine synthetase forms in *S. pombe*, as has been found in prokaryotes [7] and other lower eukaryotes [25]. GS activity followed Michaelis-Menten kinetics for both substrates tested ($NH_2OH \cdot HCl$ and glutamine) with K_m values 4 mM and 100 mM respectively (Fig. 2a,b; Table 1).

S. pombe cells did not show any significant differences in GS levels grown on various N sources such as adenine, allantoin, asparagine, aspartate, glutamate, glutamine, urea, uridine, various concentration of ammonium (1–100 mM) or under nitrogen starvation. In contrast, cells grown on histidine or cytosine as sole nitrogen source had very low levels of GS activity (Fig. 3). The presence of the protein synthesis inhibitor cycloheximide at the time of transfer in fresh media containing histidine or cytosine as sole nitrogen source prevented the loss of GS activity (Fig. 3).

GOGAT activity. GOGAT activity was specific for NADH, α-ketoglutarate (oxoglutarate) (α-kg) and

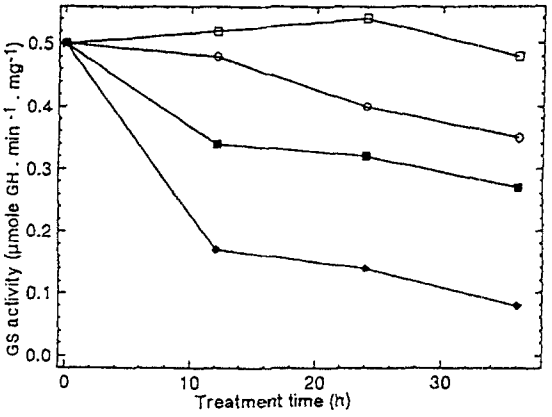

Fig. 3. The effect of cycloheximide on GS activity in the presence of histidine and cytosine. ●, His; ○, His + cycloheximide (Cyh); ■, cytosine; □, cytosine + Cyh.

glutamine. No NADP-linked GOGAT activity was observed. Temperature and pH optima were found to be 40°C and 6.35 respectively (Table 1). GOGAT activity followed Michaelis-Menten kinetics, which showed high affinity for both its substrates (Fig. 4a,b; Table 1). Growth of cells on a number of N sources as listed above, as well as transfer experiments to N starvation condition, were found to have insignificant effect on GOGAT activity levels. Here again, cells grown on histidine or cytosine as sole nitrogen sources had very low levels of GOGAT activity (about 50% of the initial), and this loss of activity was prevented by the presence of cycloheximide (for clarity, not shown on Fig. 3). Out of all the above N sources tested, only allantoin was found to have an inhibitory effect on GOGAT activity in vitro. Presence of 10 mM allantoin in the reaction mixture caused more than 25% reduction of the activity. This result possibly explains the inability of NADP-GDH-deficient mutants to grow on MM + allantoin, as described in a recent report [24].

Isolation of glutamine auxotrophs. Colonies from mutagenized cells, growing on MM containing ammonium and glutamine, were velvet replicated on solid MM containing 10 mM ammonium as sole nitrogen source. After screening 10^6 individual colonies, six were identified that were unable to grow on the screening plates. The growth of these mutants was not repaired by the addition of alanine, arginine, asparagine, aspartate, glutamate, leucine, proline, or ornithine.

The six glutamine auxotrophic isolates were crossed pairwise with each other. A very large number of progeny were analyzed in each cross, and a recombination frequency by free-spore analysis was

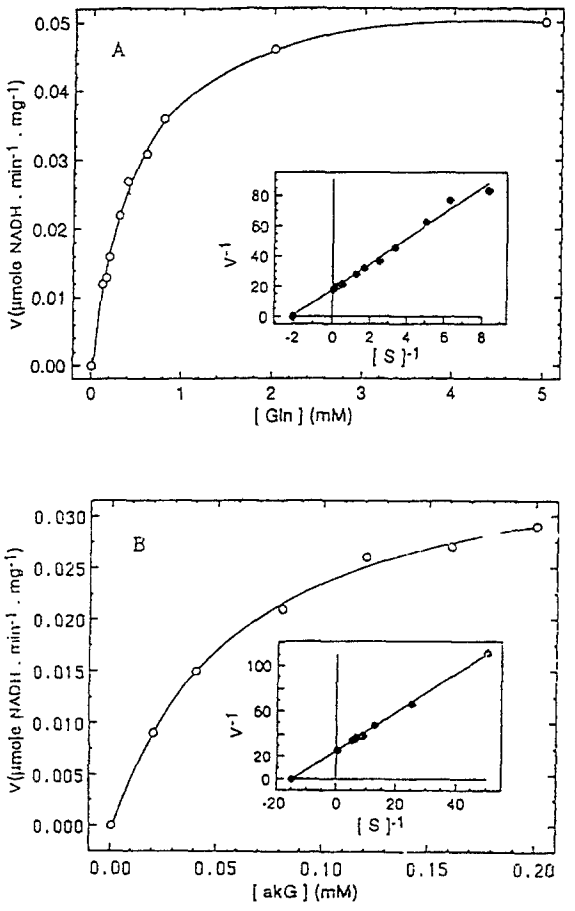

Fig. 4. The influence of glutamine (panel A) and α-ketoglutarate (panel B) concentration on GOGAT activity.

estimated as 0.2–4 × 10^{-4} (Table 2). The reversion frequency for each mutant isolate was lower than 10^{-9}. These results indicate that all mutant isolates most likely map in a single genetic locus at sites allowing intragenic recombination.

Five of the mutants designated as *gln1-1,2,3,4,5* were found to lack GS activity but retained wild-type levels of NADP-GDH, NAD-GDH, and GOGAT activities. Specifically, NADP-GDH and GOGAT activities in wild-type and mutant cells grown on MM plus 5 mM $(NH_4)SO_4$ plus 10 mM glutamine were 1.05 ± 0.10 μmole NADPH min^{-1} mg^{-1} and 0.050 ± 0.004 μmole NADH min^{-1} mg^{-1} respectively. For assaying NAD-GDH activity, glutamate was added in the MM as an inducer (A. Perysinakis, unpublished results) and found to be 0.42 μmole NADH min^{-1} mg^{-1} in both wild-type and mutant strains. The sixth glutamine auxotroph (*gln1-6*) was found to lack GOGAT as well as GS activity. This isolate was further examined, and the results are given below.

Table 2. Recombination frequencies between *gln* mutants estimated by free spore analysis

Genetic cross	Progeny analyzed	Phenotype of progeny[a] growth	Recombination frequency[b,c]
h⁺*gln1-1* × h⁻*gln1-2*	2.0 × 10^7	396	0.4 × 10^{-4}
h⁺*gln1-1* × h⁻*gln1-5*	0.9 × 10^7	1652	4.0 × 10^{-4}
h⁺*gln1-2* × h⁻*gln1-5*	1.2 × 10^7	613	1.0 × 10^{-4}
h⁺*gln1-3* × h⁻*gln1-5*	0.7 × 10^7	353	1.0 × 10^{-4}
h⁺*gln1-6* × h⁻*gln1-2*	0.4 × 10^7	48	0.2 × 10^{-4}
h⁺*gln1-6* × h⁻*gln1-5*	0.7 × 10^7	60	0.2 × 10^{-4}
h⁺*gln1-6* × h⁻*972*	7 tetrads	14	0.5

[a] Random analysis of ascospores grown on MM plus ammonium plus glutamine and screened on MM with ammonium. Ten segregants of wild-type phenotype from the cross h⁺ *gln1-6* × h⁻*972* were assayed, and three of them were found to lack GOGAT activity.

[b] Reversion frequency of *gln1-1, gln1-2, gln-3, gln1-4, gln1-5,* and *gln1-6* was < 10^{-9}.

[c] Recombination frequencies were obtained by doubling the number of progeny with a wild-type phenotype.

Identification of a GOGAT defective mutant. The *gln1-6* mutant strain, which was shown to be impaired in GOGAT as well as GS activities, was out-crossed to the wild-type strain. Both free spore analysis and tetrad analysis revealed progeny that lacked GOGAT activity while retaining GS activity. GOGAT-deficient segregants, in the absence of the *gln1-6* mutation, grew as wild type on ammonium as a sole nitrogen source under both solid and liquid cultures. This mutation, resulting in the lack of GOGAT activity, was designated *gog1-1*.

Characteristics of GOGAT and NADP-GDH double-mutant strains. The *gog1-1* mutant was crossed to NADP-GDH-defective representative mutants at the *gdh1* and *gdh2* loci (namely *gdh1-1* and *gdh2-1* respectively). It is pertinent to point out here that the single mutant *gdh1-1* and *gdh2-1* strains grow as wild type on solid medium and at a slower rate on liquid medium with ammonium as sole nitrogen source (Table 3) [24]. The double mutants *gog1-1, gdh1-1* and *gog1-1, gdh2-1* failed to grow under these conditions (Table 3).

Discussion

We describe here certain parameters and genetic factors associated with GS and GOGAT activity in the fission yeast *S. pombe*. Previous work with chemostat cultures suggested that NADP-GDH is the important route of ammonium assimilation in cells growing

Table 3. Growth characteristics of GOGAT and NADP-GDH double mutants

Mutant	Generation time (min) (liquid medium) MM	MM + Glu	Growth on (solid medium) MM
Wild-type	220	220	+[a]
gdh1-1	390	220	+
gdh2-1	400	220	+
gog1-1	220	220	+
gog1-1, gdh1-1	NA[b]	220	−
gog1-1, gdh2-1	NA	220	−

[a] +, growth; −, no growth. Generation time is given in min. The standard error calculated from four replicas was less than 5%.
[b] NA, not applicable.

in ammonium excess, with GS/GOGAT the route under ammonium-limiting conditions [6, 14, 26]. The results of these reports in batch cultures suggest that the GS/GOGAT activities remain constant in cells grown on various concentrations of ammonium or various other nitrogen sources as sole N sources, including ammonium-limiting conditions (0.5 mM initial concentration of ammonium as sole N source). Therefore, there is some disagreement with the above earlier findings. The results presented here showed that histidine and cytosine markedly reduced GS and GOGAT activity. The effect of histidine could be indirectly connected with a transcription activation system, similar to the one described for the activation of the transcription of the *E. coli* glutamine synthetase [9, 23, 27]. In the later organism a histidine kinase and the intracellular level of acetyl phosphate regulate GS transcription. Alternatively, the effect of histidine could resemble the effect of glycine and serine on the GS of *N. crassa* [12]. In a previous communication we have reported that histidine increased NADP-GDH activity in *S. pombe* when histidine was used as the sole nitrogen source [24]. There are no other reports to our knowledge regarding the effect of histidine, and this phenomenon would require further investigation for clarification.

Our genetic studies revealed one genetic locus, *gln1*, within which mutations result in the complete loss of GS activity. This locus could be the one reported by others [1]. Previously isolated mutants have not been made available to us to check allelism. Significantly, a new locus was identified, within which mutations result in abolition of GOGAT activity. This locus, designated *gog1*, revealed that GOGAT must be able to provide glutamate at high ammonium concentrations, in the absence of NADP-GDH, sufficiently for a *gdh*-defective strain to grow on ammonium like the wild type. Since the double mutants between *gog1-1* and NADP-GDH defective strains fail to grow on ammonium, unlike the single mutants, it is suggested that (1) both systems are normally operational in *S. pombe*, (2) no other major ammonium assimilation step is present.

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