Regulation of Carbon and Nitrogen Flow by Glutamate Synthase in *Neurospora crassa*

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A glycine-resistant *Neurospora crassa* mutant (*am-132; gly^r*), derived from the *am-132* mutant, was isolated and characterized. [*am-132* itself has a deletion in the structural gene for NADP-dependent glutamate dehydrogenase (GDH).] This new mutation also conferred resistance to serine and methionine sulfoximine (MS), which are inhibitors of glutamine synthetase (GS). In addition, the mutant obtained grew better on ammonium than the *am-132* parental strain. Resistance to glycine was not due to increased synthesis of glutamine by an altered or induced GS, nor to increased glutamate synthesis by induction of the catabolic NAD-dependent GDH, nor to NADH-dependent glutamate synthase (GOGAT), which was as sensitive to inhibitors as the GOGAT from the parental strain. The glycine-resistance mutation lowered but did not abolish the carbon flow; this resulted in a lower content of tricarboxylic acid cycle intermediates. GOGAT activity was inhibited *in vitro* by several organic acids and methionine sulphone (MSF). The higher growth rate of the glycine-resistant mutant on ammonium or on ammonium plus glycine, serine or MS was explained by an increased capacity of GOGAT to synthesize glutamate *in vivo* due to a lower content of inhibitory tricarboxylic acid cycle intermediates; the higher glutamate content overcomes the effect of the GS inhibitors and explains the MSF resistance of the mutant.

INTRODUCTION

The regulation of nitrogen metabolism in *Neurospora crassa* has been investigated previously (Hummelt & Mora, 1980*a, b*; Hernández *et al.*, 1983; Lara *et al.*, 1982; Vichido *et al.*, 1978). The growth on ammonium of a *N. crassa* mutant that lacks NADP-dependent glutamate dehydrogenase (GDH) (EC 1.4.1.4) is inhibited by the amino acids glycine and serine (Fincham, 1950). This is due to the inhibition of glutamine synthetase (GS) (EC 6.3.1.2) which results in the diminution of the carbon flow (Hernández, *et al.*, 1986). When nitrogen assimilation is blocked by inhibition of glutamine synthesis, carbon catabolism from sucrose decreases (Hernández & Mora, 1986). We proposed that the drain of ATP through glutamine synthesis and the regulation of carbon metabolism by the energy charge could be considered as a point of interaction in the coordinated regulation of carbon and nitrogen metabolism.

On this basis, we now report the isolation and characterization of a glycine-resistant (*gly^r*) mutant strain of *N. crassa*, that has altered carbon and nitrogen flows.

METHODS

Strain. The *Neurospora crassa* wild-type strain 74A was from the Fungal Genetics Stock Center (Humboldt State University Foundation, Arcata, Calif., USA). The mutant strain *am-132a*, which has a deletion in the NADP-dependent GDH structural gene (Kinsey & Hung, 1981), was kindly provided by J. A. Kinsey, Department of Microbiology, University of Kansas Medical Center, Kansas 66103, USA.

Abbreviations: GDH, glutamate dehydrogenase; GOGAT, glutamate synthase; GS, glutamine synthetase; MS, methionine sulfoximine; MSF, methionine sulphone.

Growth conditions. Batch cultures of *N. crassa* were grown at 37 °C on Vogel's minimal medium (N) (Vogel, 1964) containing 1.5% (w/v) sucrose. Other nitrogen sources used in addition to ammonium nitrate are stated in the text. Growth was measured as total protein (determined by the Lowry method) as described previously (Lara *et al.*, 1982; Vichido *et al.*, 1978).

Mutagenesis and mutant selection. A conidial suspension of strain *am-132a* was prepared and adjusted to 4×10^6 conidia ml⁻¹ in sterile water. A sample of the suspension (5 ml) was placed in a Petri dish and exposed to direct UV radiation for 60 s. After the mutagenic treatment, 0.2 ml of the suspension were plated on N agar medium (Vogel, 1964) supplemented with glucose and fructose (0.05%, w/v, each), 2% (w/v) sorbose and 25 mM-glycine. Plates were incubated in the dark at 29 °C until colonies appeared (2 to 7 d), and then transferred to slants of N agar medium supplemented with 1.5% (w/v) sucrose, and 25 mM-glycine. Crosses were made in Corn meal agar (34 g l⁻¹) (Difco). Spot testing and progeny analysis were done as described by Davis & De Serres (1970).

Determination of enzyme activities. This was done in cell-free extracts of *N. crassa* prepared as described previously (Lara *et al.*, 1982; Vichido *et al.*, 1978). GS activity was measured as described by Ferguson & Sims (1974). NAD-dependent GDH (EC 1.3.1.2) was measured as described by Fincham (1957). NADH-linked glutamate synthase (GOGAT) (EC 1.4.1.14) was measured by following NADH oxidation as described by Hummelt & Mora (1980a). Enzyme activities are expressed as units (mg protein)⁻¹. One unit is the amount of activity that transforms 1 µmol substrate min⁻¹.

In the experiments where GOGAT activity was inhibited *in vitro*, the enzyme was purified as described by Hummelt & Mora (1980b), except that the DEAE column was equilibrated with 0.05 M-phosphate buffer. A 14-fold purified preparation was obtained. The final concentration of the organic acids was 10 mM and the solutions were adjusted to pH 8.6 with 1 M-KOH.

Determination of amino acids. Samples for amino acid analysis were prepared by homogenizing cells with 80% (v/v) ethanol (Hummelt & Mora, 1980a). The amino acids were separated by using an Aminco amino acid analyser, and after being coupled with orthophthalaldehyde (Sigma) were quantified in an Aminco ratio fluorimeter.

Determination of tricarboxylic acid cycle intermediates. Mycelial or conidial samples were collected on membrane filters (Millipore type HA, 0.45 µm) and washed with distilled water. Organic acids were extracted by resuspending the samples in 0.6 M-HClO₄ and neutralizing with 2.0 M-K₃PO₄; they were then determined in the clear supernatant after centrifugation. The extracts were stored at 4 °C before the determinations were done. 2-Oxoglutarate was determined by measuring the initial rate of change in A₃₄₀ using bovine GDH. A sample of the extract was incubated at room temperature with 30 mM-ammonium sulphate, 0.1 mM-NADH and 0.01 ml 2.5% (v/v) bovine liver GDH [50 units (mg protein)⁻¹] (Sigma no. G2626) in 1 ml final volume 100 mM-potassium phosphate buffer, pH 7.6.

The other tricarboxylic acid cycle intermediates were separated and quantified by HPLC [method of J. Calderón (unpublished)].

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

RESULTS

Genetic and phenotypic analysis

The *am-132* mutant has a deletion in the NADP-dependent GDH structural gene (GDH⁻) (Kinsey & Hung, 1981) and is unable to grow on ammonium plus glycine (Fig. 1) or serine, like other mutant strains which have a point mutation in the structural gene of this enzyme (Fincham, 1950; Brett *et al.*, 1976). The *am-132* mutant was used as the parental strain for mutant selection. One of the *am-132* glycine-resistant (*gly*^r) mutants isolated was crossed with the wild-type strain 74A. One *am-132;gly*^r mutant obtained from this cross was backcrossed again with the wild-type strain and the progeny were analysed: 57% were wild-type, 24% *am-132* and 19% *am-132;gly*^r. These results indicated that the *gly*^r mutation segregated as a monogenic mutation. The work we report here was done with a GDH⁻ *am-132;gly*^r double mutant obtained from the latter cross.

Fig. 1 shows the growth of the *am-132;gly*^r mutant on ammonium plus glycine compared to the *am-132* single mutant, which is unable to grow on this nitrogen source (Fincham, 1950), and the wild-type strain, which had a higher growth rate. Unexpectedly, the growth rate of the *am-132;gly*^r mutant on ammonium was higher than that of the GDH⁻ strain (Fig. 1), but lower than that of the wild-type strain. This same phenomenon was observed when the cultures were grown at 25 °C (data not shown), when the growth rate on ammonium of the GDH⁻ mutant strain (*am*) is considerably lower (Hummelt & Mora, 1980b) than at 37 °C (Fig. 1). In addition, this new

Fig. 1. Growth of the *am-132;gly^r* (●, ○) and *am-132* (▲, △) mutants and the wild-type strain (■, □) of *N. crassa* on ammonium (●, ▲, ■) or ammonium + 50 mM-glycine (○, △, □). The experiment was done four times; results from a representative experiment are shown.

Fig. 2. Intracellular content of 2-oxoglutarate of the *am-132;gly^r* (●, ○) and *am-132* (▲, △) mutants and the wild-type strain (■, □) of *N. crassa* grown on ammonium (●, ▲, ■) or ammonium + 50 mM-glycine (○, △, □). The experiment was done three times; representative results are shown.

Fig. 3. Inhibition *in vivo* of growth of the *am-132;gly^r* and *am-132* mutants by MSF. Conidia were pregerminated for 10 h in minimal medium at 37 °C with agitation; MSF was then added to each culture. ●, ■, ▲, ▼, *am-132;gly^r* mutant; ○, △, ▽, *am-132* mutant. ●, ○, Minimal medium without MSF; △, ▲, 0.01 mM-MSF; ■, 0.05 mM-MSF; ▼, ▽, 0.1 mM-MSF. The experiment was done twice; representative results are shown.

mutation also conferred resistance to serine and to methionine sulfoximine (MS) (data not shown), a specific GS inhibitor (Ronzio & Meister, 1968).

Activities of ammonium-assimilation enzymes in the *am-132;gly^r* mutant

We reported previously the isolation of a glycine-sensitive mutant that maps in the structural gene of GS and has an altered and inactive β polypeptide in its GS (Hernández *et al.*, 1986). However, the *am-132;gly^r* mutant has a wild-type GS as indicated by the M_r and isoelectric point of its α and β GS monomers (data not shown). As mentioned before, the *gly^r* mutation segregated independently from the *am-132* mutation, which indicates that this new mutation did not map in the structural gene of GS since the *am* and *gln* loci both map in the VR chromosome and are closely linked (Sober, 1970).

GS activity was not considerably increased in the *am-132;gly^r* mutant compared to the *am-132* strain, either when grown on ammonium or on ammonium plus glycine (Table 1). High GS activity was not responsible for growth on glycine as the *am-132* mutant had higher GS activity (Table 1) and was unable to grow on ammonium plus glycine (Fig. 1). The *in vitro* activity of the GS from the *am-132;gly^r* mutant was inhibited by glycine in a similar manner to the GS from the *am-132* mutant (data not shown).

Table 1. *Enzyme activities in the am-132 and am-132;gly^r mutants*

Enzyme activities (units) were determined after 12 h incubation on the nitrogen sources indicated. The experiment was done twice; representative results are shown.

Strain	Nitrogen source	GS*	GOGAT	NAD-dependent GDH
<i>am-132</i>	Ammonium	0.029	0.008	0.001
	Ammonium + 50 mM-glycine	0.116	0.004	0.001
<i>am-132;gly^r</i>	Ammonium	0.032	0.010	0.002
	Ammonium + 50 mM-glycine	0.052	0.009	0.001

* GS activity was determined by the synthetase assay.

Table 2. *Inhibition of GOGAT activity in vitro by MSF in the am-132;gly^r and am-132 mutants*

The experiment was repeated twice; representative results are shown.

MSF concn (mM)	Percentage inhibition	
	<i>am-132</i>	<i>am-132;gly^r</i>
0.05	60	67
0.20	74	80
0.40	100	100

The residual growth of the *am-132* mutant or other *am* mutants on ammonium is due to the presence of GOGAT (Hummelt & Mora, 1980*a, b*). It has also been shown that GOGAT does not completely replace NADP-dependent GDH in cultures with an excess of ammonium as the nitrogen source. (Hummelt & Mora, 1980*a, b*).

The GOGAT activity of the *am-132;gly^r* mutant was similar to that of the *am-132* mutant (Table 1). So, glycine-resistance cannot be explained by higher induction of GOGAT thus compensating for GS inhibition by glycine, which depends on glutamate concentration (Hernández *et al.*, 1986).

Methionine sulphone (MSF) is a very potent inhibitor of GOGAT from *Saccharomyces cerevisiae* (Masters & Meister, 1982). We found that 0.4 mM-MSF completely inhibited GOGAT activity in a dialysed cell extract of the *am-132* mutant grown on ammonium (Table 2). GOGAT from the *am-132;gly^r* mutant was also inhibited *in vitro* by MSF (Table 2), which suggests that the glycine-resistance mutation does not result in a structural alteration of GOGAT.

A *Saccharomyces cerevisiae* mutant that lacks biosynthetic NADP-dependent GDH activity was reported to regain its ability to grow optimally on ammonium when its catabolic NAD-dependent GDH was induced to very high levels (Grenson *et al.*, 1974). This was not the case for the *N. crassa* glycine-resistant mutant since the catabolic NAD-dependent GDH activity was very low in the *am-132;gly^r* mutant as well as in the *am-132* strain (Table 1).

Metabolic analysis of the am-132;gly^r mutant

If the *gly^r* mutation could have an effect on the utilization and/or flow of the carbon source, this could result in increased glutamate synthesis. The GDH⁻ mutant did not accumulate 2-oxoglutarate when incubated with ammonium plus glycine (Fig. 2) and the 2-oxoglutarate pools of the *am-132;gly^r* double mutant grown on this nitrogen source were slightly but significantly higher (Fig. 2). In the *am-132* mutant the pyruvate and succinate contents were higher on ammonium than on ammonium plus glycine as the nitrogen source (Table 3). In the *am-132;gly^r* double mutant the intracellular pools of pyruvate and succinate were similar in the two growth conditions, and they were lower than in the *am-132* single mutant (Table 3).

Table 3. Metabolite contents of the *am-132* and *am-132;gly^r* mutants

Metabolite contents [$\mu\text{mol (mg protein)}^{-1}$] were determined after 12 h incubation on the nitrogen sources indicated. The experiment was done twice; representative results are shown. ND, Not detected.

Strain	Nitrogen source	Pyruvate	Succinate	Glycine	Glutamate	Glutamine	Alanine
<i>am-132</i>	Ammonium	0.377	1.338	0.036	0.092	0.235	0.154
	Ammonium + 50 mM-glycine	0.219	0.881	2.570	ND	0.029	0.001
<i>am-132;gly^r</i>	Ammonium	0.157	0.593	0.056	0.057	0.185	0.093
	Ammonium + 50 mM-glycine	0.134	0.632	1.015	0.005	0.112	0.007

We conclude that the *am-132* mutant accumulated several tricarboxylic acid cycle intermediates due to the blockage in glutamate synthesis by NADP-dependent GDH; in the presence of glycine this accumulation did not occur (Fig. 2; Table 3), which may indicate that the carbon flow stops in the latter condition. In the *am-132;gly^r* double mutant grown on ammonium, there was a low content of tricarboxylic acid cycle intermediates (Fig. 2; Table 3), which suggests a decrease in carbon flow; when glycine was added the lower content of tricarboxylic acid cycle intermediates (Fig. 2; Table 3) suggested that the carbon flow was only slightly decreased as compared to the former condition. The contents of 2-oxoglutarate (Fig. 2), and pyruvate and succinate (Table 3), in the *am-132;gly^r* double mutant were closely similar to those of the wild-type strain (Fig. 2). The glycine-resistance mutation was not related to the transport of glycine into the cell since glycine was accumulated by the *am-132;gly^r* mutant grown on ammonium plus glycine (Table 3).

A correlation between the content of tricarboxylic acid cycle intermediates and amino acids was observed as expected. The *am-132* mutant contained no detectable glutamate and had a very low alanine and glutamate content on ammonium plus glycine (Table 3) where its growth was abolished (Fig. 1), while the *am-132;gly^r* double mutant had a detectable glutamate pool (Table 3) and its content of alanine and glutamine increased several-fold when grown thus.

From these data we conclude that the *am-132;gly^r* mutant synthesizes more glutamate, alanine and glutamine, which is related to its higher capacity to grow on ammonium with or without glycine, and perhaps to a reduced carbon flow.

Curiously, the amino acid pools were higher in the *am-132* mutant than in the *am-132;gly^r* double mutant when grown on ammonium alone (Table 3). However, the data shown in Table 3 represent the total metabolic pools accumulated after 12 h incubation on the media indicated, and not the nitrogen and carbon flows of this mutant strain, a result which is discussed below.

Regulation of GOGAT activity by organic acids

From the experimental data shown, it appears that due to the blockage in the *am-132* mutant the intracellular content of several tricarboxylic acid cycle intermediates increases, and this, in turn, lowers the capacity of this mutant to synthesize glutamate. We tested if the latter effect could be due to the inhibition of GOGAT by organic acids. We determined the percentage inhibition of GOGAT activity in a partial purified enzyme preparation of the *am-132* mutant grown on ammonium in the presence of different concentrations of several organic acids that are tricarboxylic acid cycle intermediates, or the oxoacids of glycine or serine (Table 4). Glycine did not inhibit *N. crassa* GOGAT activity *in vitro* (data not shown).

GOGAT from the *am-132;gly^r* mutant was inhibited by organic acids in a similar manner to the GOGAT from the *am-132* mutant (data not shown). This supports our proposition that the glycine-resistance mutation is not due to a structural mutation in GOGAT, which could give a more resistant enzyme. Glycine resistance could rather be related to an increased capacity of GOGAT to synthesize glutamate *in vivo* in the *am-132;gly^r* mutant due to a lower concentration of tricarboxylic acid cycle intermediates (Fig. 2; Table 3) that are inhibitors of GOGAT activity (Table 4).

Table 4. *Inhibition of GOGAT activity by organic acids in the am-132 mutant*

Mycelium was grown for 24 h on minimal medium; GOGAT was partially purified as described in Methods. The concentration of organic acids was 10 mM; the pH was adjusted to 8.6. The experiment was done twice; representative results are shown.

Organic acid	2-Oxoglutarate concn . . .	Percentage inhibition	
		0.05 mM	0.1 mM
None		0	0
Succinate		25	19
Malate		67	40
Citrate		25	20
Isocitrate		40	20
Fumarate		51	31
Pyruvate		25	19
Glyoxalate		40	19
β -Hydroxypyruvate		36	31

This postulated increased capacity of GOGAT to synthesize glutamate *in vivo* in the *am-132; gly^r* mutant was investigated by determining its capacity to grow on ammonium plus MSF, compared with the *am-132* single mutant. Pregerminated conidia of the *am-132; gly^r* mutant had a higher growth rate on ammonium plus 0.1 mM-MSF than pregerminated conidia of the *am-132* mutant under the same conditions (Fig. 3). A higher growth rate on ammonium plus MSF was observed in the *am-132; gly^r* mutant, which has lower concentration of tricarboxylic acid cycle intermediates than the *am-132* mutant.

We thus conclude that in the *am-132; gly^r* mutant there is higher GOGAT activity *in vivo* than there is in the *am-132* mutant, due to the lower concentration in the double-mutant of organic acids (Fig. 2; Table 3) that are GOGAT inhibitors (Table 4). As a result of this increased capacity to synthesize glutamate (Table 3) the glycine-resistant mutant is able to grow better than the parental strain on ammonium plus a GS inhibitor such as glycine (Fig. 1), serine or MS and on ammonium plus the GOGAT inhibitor MSF (Table 2; Fig. 3).

DISCUSSION

Fincham (1950) reported that the amino acids glycine or serine completely inhibit growth of *am* strains on ammonium. We have demonstrated that the effect of glycine and serine is due to inhibition of GS activity (Hernández *et al.*, 1986). It proved possible to obtain a glycine-sensitive strain from wild-type *N. crassa* by a mutation that inactivates the highly active β polypeptide of GS (Hernández *et al.*, 1986).

When growth on glutamate of a partial glutamine auxotroph was inhibited by glycine, it was reported that synthesis of glutamate *in vivo* decreased 100-fold, even though the content of glutamine only decreased by half; this suggested that the synthesis of glutamine rather than the absolute content of this amino acid, played an important role in optimum cell growth (Hernández *et al.*, 1986). As a result of GS inhibition by glycine, or MS, the carbon flow decreases, and thus growth is prevented due to the double effect of lowering glutamine synthesis and carbon flow (Hernández & Mora, 1986).

In this paper we reported the isolation of a glycine-resistant mutant from the GDH⁻ strain *am-132* that has a deletion in the structural gene for NADP-dependent GDH (Kinsey & Hung, 1981). This mutation appears to result in diminished carbon flow that cannot be decreased further by the presence of glycine; in the *am-132; gly^r* double mutant this results in a lower content of tricarboxylic acid cycle intermediates (Fig. 2; Table 3). This is different from what occurs in the GDH⁻ strain *am-132*, where the carbon flow was almost abolished and GS was totally inhibited in the presence of glycine, as shown by the decrease in glutamate, glutamine, alanine, pyruvate, succinate (Table 3) and 2-oxoglutarate contents (Fig. 2). Although in the

am-132 strain incubated with ammonium plus glycine, the 2-oxoglutarate content was more drastically reduced (Fig. 2) as compared to the pyruvate and succinate contents (Table 3), even these values represent reduced levels, since they were obtained after 12 h incubation in the absence of growth.

GOGAT from *N. crassa* was inhibited *in vitro* by different tricarboxylic acid cycle intermediates and also by the oxoacids of glycine and serine (Table 4). It has been reported that tricarboxylic acid cycle intermediates also regulate GOGAT from *E. coli* (Miller & Stadman, 1972). West *et al.* (1967) and Ashby *et al.* (1974) reported that *N. crassa* NADP-dependent GDH is activated cooperatively by several non-substrate tricarboxylic acid cycle intermediates.

The principal function of GOGAT could be related to the interconversion of glutamine to glutamate and to glutamate recycling (Calderón & Mora, 1985). This proposal is supported by the fact that GOGAT cannot completely replace NADP-dependent GDH in a GDH⁻ mutant strain that has a decreased growth rate on ammonium (Fig. 1), where a low carbon flow and an accumulation of carbon skeletons is observed (Fig. 2; Table 3).

Taking into account that some organic acids were effective inhibitors of GOGAT activity (Table 4), a lower content of these tricarboxylic acid cycle intermediates in the *am-132; gly^r* mutant would allow a higher glutamate content (Table 3). This increased glutamate content could overcome GS inhibition by glycine, which depends on glutamate concentration (Hernández *et al.*, 1986). Another observation that supports the increased capacity of GOGAT to synthesize glutamate *in vivo* in the *am-132; gly^r* mutants is that this strain had a higher growth rate on ammonium plus MSF, a GOGAT inhibitor, than the *am-132* mutant (Fig. 3).

The ability of the *am-132; gly^r* mutant to grow in the presence of glycine, serine or MS added individually to ammonium as the nitrogen source indicates that resistance is the result of a common effect of glycine, serine and MS. These three GS inhibitors had similar effects on glutamine synthesis and on sucrose catabolism, as shown here and by Hernández & Mora (1986).

We propose that in the *am-132; gly^r* mutant an increased carbon flow compared to that of the *am-132* mutant elicits increased glutamate and glutamine synthesis that is responsible for an increased growth rate on ammonium (Fig. 1). This proposal can also explain the capacity of the *am-132; gly^r* mutant to grow in the presence of glycine, serine, MS or MSF.

By the same token, the slow growth of the *am-132* mutant on ammonium as the sole nitrogen source (Fig. 1) is due to the accumulation of tricarboxylic acid cycle intermediates (Fig. 2; Table 3); this accumulation results in an inhibition of GOGAT that limits glutamine synthesis, which then reduces the carbon flow (Table 3). This proposal is supported by the accumulation of glutamate and other amino acids (Table 3), observed in the *am-132* mutant after 12 h slow growth on ammonium (Fig. 1); we assume that this limited growth is due to a reduced carbon flow. During the first hours of growth on ammonium, glutamate and glutamine pools were lower in the *am-132* mutant than in the *am-132; gly^r* mutant (data not shown).

It can be speculated that a glycolytic enzyme less sensitive to ATP inhibition may explain the *gly^r* phenotype.

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