The involvement of glutamate dehydrogenase and glutamine synthetase/glutamate synthase in ammonia assimilation by the basidiomycete fungus *Stropharia semiglobata*

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When grown on ammonia as sole nitrogen source, the basidiomycete fungus *Stropharia semiglobata* assimilated nitrogen via glutamate dehydrogenase (GDH). The two isoenzymes of GDH were both repressed by increasing concentrations of ammonia, but NADP-GDH (EC 1.4.1.4) was far more active than NAD-GDH (EC 1.4.1.2). Glutamine synthetase (GS; EC 6.3.1.2) and glutamate synthase (GOGAT; EC 1.4.7.1) were also active in *S. semiglobata* and these enzyme activities were independent of the ammonia concentration in the suspension. From enzyme activity and ¹⁵N-labelling studies of amino acids, it is concluded that both GDH and GS/GOGAT contribute to ammonia assimilation in this fungus.

Introduction

Inorganic ammonia and nitrate are the natural nitrogen sources preferred by bacteria, algae and fungi. The only known metabolic pathway for utilization of nitrate nitrogen is by reduction first to nitrite and subsequently to ammonia (Pateman & Kinghorn, 1975); 2-oxoglutarate and glutamate have unequivocally been demonstrated to be the primary organic acceptors for inorganic ammonia (Mifflin & Lea, 1977; Smith *et al.*, 1975; Tyler, 1978). Consequently, the amino acids glutamate and glutamine are formed primarily and serve as donors in transamination and amino group transfer reactions.

Ammonia assimilation in fungi is catalysed by NAD(P)H-specific glutamate dehydrogenase (GDH) with the formation of glutamate, or by glutamine synthetase (GS) with the formation of glutamine.

The amido group of glutamine is often further metabolized and incorporated into glutamate via the glutamate synthase (GOGAT) reaction.

By coupling the GS and GOGAT reactions an almost irreversible route for ammonia assimilation is achieved. Some fungi as well as higher plants form glutamate via the GS/GOGAT pathway, especially when the ammonia

supply is limited (Hummelt & Mora, 1980; Kusnan *et al.*, 1987; Mifflin & Lea, 1980).

In studies on the primary nitrogen metabolism of fungi, yeasts and ascomycetes have been investigated preferentially (Brown *et al.*, 1974; Casper *et al.*, 1985; Dunn-Coleman *et al.*, 1981; Holmes *et al.*, 1989; Kusnan *et al.*, 1987, 1989; Limon-Lason *et al.*, 1977). In this paper the primary nitrogen metabolism of the basidiomycetic fungus *Stropharia semiglobata* was investigated.

Methods

Organism and growth conditions. *Stropharia semiglobata* TA 79139 was obtained from T. Anke (University of Kaiserslautern, FRG). It was grown in a culture medium based on those of Garrett (1972) and Jennison *et al.* (1955), containing (g l⁻¹) glucose (10), K₂HPO₄/KH₂PO₄ (0.96/0.68), MgSO₄·7H₂O (0.1), NaCl (0.1), CaCl₂·2H₂O (0.1), biotin (0.001), riboflavin (0.001), thiamin (0.001) and micronutrients according to Nicholas & Nason (1954). Further details on the growth conditions of the fungus have been published previously (Kusnan *et al.*, 1987, 1989).

[¹⁵N]Ammonia incubation. The fungus was grown in 2.5 litres of nutrient solution containing 2 mM-NH₄Cl as sole nitrogen source. On reaching the experimental growth-phase mycelia were transferred, resuspended in 500 ml of fresh culture medium and diluted to about 1 g fresh wt per 100 ml. After adaptation for 2 h at 26 °C, 500 ml of fungal suspension was diluted with 500 ml of nutrient solution containing ¹⁵NH₄Cl (99% enrichment). Consequently, the ammonia content of the incubation medium was 2 mM (40–50% ¹⁵N enrichment). The culture media were aerated throughout the incubation period. The ammonia concentration of the media was regularly analysed by withdrawing samples and determining ammonia colorimetrically (Yuen & Pollard, 1952).

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Abbreviations: GDH, glutamate dehydrogenase; GOGAT, glutamate synthase; GS, glutamine synthetase.

Harvest for amino acid and total nitrogen determination. After incubation with $^{15}\text{NH}_4\text{Cl}$ 100 ml samples were harvested by filtration through Miracloth (Calbiochem). The mycelia were washed with approximately 100 ml ice-cold 0.1% (w/v) NaCl, suspended in 8 ml 95% (v/v) ethanol and stored at -20°C until further use.

Extraction and determination of nitrogenous compounds. The mycelia, the total nitrogen of which was determined as ammonia following Kjeldahl digestion, were homogenized with a pestle and mortar in ethanol, quantitatively transferred into a calibrated cylinder and diluted to 15 ml with ethanol. This suspension was diluted to 20 ml with distilled water (final ethanol concentration 70%).

Samples (2.5 ml) of the ethanolic fraction were taken to dryness for dry weight determination. The remaining suspension was centrifuged, the supernatant taken to dryness in a vacuum evaporator at 37°C and the residue dissolved in 5 ml of water (pH 3–5). Amino acids were analysed according to Kedenburg (1971) by using a Beckman Multichrome liquid chromatograph. Acidic amino acids were separated from neutral and basic amino acids on a Dowex 1X8 column (4×0.9 cm, acetate form). The neutral and basic amino acids passed through the column; after washing the column with 8.0 ml of water, these amino acids were recovered completely. The acidic amino acids were eluted with 8.0 ml 2 M-acetic acid. The water- and acid-eluates were vacuum-dried separately, and each was resuspended in 2 ml lithium hydroxide/citric acid buffer at pH 2.2 for amino acid analysis (Kusnan *et al.*, 1987). ^{15}N was determined by emission spectroscopy as described previously (Berger & Fock, 1983). By applying appropriate corrections for isotope dilution arising from ammonia introduced into the analytical procedure ^{15}N enrichment of total nitrogen in the mycelia and in extracted amino acids as well as of glutamine-amino and glutamine-amido was estimated (Kusnan *et al.*, 1989; Kusnan, 1990).

Enzyme extraction. Mycelium was collected by filtration, washed with 1 litre of water and frozen in liquid nitrogen until use. The frozen tissue was homogenized by pestle and mortar and extracted in potassium phosphate buffer as described by Dunn-Coleman *et al.* (1981). The extract was desalted on a Sephadex G-25 column equilibrated with the same buffer.

Enzyme assays. The activities of NADP-GDH (EC 1.4.1.4) and NAD-GDH (EC 1.4.1.2) were assayed according to Stewart & Moore (1974) at pH 7.9 and 8.5, respectively. GS (EC 6.3.1.2) activity was assayed as described by Boland & Benny (1977). In all cases, activity was determined by following the oxidation of NADH at 340 nm (30°C). GOGAT (EC 1.4.7.1) activity was assayed both by the transferase and by the synthetase reactions, as described by Ferguson & Sims (1971, 1974). Specific activities of the enzymes are expressed as nmol reduced coenzyme oxidized or γ -glutamylhydroxamate formed min^{-1} (mg protein) $^{-1}$. Protein was determined colorimetrically by binding to Coomassie Brilliant Blue G-250 (Sedmark & Grossberg, 1977) with bovine serum albumin as the standard.

Results

Amino acid and ammonia content

During exponential growth in 2 mM- NH_4Cl as sole nitrogen source (growth factor, μ , = 0.0514 mg dry wt h^{-1} , 48–96 h after inoculation), the mycelium was harvested and extracted, and the amino acid and ammonia content analysed (Table 1). Glutamine was present at by far the highest concentration (3643 ± 327 nmol per 100 mg dry wt). The concentrations of the free amino acids glutamate, alanine and

Table 1. Amino acids and ammonia content of *S. semiglobata* grown in 2 mM- NH_4Cl medium

Amino acid	Content (nmol per 100 mg dry wt)	Standard error, SE (%SE)	No. of replicates, n
Aspartate	457	59.9 (11.5%)	4
Glutamate	1682	256.6 (15.2%)	5
Threonine	118	5.6 (4.7%)	4
Serine	280	25.3 (9.0%)	6
Asparagine	306	37.2 (12.1%)	4
Proline	95	14.5 (15.3%)	4
Glycine	258	21.4 (8.2%)	6
Alanine	1130	145.5 (12.8%)	4
Valine	170	7.5 (4.4%)	4
Histidine	123	6.8 (5.5%)	5
Glutamine	3643	326.8 (8.9%)	5
Arginine	1185	118.5 (10.0%)	5
Lysine	257	15.6 (9.1%)	4
Ammonia	986	90.0 (9.1%)	4

arginine were also high (1200–1700 nmol per 100 mg dry wt). The ammonia content was 982 nmol per 100 mg dry wt.

During 60 min incubation with 2 mM- $^{15}\text{NH}_4\text{Cl}$, the ammonia content of the medium declined insignificantly (from 2.0 to 1.8 mM) while the ^{15}N enrichment of the medium remained almost constant (approximately 40% ^{15}N). During this period the mycelial amino acid pools remained fairly constant, and the concentrations measured were similar to those before incubation in $^{15}\text{NH}_4\text{Cl}$ (Fig. 1). Therefore, $^{15}\text{NH}_4^+$ uptake and metabolism occurred during steady-state growth of the fungus.

^{15}N Uptake

The total nitrogen content of mycelia during the incubation was 301 nmol N per 100 mg dry wt tissue (mean of five replicates). The ^{15}N enrichment of the tissue increased by $4.8 \pm 0.4\%$ after 70 min incubation with 2 mM- NH_4Cl (40% enrichment) (data not shown). Based on the average total nitrogen and ^{15}N enrichment of total nitrogen, about 344 nmol N min^{-1} per 100 mg dry wt were assimilated.

Incorporation of ^{15}N from $^{15}\text{NH}_4\text{Cl}$ into soluble amino acids is shown in Figs 2 and 3. After a 10–20 min lag phase, ^{15}N in glutamate increased steadily and approached a saturation of more than 30% enrichment after 60 min incubation. Total ^{15}N in glutamine (amino-plus amido-nitrogen) increased continuously from zero to 19% enrichment during 60 min incubation. This value is lower than the ^{15}N enrichment in glutamate. However, more ^{15}N was incorporated into the amide group than into the amino group of glutamine (Fig. 2). The ^{15}N enrichment of amide increased to 24.5% compared to a

Fig. 1. Amino acid content of *S. semiglobata* during $^{15}\text{NH}_4\text{Cl}$ feeding. The results of one of two replicates with similar trends are shown; the deviation from values shown was about 10% (a): ■, Glutamine; ▲, glutamate; ●, arginine. (b): ■, Alanine; ▲, ammonia; ●, aspartate. (c): ■, Serine; ●, glycine; ▲, histidine.

Fig. 2. ^{15}N labelling of glutamate (□), glutamine (★), glutamine-amine (●) and glutamine-amide (■) during $^{15}\text{NH}_4\text{Cl}$ feeding (2 mM, approximately 40% enrichment). The standard errors of the mean ($n = 2$ or 3) were $<10\%$.

13.5% enrichment of the amino group, which is lower than the ^{15}N enrichment of glutamate.

Aspartate and alanine also became highly ^{15}N -labelled (Fig. 3). The ^{15}N enrichment of these two amino acids increased steadily and reached values of 26.5 and 26.7% after 60 min incubation, respectively. Glycine incorporated more than 30% ^{15}N and seemed to approach ^{15}N saturation after 45–60 min incubation with $^{15}\text{NH}_4\text{Cl}$.

Fig. 3. ^{15}N labelling of amino acids during $^{15}\text{NH}_4\text{Cl}$ feeding (2 mM, 40% enrichment). The standard errors of the mean ($n = 2$ or 3) were $<10\%$.

Arginine, serine and histidine were labelled only after a lag phase. Then the ^{15}N label increased, but these amino acids incorporated less ^{15}N than did glutamine, glutamate, aspartate, alanine and glycine.

Effect of ammonia concentration on the activity of nitrogen-metabolizing enzymes

Enzyme activities were determined from mycelia grown in media supplied with different concentrations of NH_4Cl (2–50 mM). Fig. 4 shows that the activity of NADP-GDH declined with increasing concentrations of NH_4^+ in the medium. Thus 1944 nmol NADPH (mg protein) $^{-1}$ min $^{-1}$ were oxidized at 2 mM- NH_4^+ , 1120 nmol (mg protein) $^{-1}$ min $^{-1}$ at 5 mM- NH_4^+ and 368 nmol (mg protein) $^{-1}$ min $^{-1}$ at 50 mM- NH_4^+ .

The change in level of NAD-GDH in response to NH_4^+ differed greatly from that of NADP-GDH. For NAD-GDH, the specific activity was 33 nmol NADH (mg protein) $^{-1}$ min $^{-1}$ at 2 mM- NH_4^+ , and the activity increased slightly with 10 mM- NH_4^+ in the culture medium. However, the activity declined to 9 nmol NADH (mg protein) $^{-1}$ min $^{-1}$ at 50 mM- NH_4^+ .

GOGAT activity was 21 and 30 nmol NADH oxidized (mg protein) $^{-1}$ min $^{-1}$ at 2 mM and 10 mM- NH_4^+ , respectively, but declined to 20 nmol (mg protein) $^{-1}$ min $^{-1}$ at 50 mM- NH_4^+ .

When measured in the transferase assay, GS activity was about 9 times higher than in the synthetase assay;

Fig. 4. Enzyme activities of crude extracts of *S. semiglobata* grown at various ammonia concentrations. (a) NADP-GDH. (b) NAD-GDH. (c): ■, NAD-GOGAT; ▲, GS-transferase; ●, GS-synthetase. Bars represent standard errors.

the activities did not change significantly with the NH_4^+ concentration in the medium. The activities were about 12 $\text{nmol } \gamma\text{-glutamylhydroxamate formed (mg protein)}^{-1} \text{min}^{-1}$ in the transferase test and 1.2 $\text{nmol (mg protein)}^{-1} \text{min}^{-1}$ in the synthetase test with 50 mM- NH_4^+ in the medium.

Discussion

In this study the capacity of *Stropharia semiglobata* to incorporate and to metabolize NH_4^+ was investigated. In accordance with investigations from other laboratories, it has been shown that like fungi such as *Aspergillus nidulans*, *Cladosporium* sp., *Neurospora* sp. and *Ustilago* sp. (Kusnan *et al.*, 1987, 1989; Lewis & Fincham, 1970a, b; Morgan & MacMillan, 1954; Pateman *et al.*, 1967), *S. semiglobata* can utilize NH_4^+ as sole nitrogen source.

The mode of entry of ammonia into fungal cells is still obscure, although Pateman *et al.* (1974) described an active ammonia transporter which together with diffusion may be responsible for ammonia uptake into the fungal tissue.

S. semiglobata grew well in media enriched with NH_4Cl , although high concentrations of NH_4^+ (50 mM)

inhibited growth slightly (data not shown). The rate of ammonia uptake was 344 nmol N min^{-1} per 100 mg dry wt, which is 68% of the uptake rate for *A. nidulans* (Kusnan *et al.*, 1987). The slower rate of ammonia uptake by *S. semiglobata* is reflected by a slower growth rate and longer doubling time (13.5 h) as compared with *A. nidulans* (7.5 h; Kusnan *et al.*, 1989; Kusnan, 1990).

Several fungi assimilate NH_4^+ through the formation of glutamate by NADP-GDH. *S. semiglobata* also showed GDH activity (Fig. 4). The specific activity of NADP-GDH was approximately 60 times higher than that of NAD-GDH. This result was not unexpected, as *A. nidulans* (Kusnan *et al.*, 1987) and *N. crassa* (Marzluf, 1981), grown at low concentrations of ammonia, exhibited much higher activities of NADP-GDH than of NAD-GDH. Because of the different enzyme activities and affinities for ammonia, it appears that NADP-GDH contributes to ammonia assimilation, while NAD-GDH is involved more in the formation of ammonia (Smith *et al.*, 1975; Stewart *et al.*, 1980).

The alternative pathway of ammonia assimilation via GS/GOGAT has so far been described for only a few fungi. The coupled reactions of this irreversible pathway should assimilate nitrogen very efficiently at low ammonia concentrations (Stewart *et al.*, 1980). GS/GOGAT activity was found in *S. semiglobata* (Fig. 4). The GOGAT activities were similar in magnitude to the NAD-GDH activities. This agrees well with the similar activities of GOGAT and NAD-GDH found for *A. nidulans* (Kusnan *et al.*, 1987), *Saccharomyces cerevisiae* (Roon *et al.*, 1974) and *N. crassa* (Dunn-Coleman *et al.*, 1981).

GS transferase activities in *S. semiglobata* were low and the synthetase activities even lower (Fig. 4). As some unknown losses of *in vivo* activities during extraction would be expected and as GS shows a high affinity for ammonia, the measured activities for ammonia fixation appear to be sufficient to maintain the required flux in primary nitrogen assimilation even at high ammonia concentration, when NADP-GDH is strongly repressed. This is obvious as growth of the fungus under these conditions was only slightly reduced.

After 60 min incubation in 2 mM- $^{15}\text{NH}_4\text{Cl}$ (approximately 40% enrichment), glutamate, aspartate, alanine and glycine carried most of the ^{15}N label (Figs 2 and 3). This is consistent with the operation of highly active aminotransferases in primary nitrogen metabolism (Becker & Fock, 1989) and transfer of amino groups from glutamate, a primary product of ammonia assimilation, to alanine, aspartate and glycine. The observed high ^{15}N enrichments in aspartate, alanine, glycine and arginine may be explained by high rates of amino group transfer from glutamate. Aspartate formation in fungi possibly proceeds via glutamate:oxaloacetate aminotransferase

(Fincham & Boulter, 1956). Glutamate:pyruvate aminotransferase may catalyse alanine formation from glutamate and pyruvate. Arginine is synthesized from glutamate via ornithine and citrulline (Pateman & Kinghorn, 1975). Glycine is formed by transamination from alanine and glyoxylate.

After examination of the ^{15}N -labelling kinetics of amino acids, it is apparent that ^{15}N was incorporated into glutamate with a lag phase while glutamine was labelled more steadily (Fig. 2). Furthermore, much more ^{15}N was incorporated into the amide group of glutamine than the amino group (Fig. 2). While ^{15}N in glutamate tended to saturate above 30% enrichment after 60 min, ^{15}N -labelled amide increased continuously. If NADP-GDH, present at high levels, was the only enzyme operative in the fixation of ammonia, a steeper increase in [^{15}N]glutamate would have occurred. Therefore, not only NADP-GDH but also GS must have been involved in primary ammonia fixation to form [^{15}N]glutamate as well as glutamine with the amide group preferentially labelled (Rhodes *et al.*, 1980).

When metabolized via GOGAT, the large glutamine pool of *S. semiglobata* will, especially in the early phase of $^{15}\text{NH}_4\text{Cl}$ assimilation, dilute glutamate with ^{14}N and cause the observed lag phase of glutamate labelling. This appears to be the main reason for the observed near-saturation kinetics of [^{15}N]glutamate labelling below 40% enrichment. Albeit at a lower level than [^{15}N]glutamate, ^{15}N -amino-labelled glutamine showed near-saturation kinetics with a distinct lag phase in the first minutes of the feeding experiment (Fig. 2). This was because glutamine amino-nitrogen was derived slowly from [^{15}N]glutamate. The relatively low ^{15}N enrichment in glutamine amino-nitrogen may be explained by the large unlabelled glutamine pool (Table 1).

From our results we conclude that, as in other fungi, NADP-GDH and GS/GOGAT are both involved in ammonia assimilation in the basidiomycete *S. semiglobata*. The relative contribution of the GS/GOGAT pathway to ammonia assimilation increases with increasing external ammonia concentration.

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