Functional Analysis of Ammonium Assimilation Enzymes in *Neurospora crassa*

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In order to explore the function of each of the four enzymes involved in ammonium assimilation in *Neurospora crassa* [NADP-dependent glutamate dehydrogenase (GDH), NADH-dependent glutamate synthase (GOGAT) and the α and β isozymes of glutamine synthetase (GS)] growth curves, enzymic activities and intracellular pools of ammonium, glutamate and glutamine were determined in the wild-type as well as in mutants lacking one or two of these biosynthetic enzymes. These parameters were measured in ammonium-limited chemostat-type cultures, in which conditions resembling a continuous culture were achieved for the first time for a filamentous fungus, and in ammonium-excess batch cultures. NADP-dependent GDH appeared to be the main provider of cellular glutamate in the presence of limited and excess ammonium but could be partially replaced by NADH-dependent GOGAT depending upon the presence of GS β . GOGAT appeared to have the main role in the recycling of glutamine to glutamate, particularly in ammonium-limited conditions. GS β was the main provider of cellular glutamine but GS α could substitute for this activity if GDH was also present. In conditions of ammonium excess GDH and GOGAT were repressed by glutamate and GS was repressed by glutamine, as previously found. However, in conditions of ammonium limitation these enzymes appeared to escape nitrogen repression.

INTRODUCTION

The enzymes that participate in the assimilation of ammonium in *Neurospora crassa* are NADP-dependent glutamate dehydrogenase (GDH) (EC 1.4.1.4) (Brett *et al.*, 1976), NADH-dependent glutamate synthase (GOGAT) (EC 1.4.7.1) (Hummelt & Mora, 1980*a,b*) and glutamine synthetase (GS) (EC 6.3.1.2). The GS of *N. crassa* is composed of two different monomers, α and β , that differ in their electrophoretic mobility (Sánchez *et al.*, 1980). The α and β monomers form different isozymes with tetrameric or octameric forms respectively (Dávila *et al.*, 1980).

It has been reported that a mutant strain of *N. crassa* lacking NADP-dependent GDH activity is able to grow on ammonium as the sole nitrogen source (Fincham, 1950). We have reported the existence of an NADH-dependent GOGAT in *N. crassa*, and that a double mutant lacking GDH and GOGAT activities (*am*; *en-am-2*) is completely unable to grow on ammonium as the sole nitrogen source (Hummelt & Mora, 1980*a,b*). This double mutant is able to assimilate ammonium when growing in media with other amino acids like glutamate, glutamine or alanine as nitrogen sources (Calderón & Mora, 1985).

We have reported the isolation of revertants, capable of growing without glutamine, from *N. crassa* partial glutamine auxotrophs (Dávila *et al.*, 1983). The biochemical characterization of GS from these revertants showed that it contained only the α monomer (Dávila *et al.*, 1983).

Abbreviations: GDH, glutamate dehydrogenase; GOGAT, glutamate synthase; GS, glutamine synthetase.

Physiological studies of the wild-type strain have suggested that GDH and GS β are responsible for the assimilation of ammonium when it is added in excess to the growth medium (Lara *et al.*, 1982; Hernández *et al.*, 1983). We have proposed that in fed-batch ammonium-limited cultures of *N. crassa*, ammonium is assimilated mainly by the operation of GS α and GOGAT (Lara *et al.*, 1982). A similar scheme has been proposed for *Klebsiella aerogenes*, with GDH and GS responsible for assimilation of ammonium at high concentrations of the latter, and with GS and GOGAT active at low ammonium concentrations (Meers *et al.*, 1970; Tempest *et al.*, 1970). In *N. crassa*, however, the two pathways are not as exclusive as those in *K. aerogenes*; the α and β GS polypeptides are present under all nitrogen growth conditions, although the α/β ratio is greater under ammonium limitation (Lara *et al.*, 1982). Likewise, in this paper we report a sizeable activity of GDH in ammonium-limited cultures, and, reciprocally, some GOGAT activity present in ammonium-excess cultures.

In order to define more precisely the participation of each of these enzymes, we determined the behaviour of a number of different metabolic parameters in single and double ammonium-assimilation mutants, grown in batch ammonium-excess cultures as well as ammonium-limited continuous cultures that resemble a chemostat. The latter type of culture allows a more accurate description of the metabolic adaptation that takes place during ammonium limitation.

METHODS

Strains. The *N. crassa* wild-type strain 74A, as well as the *am* strain (GDH⁻), which lacks GDH activity due to a mutation in the structural gene that codes for GDH (Brett *et al.*, 1976), were obtained from the Fungal Genetics Stock Center (Humboldt State University Foundation, Arcata, Calif., USA). The mutants *en-am-2* (GOGAT⁻), which lacks GOGAT activity (Hummelt & Mora, 1980a), and *gln-1bR8* (GS β ⁻), which lacks the GS β polypeptide (Dávila *et al.*, 1983), were from the collection of J. Mora. The double mutants *am: gln-1bR8* (GDH⁻; GS β ⁻) and *en-am-2: gln-1bR8* (GOGAT⁻; GS β ⁻) were obtained by crossing the respective single mutant strains as described by Davis & De Serres (1970) and selecting the double mutant from the progeny.

Growth conditions. Liquid cultures were inoculated with a fresh suspension of conidia grown for 5 d in solid Vogel's minimal medium (Vogel, 1964), containing 0.05% (w/v) glutamate and 1.5% (w/v) sucrose. The batch cultures were grown with agitation on a modified Vogel's medium in which the nitrogen source was replaced by 25 mM-NH₄Cl. In chemostat-type cultures (BioFlo R model C30 bench top chemostat) ammonium limitation was started after an initial 6 h nitrogen deprivation. Fresh growth medium containing 1 mM-NH₄Cl as nitrogen source was injected at a dilution rate of 0.1 h⁻¹ for 14 h; the cultures were agitated and aerated.

The growth temperature for all cultures was 25 °C. Growth was measured as total protein concentration (Vichido *et al.*, 1978).

Enzyme activities. Cell free extracts were prepared as described by Vichido *et al.* (1978). GS was assayed by determining its synthetase activity (Ferguson & Sims, 1974), GDH by following NADPH oxidation (Fincham, 1950), and GOGAT by following NADH oxidation (Boland & Benny, 1977). Specific enzymic activities are expressed as $\mu\text{mol product formed min}^{-1} (\text{mg culture protein})^{-1}$.

Determination of extracellular ammonium. This was done after removing cells by filtration through membrane filters (Millipore type HA, 0.45 μm). In batch and fed-batch type cultures an Orion electrode was used as described by Espin *et al.* (1979). In chemostat-type cultures the extracellular ammonium concentration was measured as described by Middelhoven *et al.* (1976) with the following modifications. A sample (20 ml) of extracellular medium was placed in a 125 ml flask; 2 ml of saturated KOH solution were added and the flask was immediately closed with a rubber tap from which a wad of plastic fibre impregnated with 0.2 ml 1 M-HClO₄ was attached by means of a glass hook. After incubating the flask for 2 d at 37 °C, the wad of fibre was transferred to a test tube containing 0.8 ml 1 M-potassium phosphate buffer, pH 7.6, and 0.6 ml water. After centrifugation, the ammonium concentration in the supernatant was determined as the initial velocity of NADH oxidation using bovine GDH (measured as the change in A_{340}). A sample (1 ml) of each supernatant was incubated with 20 mM-2-oxoglutarate, 0.1 mM-NADH and approximately 1400 units bovine liver GDH (Sigma no. G-2626). The ammonium concentration was quantified by comparison of the initial velocity of each reaction with a standard curve.

Determination of intracellular metabolites. Samples for amino acid analysis were prepared by homogenizing conidia with 80% (v/v) ethanol (Hummelt & Mora, 1980a). The amino acids were separated with an Aminco amino acid analyser, coupled with ortho-phthalaldehyde (Sigma), and quantified in an Aminco ratio fluorimeter. Intracellular ammonium pools were determined by the methods of Tempest *et al.* (1970) and Tachiki *et al.* (1981) with the following modifications. Mycelia containing 5 mg protein were collected by filtration and washed twice with 5 ml Vogel's minimal medium lacking a nitrogen source. The mycelia were then resuspended in 1 ml cold

0.25 M-HClO₄ and centrifuged at 3000 r.p.m. for 5 min; 1 ml potassium phosphate buffer (1 M, pH 7.6) was then added to the supernatant, and the ammonium concentration measured using bovine GDH as described above.

Reproducibility of results. The experiments reported were each repeated at least once; representative results are shown.

RESULTS

Growth, nitrogen metabolites and enzymic activities in ammonium excess cultures

The growth curves (Fig. 1), intracellular ammonium and amino acid pools (Table 1) as well as the activities of ammonium assimilation enzymes (Table 2) were determined in ammonium assimilation mutant strains in order to compare them with those of the wild-type strain.

The GOGAT⁻ mutant (*en-am-2*) grew best with excess ammonium (Fig. 1*b*). It maintained a low intracellular ammonium content equal to that of the wild-type strain; however, its glutamate pool was slightly lower, and its glutamine pool higher, than that of the wild-type (Table 1). In this mutant strain, a decrease in GS activity was apparent (Table 2).

The growth curve of the GDH⁻ mutant strain (Fig. 1*c*) showed an initial lag phase. From 10 to 20 h after inoculation its growth rate was constant and was approximately half that of the wild-type strain (data not shown). This mutant strain has a 6-fold higher intracellular ammonium content (Fincham, 1950) in spite of having twice the GOGAT activity of the wild type, and it had a 2.5-fold lower glutamate content than the wild-type strain (Table 1). These data suggest that GDH is the enzyme with the highest capacity to assimilate ammonium into glutamate.

The single mutant strain lacking the GS β monomer, and therefore containing only the GS α isozyme, grew slower than the wild-type strain (Fig. 1*d*). The intracellular ammonium content was as low as in the wild-type strain, and an elevated intracellular glutamate content and a low glutamine content (Table 1) was observed. This mutant strain had a low GDH activity and no detectable GOGAT activity (Table 2). Glutamate repression of GDH and GOGAT in *N. crassa* has been reported previously (Hernández *et al.*, 1983; Hummelt & Mora, 1980*b*). These data suggest that the GS β isozyme has a greater capacity for glutamine synthesis than the GS α isozyme and that GDH has a high capacity to synthesize glutamate.

Fig. 1. Growth curves of different strains of *N. crassa* grown in batch cultures with 25 mM-NH₄Cl as the nitrogen source. (a) Wild-type strain; (b) GOGAT⁻; (c) GDH⁻; (d) GS β ⁻; (e) GOGAT⁻;GS β ⁻; and (f) GDH⁻;GS β ⁻ mutants. Protein concentration and density of conidial inoculum were proportional (10 μ g protein ml⁻¹ $\equiv$ 2 $\times$ 10⁶ conidia ml⁻¹). The inoculum used in (c) and (f) was higher in order to have enough material to determine the metabolite contents and enzyme activities in these cultures (Tables 1 and 2); the behaviour of the cultures was the same when a lower inoculum, similar to the other cultures, was used.

Table 1. *Intracellular metabolite contents of different strains of N. crassa grown on excess ammonium*

The metabolite contents were determined after 12 h growth and are expressed as $\mu\text{mol (mg protein)}^{-1}$. ND, Not detected.

Strain	Ammonium	Glutamate	Glutamine
Wild-type	0.03	0.10	0.18
GOGAT ⁻	0.03	0.07	0.22
GDH ⁻	0.19	0.04	0.16
GS β ⁻	0.03	0.32	0.01
GOGAT ⁻ ; GS β ⁻	0.02	0.28	ND
GDH ⁻ ; GS β ⁻	0.31	0.07	0.01

Table 2. *Specific enzyme activities of different strains of N. crassa grown on excess ammonium*

Specific enzyme activities [$\mu\text{mol min}^{-1} \text{ (mg protein)}^{-1}$] were determined after 12 h growth. ND, Not detected.

Strain	GDH	GOGAT	GS
Wild-type	0.55	0.005	0.04
GOGAT ⁻	0.65	ND	0.02
GDH ⁻	ND	0.010	0.03
GS β ⁻	0.23	ND	0.01
GOGAT ⁻ ; GS β ⁻	0.21	ND	0.01
GDH ⁻ ; GS β ⁻	ND	0.004	0.01

Table 3. *Specific enzyme activities of different strains of N. crassa grown in chemostat-type ammonium-limited cultures*

Specific enzyme activities [$\mu\text{mol min}^{-1} \text{ (mg protein)}^{-1}$] were determined after 14 h growth. ND, Not detected.

Strain	GDH	GOGAT	GS
Wild-type	0.56	0.002	0.031
GDH ⁻	ND	0.010	0.040
GOGAT ⁻	0.91	ND	0.027
GS β ⁻	0.62	0.005	0.008
GOGAT ⁻ ; GS β ⁻	0.62	ND	0.015

The growth rate and metabolic profile found in the GOGAT⁻; GS β double mutant was similar to that of the GS β ⁻ single mutant (Fig. 1e; Tables 1 and 2). The fact that this mutant which lacks GOGAT activity accumulated glutamate while maintaining a low intracellular ammonium pool, indicated once more that GDH has a high capacity for glutamate synthesis.

The GDH⁻; GS β ⁻ double mutant grew very slowly (Fig. 1e). Even after 20 h of culture (data not shown), the growth rate was slower than that of either parent mutant strain. This mutant strain had the largest ammonium pool compared to all other strains (Table 1), and it had a very low glutamine content (Table 1). We inferred that GDH and GS β are the enzymes with the highest capacity for ammonium assimilation in *N. crassa*, and that the capacities of GS α to synthesize glutamine and of GOGAT to synthesize glutamate depend upon the presence of GDH and GS β respectively.

Growth, nitrogen metabolites and enzyme activities in ammonium-limited chemostat-type cultures

In the experiments done with chemostat-type cultures, ammonium was limited in order to maintain a growth rate of one-third of the maximum that occurs in the presence of excess

Fig. 2. Growth and metabolite contents of different strains in ammonium-limited chemostat-type cultures. Spores of the wild-type strain (a), and the GDH^- (b), GOGAT^- (c), $\text{GS}\beta^-$ (d) and $\text{GOGAT}^- ; \text{GS}\beta^-$ (e) mutants were deprived of nitrogen for 6 h before the experiment. Fresh medium containing 1 mM- NH_4Cl was injected at a dilution rate of 0.1 h^{-1} (doubling time = 6.9 h). Growth ($\circ$), extracellular ammonium concentration ($\blacksquare$), and intracellular ammonium ($\square$) glutamate ($\blacktriangle$) and glutamine ($\triangle$) contents, were determined for each strain.

ammonium, after first depriving the cells of all nitrogen for 6 h. Under these conditions the protein concentration remained nearly constant. Enzyme activities and metabolite pools were determined in the wild-type and mutant strains after 14 h growth (Table 3; Fig. 2), which was as long as this type of continuous culture could be maintained.

After 5 h growth in these conditions the wild-type strain showed a moderate increase in intracellular ammonium as well as a rise in glutamine content that coincided with a drop in protein concentration; 3 h later these measurements had returned to their original values (Fig. 2a). After 8 h all ammonium had been incorporated into organic molecules and the glutamine pool was low, whereas the glutamate pool was twice its original size (Fig. 2a). A slight increase in extracellular ammonium was observed during the last 3 h (Fig. 2a).

During the first hours of growth, the GDH^- mutant showed a dramatic increase both in intracellular and extracellular ammonium content and in its glutamine pool (Fig. 2b), whereas it maintained a steady glutamate pool slightly lower than that of the wild-type strain (Fig. 2a,b). Only after 11 h did the metabolite contents return approximately to their original values (Fig. 2b).

It is clear that the wild-type strain adapted more rapidly than the GDH^- mutant in order to assimilate the limiting amounts of ammonium present in this type of culture. At the end of the experiment, the GDH^- mutant had a GOGAT activity 5-fold that of the wild-type strain (Table 3). This enzyme activity is able to replace GDH in glutamate synthesis. By this time the ammonium accumulated during the first hours by the GDH^- mutant had been fully assimilated, and a higher glutamate/glutamine ratio, similar to that of the wild-type strain, had been reached (Fig. 2a,b).

The GOGAT^- mutant did not accumulate ammonium, but had a very low glutamate pool and a high glutamine pool (Fig. 2c) indicating the involvement of GOGAT activity in glutamine recycling to glutamate. The activity of GDH in this mutant was almost twice that found in the wild-type (Table 3). Despite the high glutamine content no difference was found in GS activity

(Table 3), contrary to the previously reported effect of glutamine as a repressor of GS (Vichido *et al.*, 1978; Lara *et al.*, 1982) and nitrogen catabolism (Marzluf, 1981) when *N. crassa* is grown on excess substrate.

The GS β ⁻ mutant showed a lower protein yield than the wild-type strain when grown in ammonium-limited chemostat-type cultures (Fig. 2*d*). It did not accumulate ammonium, its glutamate content was highly elevated and its glutamine pool was very low (Fig. 2*d*). In these conditions the GS β ⁻ mutant had high GOGAT activity (Table 3), which differed from the undetectable level of GOGAT when the strain was grown on excess ammonium. This difference occurred despite the fact that the glutamate content of the GS β ⁻ mutant in ammonium-limited cultures (Fig. 2*d*) was slightly higher than that found in ammonium-excess cultures (Table 2).

The GOGAT⁻; GS β ⁻ double mutant grew with a low protein yield in ammonium-limited chemostat-type cultures (Fig. 2*e*) similar to the GS β ⁻ parent single mutant. This double mutant had a high intracellular glutamine content, similar to that observed in the GOGAT⁻ parental strain, despite the fact it had low GS activity (Table 3) due to the absence of GS. These data indicate once more the importance of GOGAT for the maintenance of a low glutamine/glutamate ratio.

DISCUSSION

The presence in several micro-organisms of more than one enzyme able to assimilate ammonium into glutamate (Tempest *et al.*, 1970; Meers *et al.*, 1970; Brencley *et al.*, 1976; Pahel *et al.*, 1978; Ledingger *et al.*, 1980; Senior, 1975; Brown *et al.*, 1973; Roon *et al.*, 1974) and glutamine (Darrow & Knotts, 1977; Keistler & Fuchs, 1980; Wedler *et al.*, 1981) has been described. Only in *K. aerogenes* has the role of the enzymes that synthesize glutamate been elucidated (Tempest *et al.*, 1970; Meers *et al.*, 1970). An integral study of the role of individual enzymes of ammonium assimilation in other micro-organisms, as well as their role in relation to each other, has not previously been reported. For instance, in *E. coli* GDH and GOGAT activities are both present in ammonium-excess cultures (Senior, 1975) but their precise functions are not known.

Our results suggest that despite having a lower affinity for ammonium than GS (Wootton, 1983; J. Guzmán and J. Mora, unpublished), GDH has the higher enzymic capacity for nitrogen assimilation and is the main provider of cellular glutamate when *N. crassa* is grown either on excess or limiting ammonium.

The data obtained in the experiments using chemostat-type ammonium-limited cultures (Fig. 2; Table 3) suggest that GDH and GOGAT activities are both involved in glutamate synthesis under such conditions.

We propose that the principal function of GOGAT in *N. crassa* is the recycling of some organic nitrogen from glutamine to glutamate. This is indicated by the low glutamate/glutamine ratio present in the GOGAT⁻ mutant, both in ammonium-excess (Table 1) and even more dramatically in ammonium-limited cultures (Fig. 2*c*). The high glutamate pools found in the wild-type strain when grown on limited ammonium (Fig. 2*a*) may provide substrate availability for the efficient operation of the reversible transaminase reactions. This is not necessary in the case of glutamine, low levels of which (Fig. 2*a*) may be sufficient to drive irreversible transamidase reactions.

We propose that the role of GOGAT in the recycling of glutamine is more relevant in ammonium-limited conditions, in which GS could efficiently assimilate ammonium in an irreversible way (since GS has a higher affinity for ammonium than does GDH), with consequent glutamate deprivation. Thus GOGAT, under these conditions, could recycle glutamine to glutamate avoiding glutamate deprivation and maintaining the proper glutamate/glutamine ratio needed for the distribution of amino and amido nitrogen to other cellular components.

Another possible function of GOGAT might be the prevention of glutamine accumulation and the consequent nitrogen repression. However, the glutamine accumulated in the GOGAT⁻ mutant strain grown on limited ammonium (Fig. 2*c*) had a minimal effect on GS (Table 3) as compared to the effect of glutamine accumulated under ammonium-excess conditions (Table 2).

Furthermore, the glutamine-repressible enzyme L-amino acid oxidase (Sikora & Marzluf, 1982) from the same GOGAT⁻ mutant was not repressed by glutamine under ammonium-limited fed-batch conditions (data not shown). We have also demonstrated that the intracellular glutamine present in ammonium-limited conditions was not compartmentalized in vesicles (data not shown), as opposed to other amino acids like arginine that have been reported to be compartmentalized in vesicles in *N. crassa* (Weiss & Davis, 1977).

As expected (Hernández *et al.*, 1983; Hummelt & Mora, 1980b) GDH and GOGAT were both repressed in the GSβ⁻ mutant strains which had high glutamate contents when grown on excess ammonium (Tables 1 and 2). However this repression again was not observed in ammonium limitation (Fig. 2; Table 3).

The unexpected result showing the lack of nitrogen repression by accumulated glutamine of some enzymes in ammonium-limited chemostat-type cultures suggests a possible relation between efficient nitrogen repression and an optimum cellular growth rate and carbon flow, which does not occur in limited growth conditions.

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