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¹⁵N-NMR study of ammonium assimilation in *Agaricus bisporus*

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Abstract

Ammonium assimilation was studied by feeding [¹⁵N]ammonium to actively growing mycelium of *Agaricus bisporus*. Products of ammonium assimilation were analysed using ¹⁵N-NMR. Participation of glutamine synthetase, glutamate synthase and NADP-dependent glutamate dehydrogenase was determined by inhibiting glutamine synthetase with phosphinothricin and glutamate synthase with azaserine. Our results clearly indicate that, under the conditions used, ammonium assimilation is mainly catalysed by the enzymes of the glutamine synthetase/glutamate synthase pathway. No indications were found for participation of NADP-dependent glutamate dehydrogenase. Furthermore, ¹⁵N-labelling shows that transamination of glutamate with pyruvate to yield alanine is a major route in nitrogen metabolism. Another major route is the formation of N-acetylglucosamine. Compared to the formation of N-acetylglucosamine there was only a limited formation of arginine.

Keywords: Nuclear magnetic resonance spectroscopy; Mushroom; *Agaricus*; Ammonium assimilation

1. Introduction

A large number of studies [1,2] have demonstrated the existence of two systems for nitrogen assimilation in microorganisms: the reductive amination of 2-oxoglutarate catalysed by NADP-dependent glutamate dehydrogenase (NADP-GDH; EC 1.4.1.4.) with the synthesis of glutamate (GDH-pathway), and the amidation of glutamate catalysed by glutamine synthetase (GS; EC 6.3.1.2.) with the formation of glutamine. Consequently, the amino acids glutamine and glutamate are primary products of nitrogen assimilation and serve as donors of amide nitrogen and in transamination reactions.

An alternative to the NADP-GDH pathway for glutamate production is offered by the reaction catalysed by glutamate synthase (GOGAT; EC 1.4.7.1.). This reaction involves the reductive transfer of the amide nitrogen of glutamine to 2-oxoglutarate. By coupling the GOGAT

reaction with GS an essentially irreversible pathway (GS/GOGAT-pathway) for the formation of glutamate is achieved.

Until 1980 ammonium assimilation in fungi was believed to be catalysed by NADP-GDH [1]. However, more recent studies on *Neurospora crassa* have shown that ammonium assimilation in fungi can also be mediated by the joint operation of GS and GOGAT [3–5]. Furthermore, studies on *Cenococcum geophilum* [6], *Aspergillus nidulans* [7] and *Stropharia semiglobata* [8] have shown that ammonium assimilation in fungi can be catalysed by the concurrent activity of the NADP-GDH pathway and the GS/GOGAT pathway.

Although the cultivation of the commercial mushroom *A. bisporus* has developed into an industry over the past decades, only a limited amount of basic knowledge on its nitrogen metabolism is available. Recently Baars et al. [9] made an inventory of the nitrogen-assimilating enzymes in *A. bisporus*. Besides high transaminating activities, NADP-GDH, GS and GOGAT activities could be demonstrated in cell-free extracts. So *A. bisporus* has the enzymic potential to produce glutamate by either the NADP-

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GDH pathway or the GS/GOGAT pathway. In order to determine the relative contribution of both pathways for ammonium assimilation in more detail, the incorporation of [^{15}N]ammonium by *A. bisporus* was studied by nuclear magnetic resonance spectroscopy (^{15}N -NMR).

2. Materials and methods

Organism and culture conditions. *Agaricus bisporus* strain Horst® U1 was used throughout this study. Stock cultures were stored at 4°C on slants of wheat agar. Mycelium was grown at 24°C in static cultures using Fernbach flasks containing 100 ml of liquid medium. Two slightly different media were used. Medium A contained 2 mM KH_2PO_4 , 1 mM MgSO_4 , 0.5 mM CaCl_2 , 0.134 mM Na_2EDTA , 25 μM FeSO_4 , 5 μM ZnSO_4 , 5 μM MnSO_4 , 4.8 μM H_3BO_3 , 2.4 μM KI , 52 nM Na_2MoO_4 , 4 nM CuSO_4 , 4 nM CoCl_2 , 0.5 μM thiamine-HCl and 0.1 μM D(+)-biotin. In most cases a modification of the medium described by Dijkstra et al. [10] was used (medium B). In this medium the amino acids were omitted. Both media contained 100 mM glucose. As a nitrogen source either 20 mM glutamate or 10–20 mM $(\text{NH}_4)_2\text{H}_2\text{PO}_4$ was used.

Liquid cultures were inoculated with mycelium grown for 7 days on agar plates containing compost extract medium prepared according to Rainey [11] and solidified with 1.5% (w/v) of bacto-agar. The plates were overlaid with a cellophane disk and inoculated at 7 points per petri dish. After growth the mycelium was scraped off the plates with a sterile spatula and fragmented in a Waring blender for 30 s. Aliquots of the homogenate were used as an inoculum for the liquid media. Unless stated otherwise, cultures were harvested after 22 days by filtration over nylon gauze (100 μm pore size).

^{15}N ammonium feeding procedure. Mycelium harvested from liquid cultures was dried by pressing gently between nylon gauze layered upon filter paper and parafilm. After weighing, equal portions (2–4 g fresh weight) were incubated in 50 or 100 ml medium B in which the nitrogen source was replaced by 5 or 10 mM $^{15}\text{NH}_4\text{Cl}$ (99% enriched in ^{15}N , ICN Biomedicals, Cleveland, OH, USA). In addition, penicillin G (50 $\mu\text{g} \cdot \text{ml}^{-1}$) and streptomycin (50 $\mu\text{g} \cdot \text{ml}^{-1}$) were added to prevent bacterial growth. The cultures were shaken at 50 rpm for specified periods in 1000 ml baffled flasks at room temperature. Where indicated, azaserine (AZS) or phosphinothricin (PPT) were added to the culture to final concentrations of 1 and 5 mM, respectively.

Harvest and extraction of nitrogen compounds. After [^{15}N]ammonium feeding mycelia were harvested either by filtration over nylon gauze (100 μm pore size) or by centrifugation (12000 $\times g$ for 10 min). After washing the mycelia with 0.15 M NaCl and demineralised water, nitrogen compounds were extracted as described by Martin et

al. [12]. Briefly, mycelia were ground and extracted in 25 ml of ice-cold methanol/chloroform/water (12:5:3, v/v/v). The homogenate was centrifuged (10000 $\times g$ for 15 min) and the pellet was extracted a second time. The supernatants were combined and dried at 40°C using a rotavapor. The samples were dissolved in 0.5 ml of 0.01 M HCl. The acidic solution was washed with 1 ml of ice-cold chloroform to remove compounds interfering with the ^{15}N -NMR measurement and stored at -20°C .

When amino acid compositions of the mycelia were to be determined, 1 μmol norleucine was added to the mycelia as an internal standard before the extraction procedure. The extracted pellet was dried overnight at 80°C to estimate the amount of mycelium extracted.

^{15}N -nuclear magnetic resonance. The ^{15}N -NMR spectra were obtained with a Bruker AMX-600 spectrometer operating at 60.816 MHz. Proton decoupling by the WALTZ-16 composite pulse sequence was used. The ^{15}N -NMR spectra were obtained with the following settings: 90° pulse, 2 s recycle delay, and 16 K data points were recorded per free induction decay (FID). The number of scans accumulated per FID differs from one sample to the other and is given in the legend of the figures. The FIDs were zero-filled once and multiplied by exponential window function with 10 Hz line broadening prior to Fourier transformation leading to spectra with 16 K real data points. Spectra were recorded in 20% D_2O to provide a lock signal. Chemical shifts were reported relative to liquid ammonia at 25°C, where 0 ppm has been obtained by multiplying the 0 ppm ^1H TSP frequency by 0.10132914 [13]. Temperature was maintained at 298 K. Assignments of resonances were made by comparison with published ^{15}N -NMR data [14–17] and by analysing spectra of extracts from [^{15}N]glutamate-fed mycelia.

Enzyme assays. Laccase (EC 1.14.18.1) activity of culture supernatants was used as a measure of mycelium growth and was determined spectrophotometrically according to a modification of the method of Wood [18]. Briefly, up to 0.5 ml of culture fluid was added to 2 ml substrate buffer (0.1 M Na-acetate, pH 5.6, containing 1 $\text{mg} \cdot \text{ml}^{-1}$ N,N-dimethyl-*p*-phenylenediamine sulfate) in a total volume of 2.5 ml. Absorbance of the red quinone product was measured at 552 nm. A change in absorbance of 0.1 min^{-1} was defined as one unit of enzyme activity (U). Activities of NADP-GDH and GS were determined according to the methods described by Baars et al. [9]. GS activity was measured by the transferase reaction.

Analytical procedures. Ammonium was determined by the method described by Bergmeyer and Beutler [19]. Glutamate was determined by a modification of the method described by Beutler [20]. Instead of using diaphorase and 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride (INT), we used 0.75 mM N-methyl-dibenzopyrazine methyl sulfate (PMS) and 3.75 mM (3-[4,5-dimethylthiazol]-2-yl)-2,5-diphenyltetrazolium bromide (MTT).

After recording the NMR spectra amino acid composi-

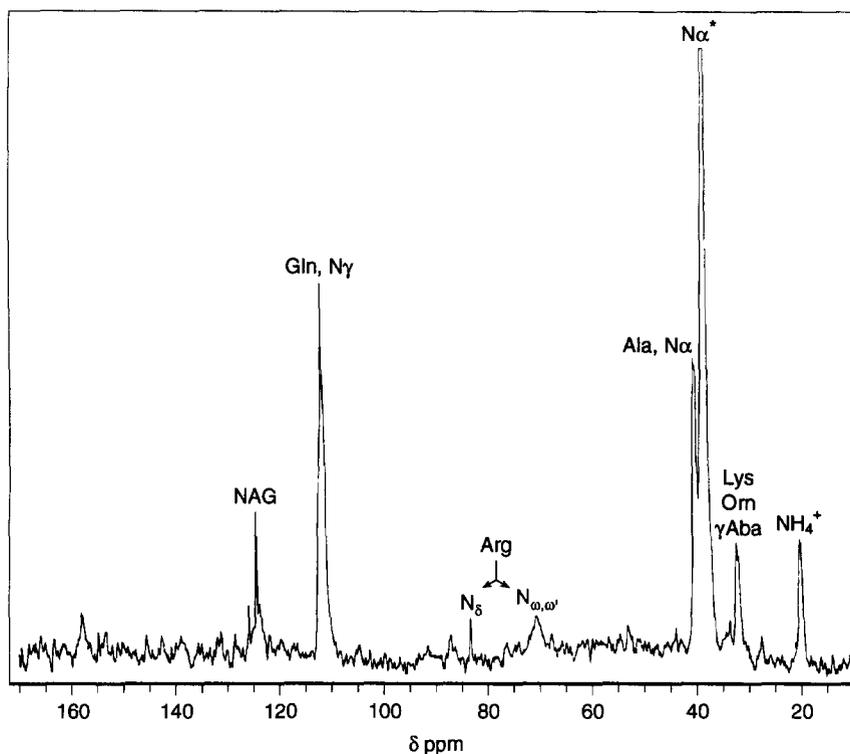


Fig. 1. ^{15}N -NMR spectrum of nitrogen compounds extracted from mycelia of *A. bisporus* growing actively on $[^{15}\text{N}]$ ammonium as a nitrogen source (27059 scans). Resonance frequencies: NH_4^+ , 20.6 ppm; lysine, ornithine or γ -aminobutyrate (γABA), 32.7 ppm; α -amino nitrogens ($\text{N}\alpha^*$) of glutamine, glutamate, lysine and arginine, 38.9 ppm; alanine, 40.74 ppm; arginine ω, ω' -N, 71.04 ppm and δ -N, 83.61 ppm; glutamine γ -N, 112.18 ppm and *N*-acetyl-D-glucosamine (NAG) 124.75 ppm.

tion of the extracts was analysed on a Varian LC-5060 Liquid Chromatograph equipped with a Varian 9095 autosampler, coupled to a Varian 9070 fluorescence detector.

After derivatization with 9-fluorenyl-methyl chloroformate (FMOC) the amino acids were separated on a TSK gel ODS 80 T_M column (250 \times 4 mm, Tosohaas, Mont-

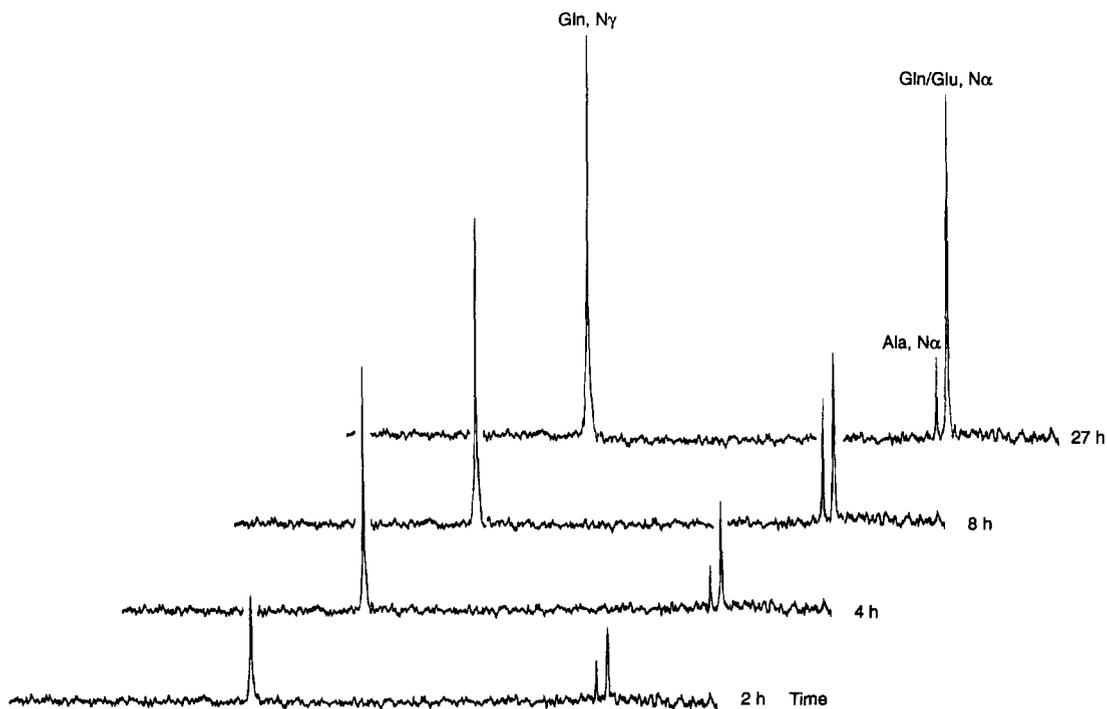


Fig. 2. ^{15}N -NMR spectra of extracts from *A. bisporus* obtained after feeding $[^{15}\text{N}]$ ammonium chloride (99% ^{15}N) at time zero (3072 scans). Resonance frequencies: α -amino nitrogens ($\text{N}\alpha^*$) of glutamine/glutamate, 40.6 ppm; alanine, 42.7 ppm; glutamine γ -N, 111.8 ppm.

gomeryville, PA, USA) according to the method described by Einarsson et al. [21] using a flow rate of $1.1 \text{ ml} \cdot \text{min}^{-1}$.

3. Results

3.1. Growth with [^{15}N]ammonium as a nitrogen source

Mycelium was grown on medium A with 20 mM $^{15}\text{NH}_4\text{Cl}$ as a nitrogen source to study the major pools of ^{15}N -labelled compounds. Actively growing mycelium (3.4 g fresh weight) was harvested after 9 days of growth. Soluble compounds were extracted and subjected to NMR

spectrometry (Fig. 1). Several peaks can be identified from the spectrum. Resonances were found at 32.7 ppm (lysine, ornithine or γ -aminobutyrate); 38.9 ppm (α -amino nitrogens of glutamine, glutamate, lysine and arginine); 40.7 ppm (alanine); 71 ppm (arginine ω, ω' -N); 83.6 ppm (arginine δ -N); 112.2 ppm (glutamine γ -N) and 124.7 ppm (*N*-acetyl-D-glucosamine).

3.2. Time-dependent incorporation of [^{15}N]ammonium

To study the uptake of $^{15}\text{NH}_4^+$ and its time-dependent incorporation, mycelium was grown in medium B, using 150 mM glucose and 10 mM $(\text{NH}_4)_2\text{H}_2\text{PO}_4$ as carbon and

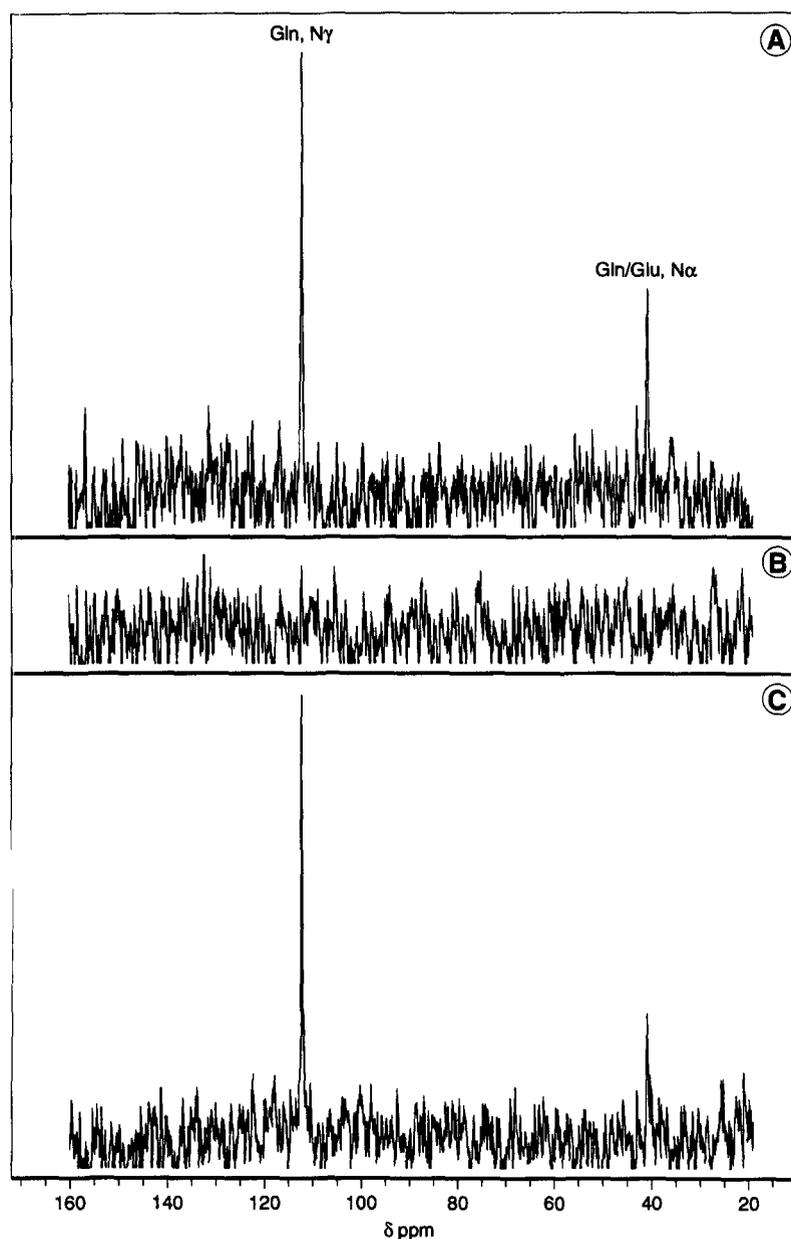


Fig. 3. Effect of 1 mM azaserine or 5 mM phosphinothricin on the incorporation of [^{15}N]ammonium by *A. bisporus* analysed by ^{15}N -NMR (5000 scans). Resonance frequencies: α -amino nitrogens ($\text{N}\alpha^*$) of glutamine/glutamate, 40.6 ppm; glutamine γ -N, 111.8 ppm. (A) no addition; (B) phosphinothricin; (C) azaserine. Extracts for ^{15}N -NMR were prepared after incubation for 19.5 h.

nitrogen source, respectively. After 20 days of culture the medium was replaced by fresh medium and laccase activity was determined to measure mycelial growth [18]. From day 20 to 23, a linear increase in laccase activity from 10 to 25 U · ml⁻¹ was observed. Thereafter laccase activity hardly increased and the mycelium was harvested for the incorporation experiment at day 24. In the freshly harvested mycelium activities of NADP-GDH and GS were 0.038 U · mg⁻¹ and 0.032 U · mg⁻¹, respectively. Equal portions of mycelium (2.8 g fresh weight) were incubated in 100 ml nitrogen-free medium B supplemented with 5 mM ¹⁵NH₄Cl and antibiotics for a limited number of time intervals. Uptake of ¹⁵N was followed by enzymatic determination of residual ¹⁵NH₄⁺ in the incubation medium. After 27 h about 1 mM was taken up by the mycelium, while after 48 h the ¹⁵NH₄⁺ concentration in the incubation medium was decreased to 0.3 mM. Mycelia were harvested at different time intervals from the incubation medium, their soluble compounds were extracted and ¹⁵N-NMR spectra were recorded (Fig. 2). Spectra of samples taken over a period of 27 h show the gradual appearance of a number of labelled compounds. Three peaks are observed: a resonance at 111.8 ppm due to glutamine γ -N and resonances at 42.7 and 40.6 ppm due to α -N from alanine and glutamate/glutamine, respectively. The resonance from glutamine γ -N is the most prominent over the complete period of labelling and increases with time. The signal-to-noise ratio of the glutamine γ -N peak increases from 19.8 after 2 h of incubation to 39.8, 47.6 and 57.4 after 4, 8 and 27 h, respectively. Labelling of glutamate/glutamine α -N lags behind but in time becomes almost as intense as the resonance of glutamine γ -N.

3.3. Inhibition of [¹⁵N]ammonium incorporation by phosphinothricin and azaserine

Ammonium assimilation can be influenced in vivo by adding specific inhibitors of GS and GOGAT [7,22]. The biosynthetic reaction catalysed by GS purified from *A. bisporus* is inhibited strongly by phosphinothricin (PPT; $K_i = 17 \mu\text{M}$, [23]). GOGAT activity in cell-free extracts was inhibited completely by 1 mM azaserine (AZS, [9]). In vitro NADP-GDH activity was not affected by addition of PPT (5 mM) to the NADP-GDH assay mixture, while addition of AZS (1 mM) caused about 25% inhibition of NADP-GDH activity. So it is unlikely that these inhibitors exert a significant effect on NADP-GDH activity in vivo. The effect of inhibitors on the ammonium assimilatory pathway was tested with mycelium pre-grown on medium B with 100 mM glucose and 20 mM (NH₄)₂PO₄. After 22 days of cultivation, actively growing mycelium, as followed from laccase activity, was harvested. At this time the residual ammonium concentration of the medium was 10 mM. For incubation with ¹⁵NH₄⁺ the harvested mycelium was divided into 3 equal portions (4.1 g fresh weight per portion) and resuspended in 50 ml of medium

Table 1

Effect of addition of azaserine or phosphinothricin on the free amino acid pool of ammonium grown mycelium

Amino acid	No inhibitor	Azaserine (1 mM)	Phosphinothricin (5 mM)
	$\mu\text{mol} \cdot (\text{g dry matter})^{-1}$		
Glutamine	13.7	24.6	1.4
Asparagine	1.0	1.9	0.5
Glutamate	6.7	8.3	10.2
Aspartate	1.2	1.5	1.5
Alanine	12.6	231.4	20.4
Serine	1.2	5.4	2.0
Glycine	2.0	4.7	3.3
γ -Aminobutyrate	3.7	4.6	3.8

Mycelium was pre-grown on medium B with 100 mM glucose and 20 mM (NH₄)₂PO₄. After 22 days of cultivation actively growing mycelium was harvested and divided in 3 equal portions (4.1 g fresh weight) and resuspended in 50 ml of medium B with 10 mM ¹⁵NH₄Cl, antibiotics and additions. After 19.5 h soluble nitrogen pools were extracted from the mycelium and amino acids were analysed by HPLC.

B with 10 mM ¹⁵NH₄Cl and antibiotics. After 19.5 h soluble nitrogen pools were extracted from the mycelium.

The addition of inhibitors of ammonium assimilation had marked effects on the uptake of ¹⁵NH₄⁺. Addition of 1 mM AZS to ammonium-grown mycelium inhibited uptake by 60%. Addition of 5 mM PPT completely inhibited uptake of ¹⁵NH₄⁺ and even resulted in an increase of extracellular ammonium of 0.05 mM. When inhibitors were absent, peaks could be observed at 40.62 ppm and 111.84 ppm in the spectra (Fig. 3A). Also some other peaks were observed, which could hardly be distinguished from the noise. These peaks became more pronounced after prolonged incubation (Fig. 1). Inhibition by AZS resulted in the accumulation of labelled glutamine γ -N, at the expense of the intensities of the α -N peak at 40.62 ppm (ratio γ -N/ α -N changed from 2.2 ± 0.1 to 3.7 ± 0.1 , Fig. 3C). No incorporation of ¹⁵NH₄⁺ was observed when PPT was added (Fig. 3B). Analysis of the free amino acid pools of the mycelium also reflects the influence of addition of inhibitors (Table 1). Levels of glutamine and alanine were influenced most by the addition of AZS or PPT. Addition of 1 mM AZS to the incubation medium increased the levels of glutamine and alanine by a factor 2 and 18, respectively. Inclusion of 5 mM PPT in the incubation medium caused a drop in the level of glutamine by a factor 10.

4. Discussion

Growth conditions of the mycelium can have a large influence on the pathway used for the assimilation of ammonium in fungi. In *N. crassa* it has been found that NADP-GDH and GS are responsible for ammonium assimilation when there is a large supply of nitrogen. However, when the ammonium concentration is low, assimilation is

mainly catalysed by the GS/GOGAT-pathway [4,5]. In *C. geophilum*, ammonium assimilation takes place via the concurrent activity of NADP-GDH and GS. However, in the N-starved mycelia N-flux through GDH is higher than the flux through GS, while in rapidly growing mycelia the opposite situation occurs [6,24].

This study describes the use of ^{15}N -NMR to study ammonium assimilation in *A. bisporus*. The main signals of the NMR spectra of extracts from [^{15}N]ammonium-grown mycelium (Fig. 1) corresponded with α -N of several amino acids and γ -N of glutamine. Some minor signals were located at 32.7, 71, 83.6 and 124.7 ppm. The resonance at 32.7 ppm presumably corresponds with γ -aminobutyrate. Since the Krebs cycle appears to be blocked at 2-oxoglutarate dehydrogenase, there is an active γ -aminobutyrate shunt [25]. The resonance at 124.7 ppm corresponds with the formation of N-acetylglucosamine. Jennings [26] proposed that a nitrogen reserve could be formed by polymerization of N-acetylglucosamine. This could be the reason why the cell wall of *A. bisporus* contains about 45% N-acetylglucosamine. The intensity of the resonances at 71 and 83.6 ppm, corresponding with ω, ω' -N and δ -N from arginine, respectively, is very low as compared to arginine resonances in *C. graniforme* [16] and *N. crassa* [14]. These fungi appear to accumulate arginine as a nitrogen reserve into their vacuoles [16,27].

Time-dependent incorporation showed that glutamine, glutamate and alanine are labelled first (Fig. 2). Nitrogen incorporated into glutamate is quite rapidly transaminated to alanine. This is consistent with the high activity of glutamate-pyruvate transaminase found in cell-free extracts of *A. bisporus* [9]. However, transamination to aspartate was not observed, despite a high activity of glutamate oxaloacetate transaminase in cell-free extracts [9]. The activities of the primary enzymes of ammonium assimilation have been determined by Baars et al. [9]. In ammonium-grown mycelium activities of GS and NADP-GDH were 0.028 and 0.013 $\text{U} \cdot \text{mg}^{-1}$, respectively. To determine their relative contribution in ammonium assimilation, inhibitor studies, including effects on $^{15}\text{NH}_4^+$ uptake/incorporation and on amino acid pools, were performed (Table 1; Fig. 3). Inhibitory effects of PPT and AZS on actively growing mycelium were reflected in the pools of free amino acids. Inhibition by PPT resulted in a 10-fold decrease of the glutamine pool and a moderate increase of the pools of glutamate and alanine. Incorporation of $^{15}\text{NH}_4^+$ was completely blocked. Moreover an excretion of ammonium was observed. This indicates a rapid intracellular turnover of unlabelled nitrogen-containing compounds. Release of ammonium was also found by Lea et al. [28] upon incubation of cyanobacteria and a number of higher plants with PPT. Inhibition by AZS resulted in a marked accumulation of alanine and a 2-fold increase of glutamine. Inhibition by AZS apparently is not absolute since labelling of glutamate is still found. No labelled alanine was observed which indicates that the increase in alanine must be de-

rived from the above-mentioned turnover. These results are compatible with the presence of a ω -amidase pathway in which unlabelled α -N of glutamine is transaminated. Such a pathway was described in *N. crassa* [29]. Based upon the complete inhibition of $^{15}\text{NH}_4^+$ -incorporation by PPT, a specific inhibitor for GS from *A. bisporus* [23], it can be concluded that ammonium assimilation in *A. bisporus* mainly proceeds via the GS/GOGAT pathway. NADP-GDH is not or only moderately affected by PPT and AZS, respectively.

Ammonium assimilation in *A. bisporus* shares a number of characteristics with ammonium assimilation in the basidiomycete fungus *S. semiglobata* [8]. The results of ^{15}N -labelling showed that, despite the high activity of NADP-GDH, ammonium assimilation is catalysed mainly by the action of GS/GOGAT. Martin et al. [12] concluded from their ^{15}N -labelling studies with ammonium-fed beech ectomycorrhizal fungi, that ammonium assimilation mainly occurs via the GS/GOGAT pathway and that NADP-GDH plays little, if any, role in this process. In other fungi such as *Cenococcum graniforme* [6,24], *A. nidulans* [22], *N. crassa* [5] and the yeasts *Candida albicans* and *Saccharomyces cerevisiae* [30], the concurrent operation of NADP-GDH and GS/GOGAT is reported. Relative participation of the GS/GOGAT pathway is calculated to be 1.6% in *S. cerevisiae*, 78% in *C. albicans* [30] and about 50–60% in *A. nidulans* [7,22].

In the past, the elucidation of the pathway of ammonium assimilation in fungi has relied heavily on the use of mutants and on measurement of the levels and kinetic properties of the enzymes involved, particularly NADP-GDH and GS. In recent years ^{15}N -NMR has been added to these methods of studying metabolism (for reviews see Martin [31] and Lundberg et al. [32]). Present results have shown a distinct role for GS/GOGAT in nitrogen assimilation in *A. bisporus*. NADP-GDH appears to play a minor role in the assimilation of ammonium. Studies with mutant strains of *A. bisporus* may help in obtaining insight in the precise role of this enzyme in nitrogen metabolism. More detailed information could further be obtained from ^{15}N -labelling experiments followed by gas chromatography-mass spectrometry (GC/MS) analysis.

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