Metabolism of [^{14}C]glutamate and [^{14}C]glutamine by the ectomycorrhizal fungus *Paxillus involutus*

Michel Chalot, Annick Brun, Roger D. Finlay and Bengt Söderström

Author for correspondence: M. Chalot. Tel: +46 46 10 86 14. Fax: +46 46 10 41 58.

Department of Microbial Ecology, University of Lund, Ecology Building, S-223 62 Lund, Sweden

To examine pathways of glutamate and glutamine metabolism in the ectomycorrhizal fungus *Paxillus involutus*, tracer kinetic experiments were performed using L-[U- ^{14}C]glutamate and L-[U- ^{14}C]glutamine and the enzyme inhibitors methionine sulfoximine (MSX), azaserine (AZA) and aminooxyacetate (AOA). When [^{14}C]glutamate was supplied to fungal cultures, 25% of the radioactivity of the amino acid fraction was incorporated into glutamine after 5 min feeding, but MSX inhibited incorporation of ^{14}C into glutamine by 85%, suggesting the rapid operation of glutamine synthetase. Conversely, when *P. involutus* was fed with [^{14}C]glutamine, 46% of the label was found in glutamate within 30 min of feeding and AZA inhibited glutamate formation by 90%. Taken together, these data indicate that glutamate synthase (GOGAT) is the major enzyme of glutamine degradation. In addition, the strong inhibition of glutamine utilization by AOA indicates that glutamine catabolism in *P. involutus* might involve a transamination process as an alternative pathway to GOGAT for glutamine degradation. The high $^{14}\text{CO}_2$ evolution shows that glutamate and glutamine are further actively consumed as respiratory substrates, being channelled through the tricarboxylic acid (TCA) cycle and oxidized as CO_2 . It appears that synthesis of amino acid precursors during TCA cycle operation is an essential step for aspartate and alanine synthesis through aminotransferase activities in *P. involutus*.

Keywords: ectomycorrhizal fungi, glutamine metabolism, glutamate metabolism, *Paxillus involutus*

INTRODUCTION

Symbiotic associations between roots and ectomycorrhizal fungi play an integral role in the nitrogen metabolism of most forest trees. Investigations based on ^{15}N -labelling have indicated that glutamate and glutamine are the main acceptors of inorganic nitrogen in ectomycorrhizas and ectomycorrhizal fungi (Martin *et al.*, 1986; Finlay *et al.*, 1989; Chalot *et al.*, 1991b; Kershaw & Stewart, 1992). The potential enzymes for N transfer from ammonium to amino acids are NADP-dependent glutamate dehydrogenase (NADP-GDH, EC 1.4.1.4), glutamine synthetase (GS, EC 6.3.1.2) and glutamate synthase (GOGAT, EC 1.4.7.1) (Mifflin & Lea, 1980; Stewart *et al.*, 1980). Ammonium is assimilated by

sequential GDH/GS activity in spruce ectomycorrhizas (Dell *et al.*, 1989; Chalot *et al.*, 1991b) and in rapidly growing *Cenococcum geophilum* (Genetet *et al.*, 1984) whereas the GS/GOGAT cycle seems to predominate in beech ectomycorrhizas (Martin *et al.*, 1986) as well as in the ectomycorrhizal fungus *Pisolithus tinctorius* (Kershaw & Stewart, 1992). GOGAT is also the main enzyme of glutamine degradation in yeasts (Holmes *et al.*, 1989), *Neurospora crassa* (Calderon & Mora, 1985, 1989; Lomnitz *et al.*, 1987) and *Aspergillus nidulans* (Kusnan *et al.*, 1987, 1989). GDH, GS and GOGAT activities have been detected in a range of ectomycorrhizal fungi (Vézina *et al.*, 1989; Ahmad *et al.*, 1990). NADP-GDH has been purified to electrophoretic homogeneity from *C. geophilum* (Martin *et al.*, 1983) and *Laccaria laccata* (Brun *et al.*, 1992). GS has also been purified and characterized from *L. laccata* (Brun *et al.*, 1992).

One of the major alternative pathways to the GS/GOGAT cycle in *N. crassa* is the ω -amidase pathway,

Abbreviations: AOA, aminooxyacetate; AZA, azaserine; GDH, glutamate dehydrogenase; Gln-T, glutamine transaminase; GOGAT, glutamate synthase; GS, glutamine synthetase; MSX, methionine sulfoximine; TCA, tricarboxylic acid.

in which glutamine is converted into 2-oxoglutarate and ammonium by the sequential activities of glutamine transaminase and ω -amidase (Calderon *et al.*, 1985; Calderon & Mora, 1989). Glutamine transaminase activity has been reported to be the major pathway for glutamine catabolism in *Saccharomyces cerevisiae* cultured under micro-aerophilic conditions (Soberon *et al.*, 1989). In free-living mycorrhizal mycelia and ectomycorrhizas, following initial nitrogen assimilation into glutamate and glutamine, the N is incorporated into a range of amino acids, mainly alanine, aspartate and asparagine after short (Martin *et al.*, 1986; Chalot *et al.*, 1991b) or long (Finlay *et al.*, 1988, 1989) incubation periods. These findings, supported by the high aminotransferase activities measured in ectomycorrhizas and ectomycorrhizal fungi (Dell *et al.*, 1989; Chalot *et al.*, 1990), stress the central role of glutamate and glutamine as N donors.

In addition, glutamate and glutamine can support biomass production comparable to that on ammonium in different ectomycorrhizal fungi (Abuzinadah & Read, 1988; Chalot *et al.*, 1991a; Finlay *et al.*, 1992). As pointed out by Abuzinadah & Read (1989), assimilation of amino acids derived from proteolytic activity can supply up to 10% of the total C gained by the host over a period of 50 d, highlighting the importance of amino acids as a potential C source. Data on the filamentous fungus *N. crassa* (Calderon & Mora, 1989), plants (Osaki *et al.*, 1992; Muhitch, 1993), and root nodules (Ta *et al.*, 1988; Kouchi *et al.*, 1991) have clearly shown that [14 C]glutamate and [14 C]glutamine are used intensively as respiratory substrates and a carbon source for organic acids, proteins and sugars. However, little information has been obtained concerning the utilization of their carbon skeletons by ectomycorrhizal fungi or ectomycorrhizas. Indeed most of the work on ectomycorrhizal fungi or ectomycorrhizas has focused on the transfer of N from glutamate and glutamine to other amino acids using the 15 N isotope (Martin *et al.*, 1986; Finlay *et al.*, 1989; Chalot *et al.*, 1991b; Kershaw & Stewart, 1992) or on the transfer of C from carbon dioxide or glucose to amino acids using 13 C (Martin & Canet, 1986) or 14 C (France & Reid, 1983) isotopes.

The objectives of the present study were (1) to examine 14 C-incorporation into amino acids from L-[U- 14 C]glutamate or L-[U- 14 C]glutamine by the ectomycorrhizal fungus *Paxillus involutus* and (2) to determine how the transfer of C from newly-absorbed 14 C-amino acids to newly-synthesized 14 C-labelled amino acids is affected by the enzyme inhibitors methionine sulfoximine (MSX), azaserine (AZA) and aminooxyacetate (AOA).

METHODS

Organism and *in vivo* labelling. *Paxillus involutus* (Batsch) Fr. was grown on cellophane-covered agar medium containing modified Melin-Norkrans (MMN) medium from which malt extract was omitted. The MMN medium contained (mg l $^{-1}$): KH $_2$ PO $_4$ (500), (NH $_4$) $_2$ HPO $_4$ (250), CaCl $_2$ (50), NaCl (25), MgSO $_4$ ·7H $_2$ O (150), thiamin hydrochloride (0.1), FeCl $_3$ ·6H $_2$ O (1). This medium was used with 1 g glucose l $^{-1}$. Discs of fungal inoculum were cut with a 25 mm diameter cork borer from the

growing edge of 10-d-old colonies and preincubated for 1 h in a nutrient solution containing either 2.5 mM MSX, 1 mM AZA or 2 mM AOA prepared in modified MMN in which the nitrogen source was omitted. These concentrations of inhibitors were those giving complete inhibition of growth in test experiments (Botton & Chalot, 1991). Their structure, specificity and mode of action have been extensively reviewed elsewhere (Mifflin & Lea, 1980; Stewart *et al.*, 1980; Botton & Chalot, 1991). A control without inhibitor was also included. The uptake of L-glutamate and L-glutamine was strongly dependent on the external pH and was optimal at pH 4.1. The initial pH of the MMN medium was 5.5 before addition of the inhibitors and was adjusted to 4.1 after addition of the inhibitors by using HCl (in control, MSX and AZA treatments) or NaOH (in AOA treatment). Fungal discs were then washed to remove excess inhibitor and placed for between 5 and 120 min in small dishes containing 1 ml nitrogen-free MMN supplemented with either 3.7 kBq L-[U- 14 C]glutamate (specific activity 10.4 MBq μ mol $^{-1}$; New England Nuclear) or 3.7 kBq L-[U- 14 C]glutamine (specific activity 7.77 MBq μ mol $^{-1}$; New England Nuclear). At the end of the feeding period, the mycelial discs were washed for 5 min with 0.1 mM CaSO $_4$ and freeze-dried prior to analysis.

Separation of amino acids. Amino acids were extracted from lyophilized tissues in 70% (v/v) methanol. The extract was centrifuged for 20 min at 13000 g and filtered through a 0.25 μ m membrane filter (Millipore). Samples were then evaporated to dryness using a Speed Vac Concentrator (Savant, Speed Vac Plus). The residues were taken up in 80 μ l 50 mM sodium acetate, pH 5.9, and a 60 μ l aliquot was used for chromatographic separation.

Identification of amino acids. Free amino acids were analysed by reversed-phase high-performance liquid chromatography (HPLC) in the methanol-soluble fraction after derivatization with *o*-phthalaldehyde/ β -mercaptoethanol reagent according to Martin *et al.* (1986). Chromatographic separations were performed using a Novapak C18 column (39 $\times$ 150 mm). Amino acid derivatives were separated with a gradient of solvent A (water/methanol, 90:10, v/v, containing 50 mM sodium acetate, pH 5.9) and solvent B (methanol/acetonitrile, 95:5, v/v). The gradient was varied as follows (flow rate: 1 ml min $^{-1}$): 0–35% B, 26 min; 35–100% B, 1 min; 100% B, 3 min; 100–0% B, 1 min; 0% B, 4 min. The absorbance of the column eluate was monitored at 340 nm. Amino acids were quantified using the HPLC Manager Workstation (Pharmacia-LKB Biotechnology).

Determination of radioactivity. The radioactivity incorporated into amino acids was measured by liquid scintillation spectroscopy of separate fractions corresponding to each amino acid peak in the HPLC eluate collected at the outlet of the spectrophotometric detector. Radioactivity was also determined in an aliquot of the methanol-soluble fraction and in the methanol-insoluble pellet after tissue solubilization with Soluene 350 (Packard Instrument Co.). The 14 CO $_2$ evolved during [14 C]glutamate or [14 C]glutamine feeding was trapped in methanol/ethanolamine (70/30, v/v) and the radioactivity measured by scintillation spectroscopy.

RESULTS

Metabolism of [14 C]glutamate and [14 C]glutamine

Following [14 C]glutamate and [14 C]glutamine feeding, 38 and 44% respectively of the total radioactivity in the control mycelium was found in the amino acid fraction after 2 h incubation (Tables 1 and 2). Five to twelve

Table 1. Total absorbed radioactivity, mycelium-associated radioactivity and radioactivity in the amino acid pool derived from metabolism of [^{14}C]glutamate

Mycelial discs were preincubated with either 2.5 mM MSX, 1 mM AZA or 2 mM AOA and incubated with 3.7 kBq L- [^{14}C]glutamate (specific activity 10.4 MBq μmol^{-1}) for 30, 60 or 120 min. Each value is the mean $\pm$ SE of at least three replicates.

Treatment	Time (min)	$10^{-3} \times$ Radioactivity [d.p.m. (mg dry wt) $^{-1}$]		
		Total absorbed	Mycelium-associated	Amino acid pool-associated
Control	30	27.8 $\pm$ 2.4	27.1 $\pm$ 2.8	12.6 $\pm$ 1.3
	60	78.0 $\pm$ 12.3	33.3 $\pm$ 4.8	16.8 $\pm$ 5.2
	120	117.3 $\pm$ 18.0	61.8 $\pm$ 9.0	23.4 $\pm$ 8.7
MSX	30	39.7 $\pm$ 1.5	32.7 $\pm$ 1.2	16.5 $\pm$ 1.3
	60	64.0 $\pm$ 0.1	40.9 $\pm$ 1.2	20.4 $\pm$ 3.4
	120	102.0 $\pm$ 3.9	50.4 $\pm$ 5.4	21.6 $\pm$ 2.6
AZA	30	51.3 $\pm$ 6.3	24.4 $\pm$ 4.9	12.4 $\pm$ 4.0
	60	43.3 $\pm$ 5.5	33.0 $\pm$ 0.9	16.8 $\pm$ 0.6
	120	103.0 $\pm$ 6.0	59.2 $\pm$ 1.8	23.5 $\pm$ 5.4
AOA	30	34.5 $\pm$ 3.3	46.2 $\pm$ 4.6	28.8 $\pm$ 3.6
	60	70.6 $\pm$ 2.8	54.6 $\pm$ 5.1	31.8 $\pm$ 3.0
	120	77.7 $\pm$ 2.2	80.7 $\pm$ 8.4	44.4 $\pm$ 6.0

Table 2. Total absorbed radioactivity, mycelium-associated radioactivity and radioactivity in the amino acid pool derived from metabolism of [^{14}C]glutamine

Mycelial discs were preincubated with either 2.5 mM MSX, 1 mM AZA or 2 mM AOA and incubated with 3.7 kBq L- [^{14}C]glutamine (specific activity 7.77 MBq μmol^{-1}) for 30, 60 or 120 min. Each value is the mean $\pm$ SE of at least three replicates.

Treatment	Time (min)	$10^{-3} \times$ Radioactivity [d.p.m. (mg dry wt) $^{-1}$]		
		Total absorbed	Mycelium-associated	Amino acid pool-associated
Control	30	36.0 $\pm$ 3.4	38.1 $\pm$ 6.6	27.5 $\pm$ 3.9
	60	53.7 $\pm$ 2.5	44.5 $\pm$ 4.2	26.7 $\pm$ 4.6
	120	86.1 $\pm$ 6.4	51.6 $\pm$ 2.5	22.9 $\pm$ 2.1
MSX	30	43.2 $\pm$ 6.4	46.6 $\pm$ 5.7	29.1 $\pm$ 5.2
	60	35.8 $\pm$ 2.8	39.1 $\pm$ 3.3	18.1 $\pm$ 1.9
	120	92.4 $\pm$ 8.5	61.9 $\pm$ 4.0	23.8 $\pm$ 2.4
AZA	30	39.8 $\pm$ 7.8	38.1 $\pm$ 10.3	22.8 $\pm$ 4.5
	60	73.2 $\pm$ 8.8	59.5 $\pm$ 5.4	37.6 $\pm$ 4.2
	120	72.6 $\pm$ 0.7	67.5 $\pm$ 5.4	40.6 $\pm$ 1.2
AOA	30	53.5 $\pm$ 2.4	56.0 $\pm$ 2.5	34.0 $\pm$ 0.7
	60	66.4 $\pm$ 4.3	58.3 $\pm$ 7.2	32.1 $\pm$ 4.0
	120	93.1 $\pm$ 1.2	102.7 $\pm$ 9.1	56.7 $\pm$ 4.6

percent of the radioactivity was associated with the methanol-insoluble pellet fraction from fungal extracts, indicating slow incorporation of ^{14}C into proteins (data not shown). The chemical form into which the remaining activity in the methanol-soluble fraction was incorporated was not investigated further but it is possible that the activity was incorporated in carboxylic acids derived from deamination of the amino acids. In preliminary experiments the mycelium was fed with both the ^{14}C source and the inhibitor (MSX or AZA), thus blocking the uptake system(s) for amino acids competitively. Under these conditions, the level of radioactivity recovered in the amino acid fraction of the mycelium was negligible, indicating that the mycelium did not retain labelled amino acids in the apoplastic space. Part of the radioactivity removed from the feeding solution could not be found inside the mycelium (Tables 1 and 2). This proportion increased with time and may be due to formation of volatile compounds in the control. We have not studied $^{14}\text{CO}_2$ release in detail, but some observations are worth noting. We found that, after 2 h incubation, 34% and 25% of ^{14}C was lost as $^{14}\text{CO}_2$ during [^{14}C]glutamate and [^{14}C]glutamine feeding respectively. This accounted for approximately 80% of the difference between the total amount of absorbed radioactivity and the amount of radioactivity associated with the mycelium.

Feeding [^{14}C]glutamate to colonies of *P. involutus* resulted mainly in incorporation into glutamine. After 5 min, [^{14}C]glutamine accounted for 25% of the radioactivity in the amino acid pool (Fig. 1a) while [^{14}C]glutamate represented 51% of the radioactivity. These proportions did not vary greatly during the 2 h feeding period. The label was also detected in a range of amino acids including aspartate, asparagine, alanine and γ -aminobutyrate, which represented 12.5, 2.9, 1.3 and 1.2% respectively of the total radioactivity incorporated into amino acids (Fig. 1b). Serine, glycine and citrulline were slightly labelled, accounting for less than 1% of the total radioactivity (not shown). Arginine was not detected in the mycelium, either in a labelled or unlabelled form, in our growth conditions. The patterns of ^{14}C -labelling found in free amino acids in *P. involutus* were similar to those demonstrated by Finlay *et al.* (1989) using $^{15}\text{NH}_4^+$, where glutamate/glutamine, alanine, aspartate/asparagine and γ -aminobutyrate were the main acceptors of ^{15}N whereas no label could be detected in arginine. When [^{14}C]glutamine was supplied to *P. involutus* cultures, 46% of the radioactivity was found in glutamate within 30 min of feeding, [^{14}C]glutamine accounting for 41% (Fig. 1c). Twelve percent of the label in the amino acid fraction was found in aspartate, 2.4% in alanine and 1.1% in γ -aminobutyrate after 2 h feeding (Fig. 1d).

After 2 h feeding, there was no marked difference between the distribution of ^{14}C into amino acids of [^{14}C]glutamate- and [^{14}C]glutamine-fed *P. involutus*. With both ^{14}C -sources, there was a rapid equilibrium between glutamate and glutamine; glutamate accounted for 50–55% and glutamine 25–30% of the total radioactivity in the amino acid pool at the end of the experiment. However, the [^{14}C]glutamine-fed mycelium differed from the [^{14}C]gluta-

Fig. 1. Accumulation of radioactivity from (a, b) [¹⁴C]glutamate, and (c, d) [¹⁴C]glutamine into glutamate (●), glutamine (○), aspartate (■), asparagine (□), alanine (△) and γ-aminobutyric acid (▲) by *P. involutus*. Discs of fungal inoculum from 10-d-old colonies were preincubated for 1 h in modified nitrogen-free MMN and then placed in a solution containing nitrogen-free MMN supplemented with 3.7 kBq L-[U-¹⁴C]glutamate (specific activity 10.4 MBq μmol⁻¹) or with 3.7 kBq L-[U-¹⁴C]glutamine (specific activity 7.77 MBq μmol⁻¹). Data are expressed as means of triplicates. Vertical bars indicate SE.

Fig. 2. Effect of MSX on accumulation of radioactivity from (a, b) [¹⁴C]glutamate, and (c, d) [¹⁴C]glutamine into glutamate (●), glutamine (○), aspartate (■), asparagine (□), alanine (△) and γ-aminobutyric acid (▲) by *P. involutus*. Discs of fungal inoculum from 10-d-old colonies were preincubated for 1 h in a nutrient solution containing 2.5 mM MSX prepared in modified nitrogen-free MMN. Fungal discs were then washed to remove excess inhibitor and placed in a solution containing nitrogen-free MMN supplemented with 3.7 kBq L-[U-¹⁴C]glutamate (specific activity 10.4 MBq μmol⁻¹) or with 3.7 kBq L-[U-¹⁴C]glutamine (specific activity 7.77 MBq μmol⁻¹). Data are expressed as means of triplicates. Vertical bars indicate SE.

mate-fed mycelium in that, within the first 5–30 min of incubation, significantly more radioactivity was incorporated into the amino acid fraction, reflecting a higher absorption rate (Tables 1 and 2). The ¹⁴C in the amino acids (except aspartate) of the [¹⁴C]glutamine-fed colonies reached a maximum after 30 min (Table 2, Fig. 1c, d) whereas it continued to accumulate up to 2 h in the [¹⁴C]glutamate-fed colonies (Table 1, Fig. 1a, b).

Effect of MSX

Preincubation of cells with 2.5 mM L-MSX prior to the addition of [¹⁴C]glutamate resulted in an immediate 85 % inhibition of the incorporation of radioactivity into the glutamine fraction and a corresponding increase in the [¹⁴C]glutamate pool (Fig. 2a) compared to the control (Fig. 1a). However, the inhibitory effect decreased gradually throughout the ¹⁴C-feeding period (Table 3). By the end of the 2 h feeding the Glu:Gln ratio in MSX-treated mycelia was similar to that of the control. This rapid decrease in the inhibitory effect of MSX could possibly be due to an *in vivo* synthesis of GS that replaced the inhibited enzyme, or to a detoxification process. Kusnan *et al.* (1987) reported that MSX had no effect *in vivo* on

Aspergillus nidulans whereas the extracted GS could be fully inhibited by MSX, suggesting that the cells either detoxified, or did not take up the inhibitor. This latter hypothesis is not consistent with our data since high GS inhibition was found in the first 5–30 min of [¹⁴C]glutamate feeding. Further studies are needed to clarify this point. There was also a marked inhibition of ¹⁴C-incorporation into aspartate and alanine under [¹⁴C]glutamate feeding within the last 20–120 min (Fig. 2b). In contrast, synthesis of γ-aminobutyrate was not affected by this inhibitor. Under MSX inhibition and [¹⁴C]glutamine feeding, there was a 1.6-fold accumulation of [¹⁴C]glutamine after 30 min feeding whereas the [¹⁴C]glutamate remained unchanged (Fig. 2c) compared to the control mycelium (Fig. 1c). There was also a 1.6- and 2-fold decrease of aspartate and alanine synthesis respectively, after 2 h feeding (Fig. 2d).

Effect of AZA

When AZA-treated mycelia were given [¹⁴C]glutamate, total mycelium-associated radioactivity as well as the total amino acid pool radioactivity were similar to that found in

Table 3. [^{14}C]Glutamate:[^{14}C]glutamine ratios under [^{14}C]glutamate or [^{14}C]glutamine feeding and inhibition treatments

Data were calculated from Figs 1–4. ND, Not determined.

Time (min)	^{14}C source...	Control		MSX- treated		AZA- treated		AOA- treated	
		Glu	Gln	Glu	Gln	Glu	Gln	Glu	Gln
5		2.59	ND	15.64	ND	ND	ND	ND	ND
10		2.61	ND	20.85	ND	ND	ND	ND	ND
20		2.95	ND	11.51	ND	ND	ND	ND	ND
30		1.38	1.00	6.14	0.63	0.13	0.07	0.98	0.49
60		1.32	1.28	2.40	1.72	0.13	0.07	1.38	0.67
120		1.60	1.85	1.78	1.79	0.14	0.08	1.23	0.47

Fig. 3. Effect of AZA on accumulation of radioactivity from (a, b) [^{14}C]glutamate, and (c, d) [^{14}C]glutamine into glutamate (●), glutamine (○), aspartate (■), asparagine (□), alanine (△) and γ -aminobutyric acid (▲) by *P. involutus*. Incubations were as in Fig. 2 except that 1 mM AZA was used as the inhibitor. Data are expressed as means of triplicates. Vertical bars indicate SE.

the control mycelium (Table 1). However, the proportion of label incorporated into individual amino acids differed greatly to that of the control. About 76 % of the label in the amino acid fraction of AZA-treated mycelium was in glutamine whereas [^{14}C]glutamate accounted for only 11 % of the radioactivity after 2 h feeding (Fig. 3a), thus giving a Glu:Gln ratio 10-fold lower under AZA

inhibition compared to the control (Table 3). Under these conditions, the [^{14}C]aspartate and [^{14}C]alanine pools decreased by 3.5- and 4.6-fold respectively, representing only 3.4 and 0.6 % of the radioactivity after 2 h feeding (Fig. 3b). In contrast, γ -amino[^{14}C]butyrate and [^{14}C]asparagine increased by 2.3- and 1.5-fold respectively, representing 2.3 and 2.2 % of the radioactivity in the amino acid fraction, after 2 h incubation. When fed with [^{14}C]glutamine, the AZA-treated mycelium had about double the radioactivity in the amino acid pool and the total radioactivity increased by 1.6-fold at 60 min (Table 2). In addition, assuming that most of the lost radioactivity was evolved as $^{14}\text{CO}_2$, $^{14}\text{CO}_2$ release was reduced eight-fold in AZA-treated mycelium fed with [^{14}C]glutamine after 2 h feeding compared to the control (Table 2). AZA also had strong and predicted effects on [^{14}C]glutamine metabolism since only 18 % of the total [^{14}C]glutamine in the mycelium was metabolized after 2 h feeding (Fig. 3c), in contrast to the control, where 72 % was utilized (Fig. 1c). Seven percent of the amino acid label was in glutamate, 2 % in aspartate and 0.3 % in alanine after 2 h feeding (Fig. 3d). In contrast, γ -amino[^{14}C]butyrate and [^{14}C]asparagine increased by 4.1- and 3.1-fold, respectively, representing 2.6 and 2.0 % of radioactivity in the amino acid fraction after 2 h feeding (Fig. 3d).

Effect of AOA

Preincubation with 2 mM-AOA prior to [^{14}C]glutamate feeding increased the total radioactivity associated with the mycelium 1.7-fold after 30 min and doubled the amount of ^{14}C -labelled amino acid after 30, 60 and 120 min (Table 1). Assuming that most of the lost radioactivity was evolved as $^{14}\text{CO}_2$, preincubation with AOA decreased the amount of released $^{14}\text{CO}_2$ to a negligible level (Table 1). Both glutamate and glutamine accounted for the increase in radioactivity in the amino acid pool and accumulation was double that in the control (Fig. 4a). The Glu:Gln ratio did not differ greatly from the control under [^{14}C]glutamate feeding. In addition, a 3.2- and 9.2-fold decrease in the label incorporated into aspartate and alanine was observed (Fig. 4b), as expected if the reactions

Fig. 4. Effect of AOA on accumulation of radioactivity from (a, b) $[^{14}\text{C}]$ glutamate, and (c, d) $[^{14}\text{C}]$ glutamine into glutamate (●), glutamine (○), aspartate (■), asparagine (□), alanine (△) and γ -aminobutyric acid (▲) by *P. involutus*. Incubations were as in Fig. 2 except that 2 mM AOA was used as the inhibitor. Data are expressed as means of triplicates. Vertical bars indicate SE.

catalysed by aminotransferases were blocked. By contrast, synthesis of asparagine was not affected by this inhibitor and γ -amino $[^{14}\text{C}]$ butyrate increased 2.8-fold after 2 h feeding. Exposure of AOA-treated mycelium to $[^{14}\text{C}]$ glutamine gave similar results and revealed marked accumulation of label associated with the mycelium or with the amino acid pool correlated to a complete reduction in the lost radioactivity, i.e. of $^{14}\text{CO}_2$ (Table 2). The $[^{14}\text{C}]$ glutamine pool increased approximately 6-fold (Fig. 4c) and the amount of $[^{14}\text{C}]$ aspartate and $[^{14}\text{C}]$ alanine decreased 2.2- and 2.3-fold after 2 h $[^{14}\text{C}]$ glutamine feeding (Fig. 4d). However, in contrast to $[^{14}\text{C}]$ glutamate feeding, the $[^{14}\text{C}]$ glutamate pool remained unchanged (Fig. 4c) and the amount of γ -aminobutyrate increased by 4.5-fold after 2 h. As Table 3 shows, the Glu:Gln ratio under $[^{14}\text{C}]$ glutamine feeding was substantially lowered compared to the control.

DISCUSSION

The results of the ^{14}C tracer experiments suggest that the carbon skeletons derived from newly-absorbed glutamate were mainly used for the synthesis of glutamine. The accumulation of $[^{14}\text{C}]$ glutamate and the marked decrease of $[^{14}\text{C}]$ glutamine under MSX treatment are consistent with rapid utilization of glutamate by GS in *Paxillus*

Fig. 5. Possible pathways for metabolism of $[^{14}\text{C}]$ glutamate and $[^{14}\text{C}]$ glutamine by *P. involutus*. The newly-absorbed glutamate is actively metabolized by GS whereas GOGAT is the major enzyme of glutamine degradation. In addition, the Gln-T/ ω -amidase sequence, as an alternative pathway to GOGAT, may also be responsible for the production of oxoglutarate. Glutamate and glutamine carbon skeletons are further actively channelled through the TCA cycle, thus providing a carbon source for mycelial respiration and for amino acid biosynthesis through transamination reactions. AIAT, alanine aminotransferase; AAT, aspartate aminotransferase; AOA, aminooxyacetate; AZA, azaserine; GDH, glutamate dehydrogenase; Gln-T; glutamine transaminase; GOGAT, glutamate synthase; GS, glutamine synthetase; MSX, methionine sulfoximine.

involutus, and support previous studies of ectomycorrhizal fungi and ectomycorrhizas (Martin *et al.*, 1986, 1988; Chalot *et al.*, 1991b; Kershaw & Stewart, 1992). The newly-absorbed, as well as the newly-synthesized, $[^{14}\text{C}]$ glutamine were actively degraded into $[^{14}\text{C}]$ glutamate, suggesting the rapid operation of the glutamine transaminase GOGAT (Fig. 5). This is also supported by the striking accumulation of $[^{14}\text{C}]$ glutamine when colonies were preincubated with AZA. Recently, Kershaw & Stewart (1992) also suggested that GOGAT is involved in the utilization of the ^{15}N amido group of glutamine by *Pisolithus tinctorius*. The unexpected accumulation of $[^{14}\text{C}]$ glutamine under MSX inhibition and $[^{14}\text{C}]$ glutamine feeding suggests that GS might be involved in the recycling of the newly-synthesized glutamate (Fig. 5), the non-utilization of glutamate then having a feedback control effect on GOGAT activity.

The data presented also suggest direct involvement of glutamate and glutamate carbon skeletons in the respiratory pathways. Rapid $^{14}\text{CO}_2$ evolution from $[^{14}\text{C}]$ glutamate or $[^{14}\text{C}]$ glutamine indicates that glutamate

and glutamine carbon are rapidly metabolized to $^{14}\text{CO}_2$, presumably via the TCA cycle (Fig. 5). Production of the key intermediate, oxoglutarate, from the newly-synthesized glutamate is achieved by the putative anabolic GDH reported to be present in ectomycorrhizal fungi (Dell *et al.*, 1989; Vézina *et al.*, 1989). Ultimately, carbon entering the TCA cycle is metabolized to give oxaloacetate and malate, which are used for aspartate and alanine (via pyruvate) synthesis, respectively, by the aminotransferases (Fig. 5). Preincubation of *P. involutus* with AOA, leading to marked reductions in the [^{14}C]aspartate and [^{14}C]alanine pools, confirmed the rapid operation of aminotransferases. In previous studies (Finlay *et al.*, 1989), aspartate and alanine (as free amino acids) were found to have high levels of ^{15}N -enrichment when *P. involutus* was fed with $^{15}\text{NH}_4^+$, confirming the importance of aminotransferases in this fungus. In contrast, in the present study, under AOA treatment, γ -amino[^{14}C]butyrate increased, suggesting that transamination is a possible route for γ -aminobutyrate degradation in *P. involutus*, as already demonstrated in alfalfa nodules (Ta *et al.*, 1988). More surprising is the marked accumulation of γ -amino[^{14}C]butyrate under AZA treatment and [^{14}C]glutamine feeding, which suggests that γ -aminobutyrate synthesis is related to the glutamine pool. A similar relationship is suggested by previous studies on spruce ectomycorrhizas, in which a correlation was demonstrated between the decrease in the glutamine pool due to MSX and the decrease in γ -aminobutyrate labelling during $^{15}\text{NH}_4^+$ feeding (Chalot *et al.*, 1991b). This hypothesis is supported by other findings that demonstrate a good correlation between glutamine synthesis and γ -aminobutyrate synthesis in cultured rice cells (Kishinami & Ojima, 1980). However, the mechanism involved remains unclear. Similarly, the unexpectedly large accumulation of [^{14}C]asparagine under AZA treatment but not under MSX treatment, from either [^{14}C]glutamate or [^{14}C]glutamine, might indicate that synthesis of asparagine is glutamine-dependent but not sensitive to AZA in *P. involutus*. Snapp & Vance (1986) also reported that AZA had little effect on asparagine synthesis in alfalfa root nodules. Our data show that [^{14}C]asparagine is synthesized in higher quantities under conditions where the necessary N donor, glutamine, is not rapidly used in competing pathways, i.e. under AZA treatment. This is also supported by the larger decrease in [^{14}C]aspartate pool, the carbon skeleton donor for asparagine synthesis, under AZA inhibition.

In addition to having the predicted effects on [^{14}C]aspartate and [^{14}C]alanine pools, AOA substantially decreased $^{14}\text{CO}_2$ release from [^{14}C]glutamate or [^{14}C]glutamine and increased the [^{14}C]glutamate or [^{14}C]glutamine pools. Similar effects of AOA on the utilization of [^{14}C]glutamate as a respiratory substrate have been observed in bacteroids isolated from soybean root nodules (Kouchi *et al.*, 1991). The addition of AOA to bacteroid suspensions resulted in a 60 % decrease in $^{14}\text{CO}_2$ evolution from glutamate. It was concluded that the degradation of glutamate might have involved a transamination process as an essential step. In *P. involutus*, it seems rather that glutamine degradation

itself was inhibited by AOA since accumulation of [^{14}C]glutamine but not [^{14}C]glutamate was observed in AOA-treated and [^{14}C]glutamine-fed mycelium. This led us to the hypothesis that glutamine degradation can be achieved by a glutamine transaminase (Gln-T) reported to be present in *Neurospora crassa* (Calderon *et al.*, 1985; Calderon & Mora, 1989). In this pathway glutamine is transaminated to yield oxoglutarate through the participation of a Gln-T and oxoglutarate is further hydrolysed to oxoglutarate and ammonium by the action of an ω -amidase which has been reported to be inhibited by amidotransferase inhibitors (Calderon *et al.*, 1985) and possibly also by AZA, a potent inhibitor of a wide range of glutamine-utilizing enzymes that transfer amide groups (Mifflin & Lea, 1980). The accumulation of [^{14}C]glutamine observed in AOA-treated and [^{14}C]glutamate- or [^{14}C]glutamine-fed mycelium might be explained by inhibition of Gln-T. Similar observations have been reported in *N. crassa*, where addition of AOA to [^{14}C]glutamine-fed cultures reduced the $^{14}\text{CO}_2$ release by 82 % (Calderon & Mora, 1989). The Gln-T/ ω -amidase sequence thus appears to be an alternative pathway to GOGAT for oxoglutarate production. However, if the Gln-T/ ω -amidase pathway was solely responsible for the degradation of glutamine, we would have expected complete inhibition of glutamine degradation and glutamate synthesis following AOA treatment, which did not occur. The presence of enzymes involved in glutamate and glutamine utilization (GS, GDH, GOGAT, Gln-T, ω -amidase) in *P. involutus* remains to be determined by *in vitro* measurement of their activities. Using protein immunoblots (with GS and NADP-GDH antibodies raised against the enzyme from the ectomycorrhizal fungus *Laccaria laccata*), we were able to demonstrate the presence of GS in *P. involutus* whereas no NADP-GDH could be detected (unpublished results). Moreover, some GS activity has been detected in *P. involutus*, either free-living or associated with *Pinus sylvestris* (Sarjala, 1993). However, the presence of abundant polyphenols is likely to have been an obstacle to the detection of the enzyme in several ectomycorrhizal fungi (Botton & Chalot, 1991). For instance, GOGAT activity was not detected in *Pisolithus tinctorius* by Vézina *et al.* (1989) whereas, using the ^{15}N isotope, the enzyme has been shown to be essential for glutamate synthesis (Kershaw & Stewart, 1992).

Our results suggest also that the newly-absorbed [^{14}C]glutamate makes little contribution to the synthesis of aspartate and alanine. Indeed when AZA-treated mycelia were given [^{14}C]glutamate, it failed to accumulate and [^{14}C]aspartate and [^{14}C]alanine pools were substantially lowered. In these conditions, where only the [^{14}C]glutamate pool from glutamine is reduced but not the newly-absorbed [^{14}C]glutamate, no reduction of the [^{14}C]aspartate and [^{14}C]alanine pools would have occurred if the newly-absorbed glutamate pool had been involved in the synthesis of those two amino acids. Similar results have been obtained from enzymic studies on *Cenococcum geophilum*, where aspartate aminotransferase was inhibited in the presence of albizziine, an inhibitor of GOGAT

(B. Botton & A. Khalid, personal communication). It is then possible that a very small but metabolically active pool of glutamate, serving as a substrate in glutamine synthesis, is tightly compartmentalized, away from the other glutamate pool which channels the carbon flow from catabolism of glutamine and serves as a source of carbon skeletons in the synthesis of organic acids and amino acids. This is consistent with previous work showing clear compartmentation of GS in *L. laccata* (Brun *et al.*, 1993). However, conflicting results were obtained from MSX experiments. When MSX-treated mycelia were given [^{14}C]glutamate there was no reduction of the [^{14}C]aspartate and [^{14}C]alanine pools during high GS inhibition, i.e. within the first 5–20 min, as would be expected if glutamine synthesis was required for [^{14}C]aspartate and [^{14}C]alanine synthesis. We suggest that the slow but increasing glutamine synthesis rate might be sufficient to provide carbon skeletons for the synthesis of aspartate and alanine.

In conclusion, the present results provide direct evidence for the utilization of glutamate and glutamine carbons via GS and GOGAT activities by *P. involutus*. The Gln-T/ ω -amidase sequence, as an alternative pathway to GOGAT, may also be responsible for the production of oxoglutarate, the key intermediate between amino acids and oxoacids. Glutamate and glutamine carbon skeletons are actively channelled through the TCA cycle, thus providing a carbon source for mycelial respiration and for amino acid biosynthesis through transamination reactions.

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