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ω-Amidase Pathway in the Degradation of Glutamine in *Neurospora crassa*

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Evidence for the participation of the glutamine transaminase-ω-amidase pathway in the utilization of glutamine in *Neurospora crassa* was obtained. Its participation is indicated by (i) the in vitro activities of glutamine transaminase and ω-amidase, (ii) the in vivo accumulation of α-ketoglutaramate when an inhibitor of transamidases is present, and (iii) the inhibition by aminooxyacetic acid and 6-diazo-5-oxo-L-norleucine of the ammonium excreted in the presence of glutamine by a mutant strain that lacks glutamate dehydrogenase and glutamate synthase.

Glutamine has a central role in nitrogen metabolism in microorganisms. It is used as a donor of amido nitrogen in transamidation reactions, and it is also a corepressor of nitrogen catabolism (15, 19).

We studied the enzymes that participate in glutamate and glutamine synthesis in *Neurospora crassa*: glutamate dehydrogenase (GDH) (NADPH dependent, EC 1.4.1.4) (10), glutamate synthase (GOGAT) (NADH dependent, EC 1.4.7.1) (12, 13), and glutamine synthetase) (EC 6.3.1.2) (17).

We reported that glutamine, arginine, and other amino acids are accumulated when *N. crassa* is deprived of an amino acid or at the end of exponential growth (6, 18). Mutant strains impaired in the assimilation of ammonium (5, 8) are unable to use the nitrogen atoms of ammonium or glutamine for arginine synthesis (7). To explain the effect of these mutations, we proposed that glutamine is first degraded to ammonium and α-ketoglutarate, which are sequentially converted to glutamic acid, glutamine, and arginine (7).

Evidence for the degradation of glutamine by the ω-amidase pathway in other cell systems has been amply documented by Cooper and Meister (3, 4). In this pathway glutamine is transaminated to yield different amino acids and α-ketoglutaramate through the participation of a glutamine transaminase; subsequently, the α-ketoglutaramate is hydrolyzed to α-ketoglutarate and ammonium by the action of an ω-amidase. Since the presence of a glutamine transaminase had been reported in *N. crassa* (16), we suggested the operation of the ω-amidase pathway in this fungus (7). In this paper we present evidence of the operation of glutamine transaminase and ω-amidase as the enzymes responsible for the conversion of glutamine to α-amino nitrogen, ammonium, and carbon skeletons.

Glutamine transaminase and ω-amidase activities were determined in cell-free extracts from *N. crassa* wild-type strain (74-A) cultures grown on Vogel minimal medium (21) supplemented with 1.5% sucrose at 30°C with shaking at 250 rpm.

For glutamine transaminase activity cell-free extracts were prepared from acetone powders (20) homogenized in extraction buffer (50 mM PP_i [pH 8.5]); the homogenates were centrifuged and dialyzed. The stoichiometry of glutamine transaminase activity is shown in Table 1. The reaction mixture was incubated for 10 min at 37°C (see Table 1); the disappearance of the substrate phenylpyruvate (1) and the formation of the product phenylalanine (7) were determined as reported. The formation of α-ketoglutaramate was also measured quantitatively by a modification of the method described by Cooper et al. (2). The transaminase was measured in the presence of 6-diazo-5-oxo-L-norleucine (DON), an inhibitor of the ω-amidase activity, which allows optimal accumulation of α-ketoglutaramate (Table 1; see below). When [^{14}C]glutamine and phenylpyruvate were used as substrates in the presence of DON, the label incorporated into α-ketoglutaramate (1.9 μmol) corresponded to the α-ketoglutaramate measured colorimetrically (2.0 μmol). The activity and stoichiometry of glutamine transaminase was also assayed as reported by Monder and Meister (16) with glutamine and α-ketosuccinamate as substrates. These authors found that in *N. crassa* only glutamine functions as an α-amino donor with α-ketosuccinamate. The specific activity with α-ketosuccinamate was similar to that assayed with phenylpyruvate (Table 1), and the amount of α-ketoglutaramate formed was the same with and without DON (data not shown). Only one transaminase was apparently involved in both assays, since the activities were not additive in the presence of both α-ketoacids (Table 1).

For the ω-amidase activity the extraction buffer used contained 0.1 mM EDTA, 5 mM 2-mercaptoethanol, and 50 mM Tris-hydrochloride (pH 8.5). The stoichiometry of ω-amidase activity is shown in Table 2. The incubation mixture (Table 2) was incubated for 30 min at 37°C, and the reaction was stopped with 0.1 ml of 17% trichloroacetic acid. After the supernatant was neutralized with NaOH, ammonium and α-ketoglutarate were determined as described (11, 22). DON completely inhibited the ω-amidase activity, and aminooxyacetic acid (AOA) did not have any effect on it. Since α-ketosuccinamate is an inhibitor of the ω-amidase activity and not a substrate (Table 2), the α-ketoglutaramate formed by the transamination of glutamine with this α-ketoacid could be detected in the absence of DON. The specific activity of *N. crassa* ω-amidase was threefold higher than the glutamine transaminase activity, and it was twofold lower than the highest value of ω-amidase activity reported for animal cells (3). We found that the ω-amidase from animal cells was also inhibited by DON (data not shown).

The presence of α-ketoglutaramate in vivo was tested from *Neurospora* wild-type strain conidia or mycelia incubated for 5 h on either ammonium or glutamine as nitrogen sources. Samples were prepared by homogenizing recently harvested conidia or mycelium in 80% (vol/vol) ethanol. The

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TABLE 1. Stoichiometry of *N. crassa* glutamine transaminase with phenylpyruvate

Assay condition	Sp act[a]	Change of concn (nmol)		
		Phenyl-pyruvate	Phenylal-anine	α-Ketoglu-taramate
Complete system[b]	5.5	−205.0	+219.4	+205.7
Without glutamine	0.6	−21.8	+17.4	ND[c]
Without phenylpyruvate	ND	ND	ND	ND
Without extract	ND	ND	ND	ND
Without DON	5.5	−205.0	+219.4	+86.2
With 10 mM AOA	ND	ND	ND	ND
With α-ketosuccinamate, without phenylpyru-vate	6.0[d]			+223.1
With α-ketosuccinamate	6.0[d]			+222.0

[a] Expressed as nanomoles of phenylpyruvate per minute per milligram of protein.
[b] The assay mixture contained (in 1.0 ml) 20 mM glutamine, 0.4 mM phenylpyruvate or 0.8 mM α-ketosuccinamate, 0.2 mM pyridoxal phosphate, 150 mM sodium borate buffer (pH 8.5), 0.3 mM DON, and 3.7 mg of dialyzed protein extract.
[c] ND, Not detected.
[d] Expressed as nanomoles of α-ketoglutaramate per minute per milligram of protein.

homogenates were filtered, lyophilized, resuspended in water, and applied to a Dowex 50 column (H^+ form; 7 by 0.5 cm). The column was eluted with 10 ml of water, and the effluent was added to a Dowex-2 column (Cl^- form; 4 by 0.5 cm). After the column was washed with water, the ketoacid was eluted with 0.1 N HCl. The fractions were neutralized and lyophilized and the α-ketoglutaramate was determined as described above. The α-ketoglutaramate was only detected in the presence of 1 mM DON, and it was fivefold higher on glutamine (10 nmol/h per mg of protein) than on ammonium as the nitrogen source. When 10 mM AOA and 1 mM DON were present in the medium, no α-ketoglutaramate was found, and the activities of glutamine transaminase and ω-amidase determined in vitro were also found to be completely inhibited. The in vivo accumulation of α-ketoglutaramate in the presence of DON and its absence in the presence of AOA and DON indicate that the ω-amidase pathway participates in glutamine degradation.

The proposal that in *N. crassa* glutamine is degraded to ammonium by the operation of the ω-amidase pathway and

TABLE 2. Stoichiometry of ω-amidase

Assay conditions	Sp act[a]	Change of concn (μmol)		
		α-Ketoglu-taramate	α-Ke-toglu-tarate	Ammonium
Complete system[b]	15.5	−1.43	+1.43	+1.58
Without α-ketoglutaramate	0.7	ND[c]	ND	0.07
Without extract	ND	ND	ND	ND
With 0.3 mM DON	0.7	ND	ND	+0.07
With 10 mM AOA	12.0	−1.12	—[d]	+1.22
With 10 mM α-ketosuccina-mate	3.8[e]		+0.39	

[a] Expressed as nanomoles of ammonium per minute per milligram of protein.
[b] The assay mixture contained (in 1.0 ml) 10 mM α-ketoglutaramate, 2 mM 2-mercaptoethanol, 50 mM Tris-hydrochloride buffer (pH 8.5), and 3.4 mg of dialyzed protein extract.
[c] ND, Not detected.
[d] AOA interfered with the determination of α-ketoglutarate.
[e] Expressed as nanomoles of α-ketoglutarate per minute per milligram of protein.

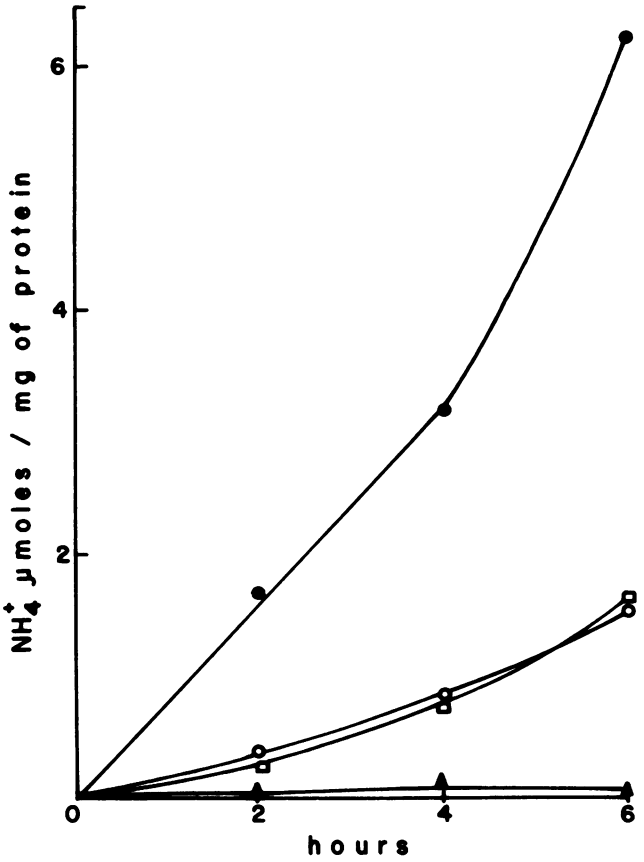

FIG. 1. Inhibition by AOA and DON of ammonium excretion by the double-mutant strain lacking GDH and GOGAT activities in glutamine as nitrogen source. Conidia of the GDH^- $GOGAT^-$ mutant strain were germinated for 6 h in a medium supplemented with 10 mM alanine as nitrogen source. The mycelium was collected and transferred to different media: 10 mM glutamine (●), 10 mM glutamine plus 25 mM AOA (□), 10 mM glutamine plus 1 mM DON (△), and without nitrogen (○).

that this ammonium could be reassimilated predicts that a mutant which lacks GDH activity might excrete ammonium to the medium when grown on glutamine as nitrogen source.

After 6 h of growth on 1 mM glutamine, the culture medium of the mutant lacking GDH activity contained 0.25 mM of ammonium, whereas only 0.02 mM ammonium was present in the wild-type strain culture. The ammonium in the medium was measured as described (7). The ammonium excreted by the G mutant lacking GDH in the presence of glutamine is the result of the lack of GDH activity and of the operation of the ω-amidase pathway enzymes. The latter is shown by the in vivo effect of inhibitors of ammonium excretion from glutamine in the double-mutant strain lacking GDH and GOGAT (Fig. 1). This mutant strain was used to prevent glutamine assimilation by GOGAT (manuscript in preparation). As shown in Fig. 1, AOA inhibits the liberation of ammonium from glutamine, and DON has a similar and more powerful effect, since it completely abolishes the production of ammonium, including the small amount of ammonium excreted in the absence of nitrogen.

We demonstrated the presence of a transaminase activity between glutamine and phenylpyruvate that leads to the formation of phenylalanine and α-ketoglutaramate (Table 1), and we detected an ω-amidase that hydrolyzes α-ketoglu-

taramate into α-ketoglutarate and ammonium (Table 2). We have also shown that the ω-amidase pathway has a role in the degradation of glutamine.

In contrast with other microorganisms (14), *N. crassa* assimilates glutamine through enzymes which do not include the participation of a glutaminase. It should be mentioned that the glutamine transaminase must be considered as an irreversible transaminase, since the α-ketoglutaramate formed in this reaction could be either hydrolyzed by the ω-amidase or spontaneously cyclize (3).

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