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NADH-DEPENDENT GLUTAMATE SYNTHASE AND NITROGEN METABOLISM IN *Neurospora crassa*

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Summary

The GDH (NADPH) mutant strain am-1 of N. crassa has sizable pools of glutamine and glutamate under ammonium-limited conditions for which requires an elevated glutamine synthetase activity. Glutamine in the presence of 2-oxoglutarate, stimulated nicotinamide nucleotide oxidation by crude and purified extracts of the am-1 strain and led to a reductant dependent formation of two molecules of glutamate. Aminooxyacetate did not have any effect on the reaction, whereas azaserine inhibited it completely. It is concluded that in N. crassa glutamine synthetase and glutamate synthase are responsible for the assimilation of low ammonium concentrations.

Introduction

We have previously reported that the limitation of ammonium in Fed-batch cultures of *N. crassa* leads to an elevation of glutamine synthetase (E.C.6.3.1.2). We also found that the strain *am-1* completely lacking the biosynthetic glutamate dehydrogenase (GDH) (NADPH-dependent, E.C.1.4.1.4) grows as well as the wild-type in limited ammonium (1). The catabolic glutamic acid dehydrogenase (NAD-dependent, E.C.1.4.1.2) has low affinity for ammonia (2) and thus cannot be expected to fix ammonium in this condition. The enzyme system comprising glutamine synthetase (GS) and glutamate synthase (E.C.1.4.7.1) has been demonstrated to participate in the assimilation of low ammonium concentrations in procaryotes (3). Furthermore glutamate synthase has been detected in certain genus of *Schizosaccharomyces* (4), in *Saccharomyces cerevisiae* (5), and in higher plants (6,7,8). In this paper we report the existence and the purification

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of a NADH-dependent glutamate synthase (GOGAT) in Neurospora crassa. In addition the role of this enzyme together with glutamine synthetase (GS) in the growth of the fungi in limited ammonium, will be discussed.

Material and Methods

Stocks. - All stocks came from the Fungal Genetics Stock Center at Humboldt State University Foundation, Arcata, Calif. U.S.A. or from the collection of J. Mora. The basic stocks were wild-type strain 74-A, glutamic acid dehydrogenase deficient mutant am-1 and the glutamine auxotroph gln-1a. The double mutant am-1; gln-1a was obtained from crossing the above mentioned stocks in 1.7% (w/v) Corn meal agar (Difco).

Chemicals. - Azaserine was obtained from Calbiochem-Behring Corp., La Jolla, CA 92037. All other chemicals were obtained from Sigma. All Chemicals used were analytical grade.

Growth Conditions. - N. crassa was grown in batch cultures in minimal medium (N) of Vogel (9) supplemented with 1.5% sucrose and 25 mM KNO_3. Fed-batch cultures of N. crassa ammonium limited were achieved as previously reported (1). Growth was determined as described (10).

Determination of Glutamate and Glutamine 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 Corp., type RA 1.2 μ). The filtrates containing the amino acids were lyophilized and the samples resuspended in deionized water. The amino acids were separated using an Aminco amino acid analyzer and quantified in an Aminco Ratio Fluorometer after coupling with orthophthalaldehyde. Under these conditions 80% of the glutamic acid and glutamine were recovered.

Determination of Enzymes Activity and Purification of Glutamate Synthase (GOGAT). - Cell free extracts were prepared as previously described (10). GS measured as transferase activity was assayed as Ferguson and Sims (11), the GDH (NADPH and NAD dependent) as Fincham (12) and GOGAT was assayed and purified by the method of Boland and Benny (13), except that freshly harvested mycelia were disrupted in a Braun Cell Homogenizer MSK, type 853030. Protein was determined by the method of Lowry et al, (14), using bovine serum albumin as a standard.

Results and Discussion

A first approach in the search of GOGAT in Neurospora crassa was to look for the synthesis and accumulation of glutamate in conditions of ammonium limitation in the GDH-(NADPH) mutant strain. In Table I is shown the accumulation of this aminoacid by this strain in comparison with the

TABLE I.- Intracellular concentrations of glutamate and glutamine in the wild-type strain and 74-A and in the mutant strain am-1, under ammonium limitation*.

Strain	hs	glu	gln
Wild-type 74-A	0	0.210	0.060
	3	0.160	0.055
	6	0.085	0.215
	9	0.080	0.030
	12	0.080	0.025
am-1	0	0.149	0.007
	4	0.051	0.119
	8	0.041	0.115
	12	0.166	0.021

*glutamate (glu) and glutamine (gln) concentrations are expressed as μmoles/mg protein.

wild-type. As a second step, the requirement of GS to fix ammonium was investigated by analyzing the double mutant am-1; gln-1a. The gln-1a mutant lacks partially the activity of GS (15). In distinction with the am-1 and gln-1a single mutants, that show some growth in KNO_3, the double mutant am-1; gln-1a does not grow at all in this nitrogen source

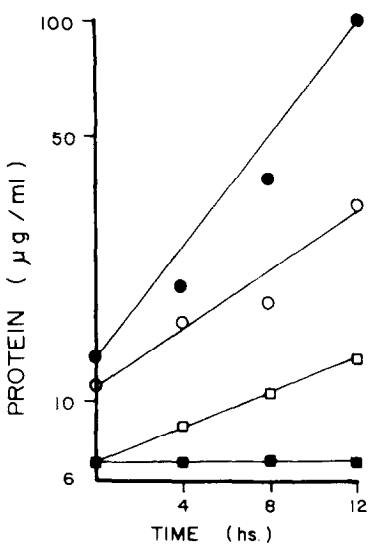

Fig. 1. Growth curves of wild-type 74-A (●) and mutants am-1 (o), gln-1a (□), and am-1; gln-1a (■) in 25 mM KNO_3 at 25°C for 12 hs.

TABLE II.- Intracellular concentrations of glutamine, activity of glutamine synthetase and glutamate dehydrogenase (NADPH and NAD dependent) in the wild-type strain 74-A and in the mutant strains am-1, gln-1a, and am-1; gln-1a grown in 25 mM KNO_3 at 25°C*.

Strain	hs	glu	gln	G.S.	glutamate dehydrogenase NADPH-dependent	glutamate dehydrogenase NAD-dependent
74-A	12	0.238	0.365	0.039	0.14	0.009
	24	0.735	2.177	0.799	0.09	0.012
am-1	12	0.072	0.156	0.300	N.D.	0.003
	24	0.337	0.463	0.282	N.D.	0.007
gln-1a	12	0.675	0.135	0.049	0.05	--
	24	0.052	0.024	0.086	0.06	--
am-1;gln-1a	12	0.009	0.008	0.050	N.D.	--
	24	0.052	0.024	0.086	N.D.	--

* glutamate (glu) and glutamine (gln) concentrations are expressed as micromoles/mg protein. Glutamine synthetase (GS) is expressed by its transferase activity (micromoles of γ-glutamyl hydroxamate produced per minute/mg protein at 30°C). Glutamate dehydrogenases are expressed as micromoles of coenzyme oxydized per minute/mg protein at room temperature. N.D. Not detected.

(Fig. 1), neither ammonium-limited, nor accumulates glutamate and glutamine (Table II). The am-1 strain grows as well as the wild-type and both have a high activity of GS. From this data we concluded that an elevated GS is required to fix ammonium. The catabolic GDH (NAD-dependent) was also assayed in conditions of low nitrogen. It was found that this enzyme did not increase under this condition (Table II), something that speaks against the participation of the catabolic GDH in the fixation of low ammonium. On the other hand the presence of an activity similar to GOGAT was detected in ammonium limited cultures of the am-1 strain mutant. The condition found for the assay of this enzyme was similar to that reported in plant nodules (13). Conditions reported for GOGAT determination in other systems (4,5-7,16-21) were unsuccessful in Neurospora. We were able to purify the GOGAT of Neurospora by the procedure used

TABLE III.- Purification of glutamate synthase of Neurospora crassa*.

Fraction	Protein (mg/ml)	Volume (ml)	Specific Activity (μmol/min/mg protein)	Total Activity (μmol/min)
Crude extract	4.51	100	0.03	13.5
Ammonium sulphate	22.04	4	0.16	14.3
Sepharose 6B	0.04	36	1.51	2.2
DEAE-Sephadex A-25	0.02	6	4.76	0.6

*Initial extract from 30 l of culture.

for the purification of the nodule enzyme (13). In Table III are presented the purification steps which resulted in a 136 fold purification.

Finally in Table IV the inhibition by azaserine and not by aminooxyacetate, is presented. The GOGAT from pea roots presents a similar pattern of inhibition (20). In the same table we show the stoichiometry of glutamate synthase in which 1 molecule of glutamine disappears, 1 molecule of NADH is oxidized and nealy 2 molecules of glutamate are produced.

TABLE IV.- Specificity and stoichiometry of purified glutamate synthase activity in Neurospora crassa*.

Assay conditions	Specific Activity	Change of concentration (μmol) of NADH	glutamine	glutamate
Complete system	4.16	-5.6	-5.4	+9.9
Absence of glutamine	N.D.			
Absence of 2-oxoglutarate	N.D.			
Presence of NH_4 (2.5 mM)	N.D.			
Presence of azaserine (2.5 mM)	N.D.			
Presence of aminooxy acetic acid (2.5 mM)	4.06			

*Purified enzyme was incubated with 2.5 mM L-glutamine, 1 mM 2-oxoglutarate and 0.1 mM NADH for 9 min at room temperature. The oxidation of NADH was determined and then ethanol was added to stop the reaction. Samples were analyzed for glutamate and glutamine content as described in Methods. Stoichiometry for 2-oxoglutarate was not determined.

In conclusion we have demonstrated that glutamate synthase is present in Neurospora in Fed-batch ammonium limited cultures, in a mutant that lacks the biosynthetic GDH. This enzyme together with the glutamine synthetase plays a role in the fixation of ammonium in low concentrations. Since we have reported that a tetrameric GS, instead of an octameric, is present in low ammonium (1), it is essential to determine the affinity for ammonium of this oligomer versus octameric GS present in excess of nitrogen. Recent evidence demonstrates that two different monomers are responsible for GS activity (Sánchez, et al, submitted for publication, 1979), and that each of these monomers is present mainly as the tetrameric or the octameric enzyme (Dávila et al, submitted for publication, 1979).

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