Glutamine Metabolism in Nitrogen-starved Conidia of *Neurospora crassa*

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During nitrogen deprivation, *de novo* synthesis of glutamine synthetase was induced in non-growing conidia of *Neurospora crassa*. When ammonia or glutamine was added to conidia which had been deprived of nitrogen, glutamine and arginine accumulated at a higher rate than in conidia not deprived of nitrogen. The degradation of exogenous glutamine to glutamate is apparently a necessary step in the accumulation of glutamine and arginine within the conidia. In non-growing conidia, a cycle probably operates in which glutamine is degraded and resynthesized. The advantages of such a cycle would be that the carbon and nitrogen could be used to synthesize amino acids in general, as well as for the synthesis and accumulation of arginine and/or glutamine in particular.

INTRODUCTION

Conidia of *Neurospora crassa* accumulate glutamine and arginine under conditions of amino acid deprivation. This accumulation requires a supply of carbon and nitrogen sources, an intact arginine biosynthetic pathway, *de novo* synthesis of pyrimidines and protein synthesis (Espín & Mora, 1978). Mycelium also accumulates glutamine and arginine when deprived either of an amino acid or pyrimidines, in the presence of cycloheximide or at the end of exponential growth. A large proportion of the accumulated arginine is sequestered in an osmotically sensitive compartment (Mora *et al.*, 1978). In this paper, we report the effect of nitrogen deprivation on the accumulation of glutamine and arginine by non-growing conidia. A positive correlation has been established between this accumulation and the induction of glutamine synthetase as a result of *de novo* synthesis of this protein. Evidence is also presented regarding the pathway by which the nitrogen of ammonia or glutamine present in the medium is utilized to synthesize and accumulate arginine and/or glutamine. The degradation of glutamine and its subsequent resynthesis by resting cultures has previously been studied with mutants defective in the synthesis of glutamate (Fincham, 1950) or glutamine (Dávila *et al.*, 1978) or by using methionine sulfoximine to inhibit glutamine degradation (Cooper *et al.*, 1976).

METHODS

Cultures. All stock cultures came from the collection of J. Mora or from Fungal Genetics Stock Center at Humboldt State University Foundation, Arcata, California, U.S.A. The basic strains were: wild-type strain 74-A, the proline auxotrophs *prol-3* and *prol-4*, the tryptophan auxotroph *tryp-2*, the glutamine auxotrophs *gln-1a* and *gln-1b*, the amino nitrogen auxotrophs *am-1* and *en-am-1* and the uridine auxotroph *pyr-3a*. All double or triple mutants were obtained from appropriate crosses of these stocks.

Growth conditions. Conidia obtained as previously reported (Sánchez *et al.*, 1972) were incubated in an aerated culture at 25 °C in minimal medium N (MM) of Vogel (1964) supplemented with 1.5% (w/v)

sucrose, unless otherwise stated. In some experiments, NH_4NO_3 was omitted or replaced by other nitrogen sources as indicated. Crosses were made on 1.7% (w/v) corn meal agar (Difco) in the dark at 25 °C.

Determination of glutamic acid, glutamine and arginine pools. Samples for glutamic acid and glutamine analysis were prepared by homogenizing conidia with 80% (v/v) ethanol. The homogenates were boiled for 10 min, cooled and filtered through membrane filters (Millipore, type RA, 1.2 μm). The filtrates containing the amino acids were lyophilized and the samples were resuspended in deionized water. The amino acids were separated using an Aminco (Silver Springs, Md, U.S.A.) amino acid analyser and quantified in an Aminco Ratio Fluorometer after coupling with *o*-phthalaldehyde. Under these conditions more than 80% of the glutamic acid and glutamine were recovered. Arginine was extracted from filtered conidia with 2 ml 5% (w/v) trichloroacetic acid. After centrifugation, arginine was determined in the supernatant fluid by the method of Van Pilsum *et al.* (1956).

Ammonia determination. Samples (10 ml) of medium were collected by filtration through membrane filters (Millipore, type HA, 0.45 μm) and, after addition of 0.1 ml 10 M-NaOH, ammonia was measured in an Orion (Cambridge, Mass, U.S.A.) electrode with a membrane specific for this compound. No interference by glutamine was detected if assays were completed within 10 min of the addition of NaOH.

Glutamine synthetase activity [L-glutamate:ammonia ligase (ADP-forming; EC 6.3.1.2)]. Conidia were separated from the culture medium by filtration, washed with water and then acetone, and finally dried under vacuum. All subsequent steps were performed at 4 °C. Acetone powders were ground with dry ice and homogenized in a motor-driven homogenizer with 10 vol. extraction buffer (5 mM- KH_2PO_4 , 50 mM- K_2SO_4 , 0.5 mM-EDTA, pH 7.2). The preparation was centrifuged for 20 min at 12000 g and the extraction procedure was repeated. Transferase activity of glutamine synthetase was measured in the combined supernatants and in the pellet as described by Ferguson & Sims (1974). Specific activity (μmol γ -glutamyl hydroxamate produced min^{-1} at 30 °C) was calculated by dividing the total activity in the supernatant and the pellet by the total protein of the culture.

Preparation and characterization of anti-glutamine synthetase antibodies. Glutamine synthetase was purified to homogeneity by chromatography on DEAE-cellulose, followed by affinity chromatography on anthranilate-bound Sepharose (Palacios, 1976). Rabbits were immunized and the serum was fractionated with ammonium sulphate to obtain the total γ -globulin fraction; the specific antibody was further purified by affinity chromatography on a matrix of purified glutamine synthetase bound to Sepharose. Different experimental criteria indicate that this antibody fraction is monospecific for *N. crassa* glutamine synthetase (Palacios *et al.*, 1977). Goat anti-rabbit γ -globulin was prepared as described by Palacios *et al.* (1977).

Immunoprecipitation of in vivo-labelled glutamine synthetase. Extracts of conidia labelled *in vivo* with [^3H]leucine were incubated in the presence of purified anti-glutamine synthetase antibody followed by addition of anti-rabbit γ -globulin. To separate the immunoprecipitates, the preparation was centrifuged through a buffer solution containing 20% (w/v) sucrose (Quinto *et al.*, 1977). The immunoprecipitates were subjected to acrylamide gel electrophoresis in the presence of sodium dodecyl sulphate followed by fluorography as described by Bonner & Laskey (1974). To measure the relative rates of glutamine synthetase synthesis, ^3H -labelled conidial extract was mixed with ^{14}C -labelled mycelial extract and samples of the mixture were either immunoprecipitated or precipitated with trichloroacetic acid. The samples were then processed for electrophoresis as described above. After fluorography, the gels were cut into 0.5 cm slices and each slice was counted for $^3\text{H}/^{14}\text{C}$ double isotope label in a Packard Tri-Carb model 3390 liquid scintillation spectrometer equipped with a model 544 absolute activity analyser. The ratio $^3\text{H}/^{14}\text{C}$ in the immunoprecipitate divided by that of the total protein precipitated indicates the relative rate of enzyme synthesis under both conditions (Quinto *et al.*, 1977).

Protein determination. Samples of conidia were collected on membrane filters (Millipore, type HA, 0.45 μm) washed with 2 vol. distilled water and placed in 2 ml 5% (w/v) trichloroacetic acid. After centrifugation, the precipitate was resuspended in 1 M-NaOH and protein was determined by the Lowry method, using bovine serum albumin as standard.

Chemicals. Amino acids, uridine, bovine serum albumin, L-methionine-DL-sulphoximine and *o*-phthalaldehyde were obtained from Sigma, cycloheximide was from Calbiochem and L-[4,5- ^3H]leucine and L-[U- ^{14}C]leucine were from New England Nuclear.

RESULTS

Effect of nitrogen deprivation on the accumulation of glutamine and arginine

When conidia of *Neurospora crassa* are deprived of certain amino acids in the presence of NH_4NO_3 , glutamine and arginine accumulate (Espín & Mora, 1978). The rate of accumulation increased when conidia were nitrogen-starved before amino acid deprivation (Table 1).

Table 1. *Effect of preincubation in nitrogen-free medium on the accumulation of glutamine and arginine by conidia of the prol-3 strain*

Conidia with or without 12 h preincubation in nitrogen-free MM were incubated in MM with NH_4NO_3 (25 mM) as nitrogen source.

Culture conditions	Time (h)	Concn [$\mu\text{mol (mg protein)}^{-1}$]	
		Arginine	Glutamine
Not starved	0	0.032	0.068
	6	0.290	0.530
	12	0.520	1.035
Starved	0	0.015	0.031
	6	0.998	1.840
	12	1.120	3.190

Furthermore, the *prol-4* auxotroph accumulated arginine at a rate lower than those shown by other auxotrophs (Espín & Mora, 1978), but when this strain had been nitrogen-starved, its capacity to accumulate arginine increased (Fig. 1*a*).

We previously reported that only low concentrations of arginine accumulate in the absence of *de novo* synthesis of pyrimidines, in the presence of cycloheximide, or in the presence of glycerol as carbon source instead of sucrose (Espín & Mora, 1978). In the present experiments, arginine accumulation following nitrogen starvation was stimulated in the *pyr-3a* uridine auxotroph (Fig. 1*b*) and in the *prol-3* strain in the presence of cycloheximide (Fig. 1*c*) or with glycerol as carbon source instead of sucrose (Fig. 1*d*).

Effects of mutations that impaired ammonia assimilation and/or glutamine synthesis

The *am-1* strain lacks the activity of the biosynthetic glutamate dehydrogenase (Fincham, 1950) and grows in ammonium as nitrogen source after a lag of 12 h. However, if in addition to the *am-1* mutation the *en-am-1* mutation is also present, no growth occurs on inorganic nitrogen (unpublished results). No glutamate, glutamine or arginine accumulated when the triple mutant *tryp-2 am-1 en-am-1* was incubated with NH_4NO_3 after nitrogen deprivation. If glutamine was added instead of NH_4NO_3 , only this amino acid accumulated. A lack of accumulation of amino acids was expected in the presence of NH_4NO_3 , but not with glutamine as nitrogen source. This could be explained if ammonia fixation into glutamate is necessary for arginine to accumulate when glutamine is the nitrogen source. The triple mutant excreted ammonia into the medium when glutamine was added after a period of nitrogen deprivation, indicating that this amino acid must be degraded to α -ketoglutarate and ammonia for glutamine and arginine to accumulate in the *tryp-2* strain. After prolonged incubation, all strains tested excreted some ammonia.

With NH_4NO_3 as nitrogen source, the *tryp-2 gln-1b* mutant did not accumulate glutamine; arginine accumulated at a reduced rate and the glutamate concentration increased four to fivefold (Fig. 2*a*). A similar observation was recorded with glutamine as nitrogen source, except that this amino acid accumulated intracellularly during the first 3 h of incubation and subsequently disappeared (Fig. 2*b*). Nitrogen deprivation before the addition of NH_4NO_3 did not affect the accumulation of glutamine and arginine, and again, only the glutamate pool increased (Fig. 2*c*). Similar results were observed after addition of glutamine except that glutamine did not accumulate (Fig. 2*d*). Comparable results were also obtained with the *tryp-2 gln-1a* and the *prol-4 gln-1a* double auxotrophs. This is in contrast to the single *tryp-2* mutant which mainly accumulated glutamine and arginine with NH_4NO_3 or glutamine as nitrogen source (Fig. 3*a, b*). When NH_4NO_3 or glutamine was added to conidia of *tryp-2* previously deprived of nitrogen, glutamine and arginine accumulated to levels higher than those shown in Fig. 3*(a, b)*; glutamate decreased during starvation and increased on

Fig. 1. Effect of nitrogen deprivation on the accumulation of arginine. Conidia were incubated as follows: (a) the *prol-4* strain in MM, (b) the *pyr-3a* strain in MM, (c) the *prol-3* strain in MM in the presence of cycloheximide (10 μg ml⁻¹), (d) the *prol-3* strain in MM with 2% (w/v) glycerol as carbon source, in each case with (●) or without (○) 12 h preincubation in nitrogen-free MM (with sucrose as carbon source).

Fig. 2. Accumulation of arginine (○), glutamine (Δ) and glutamic acid (□) by conidia of the *tryp-2 gln-1b* strain incubated in MM (25 mM-NH₄NO₃) (a, c) or with glutamine (100 μg ml⁻¹) (b, d), either with (c, d) or without (a, b) 12 h preincubation in nitrogen-free medium.

Fig. 3. Accumulation of arginine (○), glutamine (△) and glutamic acid (□) by conidia of the *tryp-2* strain incubated in MM (25 mM-NH₄NO₃) (a, c) or with glutamine (100 μg ml⁻¹) (b, d), either with (c, d) or without (a, b) 12 h preincubation in nitrogen-free medium.

addition of NH₄NO₃ or glutamine, reaching within 3 h concentrations similar to those found in non-deprived cultures (Fig. 3c, d).

Taken together, these results indicate that in non-growing conidia, glutamine is degraded to α -ketoglutarate and ammonium, and that these compounds are in turn converted to glutamic acid and glutamine.

Effect of methionine sulfoximine

To determine the role of the synthesis and degradation of glutamine in the accumulation of glutamine and arginine by non-growing conidia, the effect of methionine sulfoximine (MS) was studied. This compound is a specific irreversible inhibitor of glutamine synthetase (Ronzio & Meister, 1968) and also competes with glutamine as a substrate for glutamine transaminase (Cooper *et al.*, 1976). Through the action of the latter enzyme, glutamine is transaminated to α -ketoglutarate which is then hydrolysed to ammonium and α -ketoglutarate by an ω -amidase. The occurrence of these enzymes in *N. crassa* has been reported (Monder & Meister, 1958).

When conidia of the *prol-3* strain were incubated in a nitrogen-free medium in the presence of MS, this compound was degraded and glutamine and arginine accumulated (Table 2). This accumulation was observed only after 12 h incubation and no glutamine synthetase activity was detected during this lag phase. It is probable that this period was required for MS degradation. Similar effects were observed in the presence of MS plus NH₄NO₃, but glutamine and arginine accumulated on addition of glutamine plus MS to nitrogen-deprived *prol-3* cultures (Fig. 4).

Table 2. *Effect of L-methionine-DL-sulphoximine on amino acid accumulation by the prol-3 strain incubated in nitrogen-free MM*

Culture condition	Time (h)	Concn [$\mu\text{mol (mg protein)}^{-1}$]		
		Arginine	Glutamine	Glutamic acid
No addition	0	0.025	0.075	0.287
	12	0.023	0.028	0.113
	24	ND	0.009	0.058
Methionine sulphoximine (5 mM)	12	0.050	0.041	0.177
	24	0.290	0.261	0.146

ND, Not detectable.

Fig. 4. Effect of L-methionine-DL-sulphoximine on the accumulation of arginine (○), glutamine (△) and glutamic acid (□). Conidia of the *prol-3* strain were incubated in MM without NH_4NO_3 but supplemented with glutamine ($100 \mu\text{g ml}^{-1}$) and methionine sulphoximine (5 mM), after 12 h preincubation in nitrogen-free MM.

As shown above, double mutants lacking glutamine synthetase activity are unable to utilize the nitrogen of glutamine to synthesize and accumulate arginine (Fig. 2). The conversion of glutamine into arginine under conditions in which the activity of glutamine synthetase is completely inhibited (Fig. 4) indicates that MS is being degraded. Because of its competition as a substrate for the transaminase, MS probably spares glutamine for arginine synthesis. The degradation of MS could provide the glutamic acid required for arginine synthesis.

Effect of nitrogen deprivation and the gln-1 mutation on the catabolism of arginine

We have previously reported that glutamine prevents the catabolism of arginine by repressing arginase synthesis; thus proline auxotrophs will not grow on arginine if glutamine is simultaneously present in the culture medium (Vaca & Mora, 1977). This glutamine effect was not observed when spores of the *prol-4* mutant were deprived of nitrogen before incubation with glutamine and arginine. In the *prol-4 gln-1a* double mutant, however, the glutamine effect was observed even if the mutant had previously been deprived of nitrogen.

Glutamine synthetase activity

The *prol-3* mutant was incubated in medium devoid of proline under conditions in which different amounts of glutamine and arginine accumulated. There was a direct correlation

Table 3. Intracellular concentrations of glutamine and arginine, and glutamine synthetase activity in conidia of the *prol-3* strain under different conditions of incubation

Glutamine synthetase activity is expressed as $\mu\text{mol } \gamma\text{-glutamyl hydroxamate formed at } 30^\circ\text{C min}^{-1}$ (mg protein) $^{-1}$.

Condition	Concn [$\mu\text{mol (mg protein)}^{-1}$]		Glutamine synthetase activity
	Glutamine	Arginine	
Not incubated	0.068	0.032	0.004
Incubated 12 h in MM	1.035	0.520	0.035
Incubated 12 h in MM plus cycloheximide ($10 \mu\text{g ml}^{-1}$)	0.400	0.163	0.010
Preincubated 12 h in nitrogen-free MM and then NH_4NO_3 (25 mM) added for additional 12 h	3.190	1.120	0.149*

* Activity was measured after 12 h incubation in nitrogen-free MM, just before NH_4NO_3 was added.

between the quantities of these amino acids accumulating and glutamine synthetase activity (Table 3). Measurements of glutamine synthetase activity in samples taken at intervals during incubation of the *prol-3* strain in a nitrogen-free medium showed that it had increased 20-fold after 9 h. Enzyme induction was partially prevented by adding glutamine as nitrogen source to the culture medium. Results similar to those obtained with the *prol-3* auxotroph were found with the *tryp-2* auxotroph (results not shown).

De novo synthesis of glutamine synthetase

To determine whether glutamine synthetase induction was the result of *de novo* protein synthesis, a pulse of radioactive leucine was given to the *prol-3* mutant incubated either with glutamine as nitrogen source or in a nitrogen-free medium. Glutamine synthetase was precipitated with specific antiserum and total proteins with trichloroacetic acid. Figure 5 (A to D) shows the fluorography of total proteins and the specific immunoprecipitates after acrylamide gel electrophoresis in the presence of sodium dodecyl sulphate. The different intensities of the radioactive glutamine synthetase band from the two cultures indicated a positive correlation between the activity of the enzyme and the *de novo* synthesis of protein; the higher the band intensity, the higher the enzyme specific activity.

To measure the relative rates of enzyme synthesis, double label experiments were performed as described by Quinto *et al.* (1977). An extract from *prol-3* conidia incubated in nitrogen-free medium and labelled with [^3H]leucine was mixed with an extract from mycelium of the wild-type strain 74-A grown with glutamate as nitrogen source and labelled with [^{14}C]leucine. The $^3\text{H}/^{14}\text{C}$ ratio in the immunoprecipitate divided by the $^3\text{H}/^{14}\text{C}$ ratio of the total protein indicated the relative rate of synthesis of the enzyme in conidia incubated without nitrogen source compared with that in mycelium with glutamate as nitrogen source. Ratios of 0.75 and 1.0 were found in two experiments performed (Fig. 5, E, F) indicating that the relative rates of synthesis of the enzyme were similar under both conditions. Previous experiments have shown that optimal *de novo* synthesis of the enzyme occurs in *N. crassa* growing on glutamate as nitrogen source (Vichido *et al.*, 1978; Quinto *et al.*, 1977). The $^3\text{H}/^{14}\text{C}$ ratio of conidia incubated with glutamine compared with mycelium grown with glutamate was not determined due to the low amount of ^3H radioactivity incorporated into glutamine synthetase under the former condition.

Fig. 5. Conidia of the *prol-3* strain were incubated either in nitrogen-free MM or in MM containing 5 mM-glutamine as sole nitrogen source. After 6 h incubation, a 1 h pulse of [^3H]leucine ($5 \mu\text{Ci ml}^{-1}$) was given to each culture and extracts were prepared.

A to D. Total protein (A, C) and glutamine synthetase (B, D) were isolated by precipitation with trichloroacetic acid or by specific immunoprecipitation as described in Methods. The samples were then subjected to acrylamide gel electrophoresis and treated for fluorography. Conidia had been incubated in nitrogen-free MM for A and B, and in MM containing glutamine as sole nitrogen source for C and D.

E to H. Extracts from either culture were mixed with an extract from 74-A strain grown for 6 h on MM containing 5 mM-glutamate as sole nitrogen source and pulsed with [^{14}C]leucine ($0.5 \mu\text{Ci ml}^{-1}$), as above. Total protein (E, G) and glutamine synthetase (F, H) from both mixtures were isolated and processed as above. Conidia had been incubated in nitrogen-free MM for E and F, and in MM containing glutamine as sole nitrogen source for G and H.

DISCUSSION

When conidia of *N. crassa* are deprived of certain amino acids in the presence of NH_4NO_3 , glutamine and arginine accumulate. Such accumulation was not found in the absence of *de novo* synthesis of pyrimidines, in the presence of cycloheximide or in the presence of glycerol as carbon source (Espín & Mora, 1978). The rate of accumulation of glutamine and arginine also increased when ammonia was added to conidia which had been preincubated without a required amino acid or pyrimidine (Table 1; Fig. 1*a, b*) and this accumulation was no longer prevented by cycloheximide or glycerol as carbon source (Fig. 1*c, d*).

The increased accumulation of these amino acids was paralleled by an increased specific activity of glutamine synthetase which, in one case at least, involved *de novo* enzyme synthesis (Table 3, Fig. 5). It is likely, therefore, that the accumulation of glutamine and arginine which occurs when the exponential growth of mycelium stops is also due to an increase in the rate of synthesis of glutamine synthetase (Mora *et al.*, 1978).

Amino acid deprivation in *Dictyostelium discoideum* also triggers cell aggregation and differentiation (Schindler & Sussman, 1977).

The presence of mutations that impaired the assimilation of ammonia or the synthesis of glutamine in amino acid-deprived conidia prevented the utilization of the nitrogen atoms of exogenous glutamine for the synthesis and accumulation of arginine (Fig. 2). To explain these effects, we propose that glutamine is first degraded to ammonia and α -ketoglutarate which are then sequentially converted to glutamic acid, glutamine and arginine.

Fig. 6. Schematic diagram of the proposed glutamine cycle.

Evidence for the degradation of glutamine by the ω -amidase pathway in other cell systems has been recently reviewed (Cooper & Meister, 1977); these enzymes have also been detected in *N. crassa* (Monder & Meister, 1958). The liberated ammonium ions could be used to resynthesize glutamine through the concerted action of glutamate dehydrogenase and glutamine synthetase.

The above observations can be explained on the basis of a glutamine cycle (Fig. 6), the first step of which is the synthesis of different amino acids through an irreversible transamination, the second is the hydrolysis of α -ketoglutaramate to α -ketoglutarate and ammonium by an ω -amidase, the third being the formation of glutamic acid and the fourth the resynthesis of glutamine. The accumulation of arginine in the presence of L-methionine-DL-sulphoximine and glutamine, when glutamine synthetase is totally inactive, is consistent with the postulated role of glutamine transaminase in the cycle. Alternatively, glutamine might be degraded by the concerted action of an L-amino acid oxidase (Thayer & Horowitz, 1951) and an ω -amidase to yield ammonia and α -ketoglutarate. These, in turn, could lead to glutamine resynthesis. Although the participation of an L-amino acid oxidase could account for glutamine and arginine accumulation, it does not explain why other amino acids accumulate when amino acid-deprived conidia are incubated with either NH_4NO_3 (Espín & Mora, 1978) or glutamine (unpublished results) as the sole nitrogen sources. The requirement for a good carbon source for a variety of amino acids to accumulate is also more consistent with the involvement of a specific glutamine transaminase rather than an L-amino acid oxidase (Espín & Mora, 1978). Experiments are in progress to determine how glutamine is degraded by amino acid-deprived conidia.

Evidence indicating that the fixation of ammonia into glutamic acid is a necessary step before the transfer of the amide nitrogen of glutamine to arginine is given by the excretion of ammonia by strains that lack the activity of the enzymes that assimilate ammonia into

glutamic acid. The synthesis of glutamine is also required because *gln* auxotrophs are unable to accumulate arginine in the presence of glutamine but accumulate glutamic acid instead (Fig. 2). In contrast to the *prol-4* single mutant, the inability of the *prol-4 gln-1a* strain to grow on arginine plus glutamine after nitrogen deprivation can be explained as the result of degradation of glutamine in the absence of its resynthesis. The proposed cycle would therefore provide a mechanism for non-growing cells to utilize and distribute the carbon and nitrogen from the medium towards the synthesis and accumulation of amino acids, mainly glutamine and arginine.

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