Isolation and Characterization of a *Neurospora crassa* Mutant Altered in the α Polypeptide of Glutamine Synthetase

JORGE CALDERÓN, LUZ MARÍA MARTÍNEZ, AND JAIME MORA*

Departamento de Ecológia Molecular, Centro de Investigación sobre Fijación de Nitrógeno, Universidad Nacional Autónoma de México, Apartado Postal 565-A, Cuernavaca, Morelos, Mexico

Received 22 September 1989/Accepted 13 June 1990

We report the isolation and characterization of a *Neurospora crassa* glutamine synthetase (GS) mutant altered in one of the two polypeptides (GSα) of this enzyme. We used the *gln-lbR8* mutant strain that synthesizes only the GSα monomer and lacks the GSβ monomer and selected for growth in minimal medium in the presence of *α*-methyl-DL-methionine-SR-sulfoximine (*α*-me-MSO), an inhibitor of GS activity. The GS activity of the *gln-lbR8:α-MSO* strain drastically reduced its transferase activity and only slightly reduced its synthetase activity, and it was resistant to inhibition by *α*-me-MSO and L-methionine-DL-sulfoximine. The mutation that overcame the inhibitory effect of *α*-me-MSO also altered the antigenic, kinetic, and physical properties of GSα. The low GS activity of the *α*-me-MSO-resistant strain was compensated for by a higher glutamate/glutamine ratio and a lower glutamate synthetase activity, allowing this strain to grow as well as the parental strain. The mutation that conferred resistance to *α*-me-MSO was not linked to the *gln-lbR8* mutation, providing direct evidence of the existence of two genes involved with the structure of the two polypeptides of *N. crassa* GS.

Glutamine is the final product of ammonium assimilation and is a nitrogen donor for biosynthetic reactions (21); it has been proposed as the nitrogen metabolite responsible for nitrogen catabolite repression in several microorganisms, including *Neurospora crassa* (17, 24).

The *N. crassa* glutamine synthetase (GS) (EC 6.3.1.2), the enzyme that synthesizes glutamine, is composed of two polypeptides (α and β) that show different electrophoretic mobilities (23). Although in some conditions the α polypeptides are arranged preferentially as tetramers and the β polypeptides are arranged as octamers, hybrids with different α-β relationships are found in most conditions (6, 14, 23). The nitrogen regulates the concentration (25) and the de novo synthesis (22) of GS. In ammonium-limited cultures, a tetrameric form composed mainly of α monomers is found; in substrate excess, an octameric form composed of β monomers was the predominant state (14). It has been proposed that in ammonium-limited cultures of *N. crassa*, ammonium is assimilated mainly by the operation of glutamate dehydrogenase (EC 1.4.1.4), GSα, and glutamate synthase (GOGAT) (EC 1.4.1.14), and when ammonium is present in excess in the growth medium, glutamate dehydrogenase and GSβ are the main participating enzymes (15). The carbon source (18), glycine, and serine also regulate GS (12). It has been found that GS is involved in glutamine cycling, in which this amino acid is continually assimilated by GOGAT, degraded by the glutamine transaminase-o-oxidase pathway, and resynthetized (1–4). It has been proposed that this cycle is necessary for carbon utilization (11).

Two types of *N. crassa* glutamine auxotrophs have been isolated (5, 7). One of them grows on glutamate at 37°C but not at 25°C. These mutants have a GSα monomer with the same molecular weight and charge as the GSα monomer from the wild-type strain and do not synthesize the GSβ monomer, but instead synthesize a polypeptide (γ) with a lower molecular weight that cross-reacts with antibodies against GS monomers (5). The combination of γ and α monomers results in a hybrid GS with low activity (7). Glutamine auxotrophs of the other type grow only on glutamine and contain a β polypeptide with an altered isoelectric point (5, 12). The low frequency of recombinants found in crosses between these two types of glutamine auxotrophs indicates that these mutations are very close and possibly map in the structural gene for the β polypeptide (5).

Revertants of these mutants have been found to grow on ammonium by a GS activity composed only of α monomers assembled in a tetramer (5). These revertants have a mutation that results in the lack of GSβ monomers (5). Crosses of these revertants did not yield glutamine auxotrophic progeny, indicating that the two mutations are closely linked to the original mutation (5).

Although various selection procedures have been used to obtain glutamine auxotrophs, only mutants in the GSβ monomer have been obtained. In this work, we describe the isolation and characterization of an *N. crassa* mutant strain altered in the GSα monomer.

MATERIALS AND METHODS

**Strains.** *N. crassa* wild-type strains 74-A and 73-a came from the Fungal Genetics Stock Center; the *gln-lbR8* strain, which lacks the GSβ monomer (5) came from the collection of J. Mora.

**Growth conditions.** Batch cultures of *N. crassa* were grown at 30°C with shaking (250 rpm) on Vogel minimal medium (26) supplemented with 1.5% sucrose. The conidia used as inoculum were obtained as previously reported (25). Ammonium limitation in fed-batch cultures was achieved as previously reported (14).

**Determination of enzyme activities.** Cell extracts were prepared from mycelia as previously described (25). GS activities measured as transferase and synthetase were assayed as described by Ferguson and Sims (9). GOGAT activity was measured as previously reported (13).

**Mutagenesis and mutant selection.** A conidial suspension of

*Corresponding author.
the gln-1bR8 mutant strain was prepared and adjusted to 4 × 10⁷ conidia per ml, and 20 ml of the suspension was incubated with 0.4 mg of N-methyl-N'-nitro-N-nitrosoguanidine for 1 h at 30°C with shaking at 200 rpm. The conidia were then concentrated by centrifugation, washed twice with 20 ml of cold water, suspended in distilled water, and plated on Vogel minimal medium supplemented with glucose and fructose (0.02% each), sorbose (2%), and 10 mM α-methyl-DL-methionine-SR-sulf oxime (α-me-MSO). Plates were incubated at 29°C in the dark until colonies appeared (1 to 2 days) and then transferred to slants of Vogel minimal medium supplemented with 1.5% sucrose and 10 mM α-me-MSO. Crosses were made in cornmeal agar (34 g/liter; Difco Laboratories, Detroit, Mich.). Spot testing and progeny analysis were carried out as previously described (8).

Immunoprecipitation of GS activity. GS was purified to homogeneity by DEAE-cellulose chromatography, followed by affinity chromatography on anthranilate-bound Sepharose (19). Rabbits were immunized as previously described (20). Immunoprecipitation was done by using N. crassa extracts containing a constant amount of GS activity. The extracts were incubated for 90 min at 4°C in the presence of synthetase buffer (50 mM K₂SO₄, 50 mM imidazole, 50 mM 2-mercaptoethanol, pH 7.2) with anti-GS serum; the final volume was 0.5 ml. After incubation, the reaction mixture was centrifuged for 10 min at 10,000 rpm, and GS was measured in the supernatant. The amount of anti-GS required to precipitate all GS activity from the supernatant was usually between 200 and 300 μl, and this immunoprecipitate was used for gel electrophoresis.

Electrophoresis. The immunoprecipitates were subjected to polyacrylamide slab gel electrophoresis in the presence of sodium dodecyl sulfate and 7 M urea (23), stained with Coomassie blue, and scanned with a GS-300 transmittance/reflectance scanning densitometer (Hoefer Scientific Instruments).

Two-dimensional electrophoresis. Two-dimensional electrophoresis of the GS immunoprecipitates was done as described previously (12).

Determination of amino acid pools. Samples were prepared by homogenizing mycelia with 80% (vol/vol) ethanol. The homogenates were boiled for 10 min, cooled, and filtered through membrane filters (type HA, 0.45 μm; Millipore Corp., Bedford, Mass.). The filtrates were lyophilized, and the samples were suspended in lithium citrate buffer (pH 2.88). The amino acids were separated by using an Aminco amino acid analyzer and, after being coupled with o-phthalaldehyde, were quantified in an Aminco ratio fluorimeter.

Protein determination. Samples of mycelia were prepared as previously described (25). Protein was determined by the method of Lowry et al., using bovine serum albumin as a standard (16).

Chemicals. α-me-MSO was synthesized as described by Griffith and Meister (10). Amino acids, L-methionine-DL-sulfoximine (MSO), N-methyl-N'-nitro-N-nitrosoguanidine, and o-phthalaldehyde were obtained from Sigma Chemical Co. (St. Louis, Mo.). Electrophoresis reagents were from Bio-Rad Laboratories (Richmond, Calif.).

RESULTS

Physiological and biochemical characterization of the α-me-MSO-resistant mutants. In N. crassa, two different polypeptides (α and β) participate in GS activity (6, 23). Mutants either altered in or lacking the β polypeptide have been reported (5, 7, 12). However, all efforts to obtain mutants altered in the α polypeptide have been unsuccessful.

In N. crassa, MSO is degraded by L-amino acid oxidase and glutamine transaminase (data not shown). However α-me-MSO, an analog of glutamate that inhibits GS activity, is not degraded by L-amino acid oxidase, glutamine transaminase, or cystathionase (10). To select mutants altered in the GSα polypeptide, we started with a parental strain (gln-1bR8) that lacks the GSβ polypeptide and does not grow in the presence of α-me-MSO, in contrast to the wild-type strain, which grows in the presence of this compound. Several mutants resistant to α-me-MSO were obtained; GS activity was measured in some of them, and it was found that GS transferase activities were reduced severalfold (data not shown). Two of the gln-1bR8;α-me-MSOβ strains were purified by crosses with the wild-type strain and further characterized. These two mutants were altered in their GS properties in a similar way, although the mutant described here was more resistant to α-me-MSO inhibition and had a major alteration in its GS properties. This mutant was also resistant to 5 mM MSO, a concentration that prevents growth in the wild-type and parental strains. The effect of α-me-MSO was also tested during growth in liquid cultures, where it was observed that the new gln-1bR8;α-me-MSOβ mutant grew slightly less in the presence than in the absence of α-me-MSO, whereas the parental gln-1bR8 strain did not grow at all (Fig. 1). The gln-1bR8 and the gln-1bR8;α-me-MSOβ strains grew similarly in the absence of the inhibitor.
FIG. 2. Effect of GS inhibitors α-me-MSO (A) and MSO (B) on GS activity from the gln-1bR8 (○) and gln-1bR8;α-me-MSOR strains (□). GS activity was measured by the synthetase assay.

(Fig. 1) and somewhat less than the wild-type strain, which has the α and β GS polypeptides (15).

It was found that cell extracts of N. crassa from the gln-1bR8;α-me-MSOR strain had GS activity resistant to α-me-MSO inhibition (Fig. 2), in contrast to the gln-1bR8 strain, which was severely inhibited by this compound (Fig. 2). Similar results were observed with MSO (Fig. 2). As previously reported, the GSα polypeptide has high transferase activity (6). The α-me-MSO-resistant mutant had drastically (28-fold) reduced GS transferase activity; however, the synthetase activity was only slightly (2.5-fold) diminished (Table 1). The thermostability of the GSα enzyme was also affected in the α-me-MSO-resistant strain (Fig. 3). The $T_{1/2}$ values were 12.2 and 5.5 for the gln-1bR8 and gln-1bR8;α-me-MSOR strains, respectively.

An inhibitor present in the cell extract of the gln-1bR8;α-me-MSOR strain was not responsible for the lower GS activity, as shown by an experiment in which extracts from both the parental and the new mutant strains were mixed. GS activity of the extract mixture was the result expected after addition of the activities obtained separately with or without α-me-MSO.

Constant amounts of GS activity (50 nmol/min) from cell extracts derived either from the gln-1bR8 or the gln-1bR8;α-me-MSOR strain were immunoprecipitated and subjected to one-dimensional gel electrophoresis. Under these conditions, both strains showed only the α polypeptide of GS. Furthermore, the area corresponding to the GSα band was quantified by gel scanning, and it was found to be twofold larger in the gln-1bR8;α-me-MSOR strain than in the gln-1bR8 strain (Fig. 4). These results indicated that GS activity per enzyme molecule was lower in the gln-1bR8;α-me-MSOR strain than in the gln-1bR8 strain. To further characterize the GSα polypeptide of the gln-1bR8;α-me-MSOR strain, immunoprecipitates obtained as described

![Graph](http://jb.asm.org/)

**TABLE 1**. GS activities of gln-1bR8 and gln-1bR8;α-me-MSOR

<table>
<thead>
<tr>
<th>Strain</th>
<th>GS sp act*</th>
<th>S/T ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>gln-1bR8</td>
<td>17.4</td>
<td>246.6</td>
</tr>
<tr>
<td>gln-1bR8;α-me-MSOR</td>
<td>7.0</td>
<td>8.8</td>
</tr>
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</table>

a Determined after 24 h of growth on 25 mM NH₄NO₃ as a nitrogen source and expressed as nanomoles of γ-glutamyl hydroxamate produced per minute per milligram of protein at 30°C. S. Synthetase activity; T. transferase activity.

![Graph](http://jb.asm.org/)

**FIG. 3**. GS thermostability of gln-1bR8 (○) and gln-1bR8;α-me-MSOR (□) strains. Extracts from cells grown for 24 h on 25 mM NH₄NO₃ as a nitrogen source were heated at 50°C during the indicated time. GS was measured by the synthetase assay.
above were also subjected to two-dimensional gel electrophoresis. The gln-ibR8;α-me-MSO strain had the same molecular weight and isoelectric point as the GSA polypeptide of the gln-ibR8 strain; in addition, the sedimentation pattern of the GS from the gln-ibR8;α-me-MSO strain behaved as a tetramer as in the gln-ibR8 strain (data not shown).

In comparison with the GS from the gln-ibR8 strain, the GS from the gln-ibR8;α-me-MSO mutant strain increased its apparent $K_m$’s, for glutamate and NH$_2$OH two- and fourfold, respectively ($K_m$ for glutamate, 2.22 versus 4.41; $K_m$ for NH$_2$OH, 0.54 versus 2.11).

To determine whether there was any effect on GSA altered activity under ammonium limitation, the gln-ibR8 and gln-ibR8;α-me-MSO strains were grown in fed-batch ammonium-limited cultures. The two strains grew similarly (data not shown), and GS activity was higher than in ammonium excess (Tables 1 and 2). However, GS activity was threefold lower in the gln-ibR8;α-me-MSO strain than in the gln-ibR8 strain. The alteration in GS activity was reflected in a decrease of glutamate content (4.5-fold), in the accumulation of glutamate (3.5-fold), and in the decrease of GOGAT activity (2-fold) (Table 2).

To establish whether linkage occurred between the mutation in the gln-ibR8 strain and the mutation that conferred the resistance to α-me-MSO, we performed crosses between the gln-ibR8;α-me-MSO and the wild-type strains. The phenotype of the progeny was determined by the growth response to different GS inhibitors. A new phenotype resulted from the cross, which we called α-me-MSO, that grew as well as the wild-type strain on NH$_2$NO$_3$ and was more resistant to MSO and α-me-MSO inhibition. The progeny phenotype percentages obtained from the cross were 29% wild type, 21% gln-ibR8, 32% gln-ibR8;α-me-MSO, and 18% α-me-MSO. The recombination frequency was 39%, which indicates that the mutations are not linked.

**DISCUSSION**

In N. crassa, two polypeptides (α and β) are responsible for ammonium assimilation into glutamine (6, 14, 23). Each polypeptide participates in the synthesis of glutamine, depending on the ammonium concentration (6, 14, 15).

Although in N. crassa different selection methods have been used to obtain glutamine auxotrophs, only three types of mutants have been isolated in the GSB polypeptide (5, 6, 12). These mutations are very close and possibly map in the structural gene for the GSB polypeptide (5). It has been proposed that the altered GSB polypeptide in these auxotrophs blocks GS activity of the α polypeptide by participating in the formation of an abnormal nonfunctional oligomer (5). Revertants obtained from glutamine auxotrophs capable of growing on ammonium have been selected and found to synthesize only the GSA monomer in vivo and in vitro (5). The new mutation is closely linked to the original mutation, and it has been proposed as a regulatory mutation that turns off the expression of GSB monomers (5).

To obtain mutants altered in the GSA monomer, we used the gln-ibR8 mutant strain that synthesizes only the GSA monomer and selected for growth in minimal medium in the presence of α-me-MSO, an inhibitor of GS activity that is not degraded by L-amino acid oxidase, glutamine transaminase, or cystathionase (10). α-me-MSO was used in a selection procedure to obtain the strain mutated in GS, since when MSO was used in N. crassa, only mutants with increased degradation of this inhibitor were obtained (J. Calderón, L. Olivera, and G. Dávila, unpublished data), and until now all the efforts to obtain auxotrophic mutants from the gln-ibR8 mutant strain had been unsuccessful.

The gln-ibR8;α-me-MSO strain obtained in this study grows in α-me-MSO as well as in the analog MSO in a concentration that prevents growth in the wild-type and the parental gln-ibR8 strains; this property is a consequence of the fact that the GS in this new mutant is only slightly inhibited by α-me-MSO and MSO (Fig. 2). The GS activity of cell extracts of the gln-ibR8;α-me-MSO strain was drastically altered, since its transferase activity was reduced 28-fold and its synthetase activity was decreased 2.5-fold (Table 1). The in vitro inhibitory effects of α-me-MSO and MSO on GS activity were similar; however, a 100-fold higher concentration of α-me-MSO was necessary to inhibit growth of the gln-ibR8 strain. These data indicate that the mutation that confers resistance to α-me-MSO in the gln-ibR8 strain affects GS and that in N. crassa MSO is transported more efficiently than α-me-MSO.

The GS alteration in the antigenic, kinetic, and physical properties of the gln-ibR8;α-me-MSO strain, which has only the α monomer, indicates that the mutation that confers resistance to α-me-MSO affects the structure of the GSA subunit. However, other properties, such as molecular weight (Fig. 4), isoelectric point, and oligomeric structure (data not shown), were not altered. Although this effect may correspond to a mutation in the structural gene for GS, it

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**TABLE 2.** GS and GOGAT activities and glutamine and glutamate content in the gln-ibR8 and gln-ibR8;α-me-MSO strains grown on ammonium-limited cultures

<table>
<thead>
<tr>
<th>Strain</th>
<th>Activity</th>
<th>Content (μmol/mg of protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>GS$^a$</td>
<td>Glutamine</td>
</tr>
<tr>
<td>gln-ibR8</td>
<td>48.8</td>
<td>7.6</td>
</tr>
<tr>
<td>gln-ibR8;α-me-MSO</td>
<td>15.4</td>
<td>3.7</td>
</tr>
</tbody>
</table>

$^a$ Enzyme activities and amino acid content were determined after growth for 24 h at 30°C on fed-batch ammonium-limited cultures.

$^b$ Expressed as nanomoles of γ-glutamyl hydroxamate produced per minute per milligram of protein.

$^c$ Expressed as nanomoles of NADH oxidized per minute per milligram of protein.
cannot be ruled out that a gene whose product causes a modification in the enzyme may be responsible for the alteration of GS.

In ammonium-limited cultures, the lower GS activity in the gln-1bR8α-me-MSOR mutant strain reduced glutamine synthesis and increased glutamate concentration, something that represses GOGAT activity and the cycling of nitrogen between glutamine and glutamate, which in turn prevents the drastic drain of glutamine. This metabolic adjustment in reducing the cycling of glutamine results in similar growth of the parental and the α-me-MSO-resistant mutant strains in low ammonia. These data point out that an excess of GS activity ensures a higher glutamine cycling, something that allows for a wider range adjustment in the glutamate/glutamine ratio, which is also in accordance with the proposal that the function of GOGAT in N. crassa is to recycle glutamine to glutamate (1, 3, 15).

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LITERATURE CITED


