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Metabolism of ^{15}N-labelled ammonium by the ectomycorrhizal fungus *Pisolithus tinctorius* (Pers.) Coker & Couch

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Summary. Glutamine was the major product accumulated following transfer of nitrogen-limited cultures of the ectomycorrhizal fungus *Pisolithus tinctorius* to an ammonium medium. Experiments in which mycelium was transferred to $[^{15}N]H_4^+$ showed glutamine amide was the most heavily labelled product. Assimilation of ammonium into glutamate was markedly inhibited by azaserine. The kinetics of ^{15}N-labelling and the effects of azaserine and methionine sulphoximine on the distribution of ^{15}N-labelled products are entirely consistent with the operation of the glutamate synthase cycle. No evidence was found for ammonium assimilation via glutamate dehydrogenase. The labelling pattern observed in mycelium treated with aminooxyacetate suggests that transamination reactions are an important source of glutamate for the synthesis of glutamine.

Key words: Ectomycorrhiza – Ammonium assimilation – Glutamate synthase cycle

Introduction

Although it is generally accepted that higher plants assimilate ammonium via the glutamate synthase cycle (Lea et al. 1990), there is less consensus regarding the assimilatory pathway in fungi, particularly in species that form ectomycorrhizal associations (Ahmad et al. 1990). The classic investigations by Sims and Folkes (1964) demonstrated that net ammonium assimilation in the yeast *Candida utilis* occurred through NADP-linked glutamate dehydrogenase (GDH), with approximately 70% of ammonium entering glutamate-amino N and the remainder entering glutamine-amide N. Consistent with these observations are ^{15}N-labelling studies with nitrogen-limited cultures of the ectomycorrhizal fungus *Cenococcum graniforme* showing that the amide-transferase inhibitor albizzine had no effect on glutamate synthesis and thus implying that glutamate synthase was not active (Genetet et al. 1984). Glutamate synthase activity appeared also to be absent from nitrogen replete cultures, although in these a greater proportion of ^{15}N-labelled ammonium was incorporated into glutamine (Martin et al. 1988).

Studies with *Picea excelsa-Hebeloma* sp. ectomycorrhizal association found metabolic evidence for assimilation via GDH (Chalot et al. 1991) and this was consistent with enzymological studies showing high activities of NADP-GDH in this association (Dell et al. 1989). Thus in yeast and these mycorrhizal fungi ammonium is assimilated into glutamate and glutamine-amide.

However, mutant strains of *Neurospora crassa* (Dunn-Coleman et al. 1981), *Aspergillus nidulans* (Kinghorn and Pateman 1976) and *Hebeloma cylindrosporum* (Wagner et al. 1988), which lack detectable NADP-GDH, are able to grow on ammonium ions as a sole nitrogen source, suggesting an assimilatory pathway other than that catalysed by NADP-GDH. NADH-glutamate synthase (GOGAT) has been reported in some fungi, including *Saccharomyces cerevisiae* (Roon et al. 1974), *N. crassa* (Hummelt and Mora 1980a) and *Laccaria bicolor* (Vezina et al. 1989). The double mutant of *N. crassa*, which lacks both NADP-GDH and NADH-GOGAT, is unable to grow on ammonium (Hummelt and Mora 1980b). Such enzymological evidence suggests that in some fungi there is the potential for ammonium assimilation via the glutamate synthase cycle. Similarly methionine sulphoximine, an inhibitor of glutamine synthetase (GS), completely inhibited assimilation of ^{15}N-labelled ammonium and albizzine inhibited labelling of glutamate in beech mycorrhizae (Martin et al. 1986). This suggests that only the glutamate synthase cycle was operative, although the results did not identify the tissue(s) localisation of the cycle. Thus there is the possibility that fungal species associated with beech roots might have the potential for

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ammonium assimilation via the glutamate synthase cycle. Accordingly, the present investigation was carried out to determine the routes for ammonium incorporation in *Pisolithus tinctorius*, which is of widespread occurrence in ectomycorrhizal associations.

Materials and methods

Growth conditions

Pisolithus tinctorius cultures (strain 008, kindly provided by Professor D. Read, Department of Animal and Plant Biology, University of Sheffield) were inoculated with a 1-cm^2 disc of mycelium taken from the growing edge of a 28-day-old agar culture (half-strength Melin-Norkrans medium in 2% agar). They were grown in 50 ml half-strength Melin-Norkrans medium containing 2 mM ammonium as nitrogen source in an orbital shaker at 25° C for 18 days. At this time, cultures were nitrogen limited and for ^{15}N-labelling experiments they were transferred to fresh medium containing 2 mM $^{15}N[NH_4]$ (99% ^{15}N).

Extraction of amino compounds

Mycelia were harvested, rinsed with distilled water and extracted with methanol (70% v/v). After centrifugation, the supernatant was used directly for HPLC analysis or fractionated by ion-exchange chromatography before analysis by GC-MS.

Amino acid analysis

Amino acids were analysed as *o*-phthaldialdehyde (OPT) derivatives on a C-18 column using a method adapted from Joseph and Marsden (1986). OPT stock reagent was prepared as follows: 50 mg OPT (Sigma, St. Louis, Mo.) was dissolved in 1 ml methanol and made up to 7.5 ml with 0.4 M borate buffer (pH 9.5). For the working reagent (10 µl 2-mercaptoethanol was added to 1.5 ml OPT stock reagent. All samples, standards, eluents and derivatising reagents were passed through a 0.5-µm FP vericel filter prior to use. Ten microlitres of 0.25 mM homoserine and a 10 µl sample were derivatised with 60 µl working reagent. After 2 min, 8 µl derivatised sample was injected. The gradient was produced using two eluents: (A) 0.1 M phosphate buffer pH 8 with 20 ml methanol and 25 ml tetrahydrofuran per litre; (B) 65% methanol. Eluents were degassed with helium prior to use. The gradient was programmed as follows: 0–5 min 20–35% B, 5–27 min 35–100% B, 27–32 min 100% B. The HPLC was fitted with a guard column and a column containing Spherisorb 5 µm ODS2 spherical packing (Phase-Sep, Deeside, UK).

Gas chromatography-mass spectrometry

Methanolic extracts were taken to dryness by rotary evaporation, redissolved in 2 ml water and applied to a Bio-Rex sample preparation disc of AG 50W-X8 cation exchange resin washed with 5 ml water. Amino acids and amides were eluted with 5 ml 6 M NH_4OH and the amino acid fraction lyophilised and redissolved in 1 ml 50% methanol. Purified extract (0.5 ml) was taken directly into a silanised glass vial, dried under N_2 and derivatised with *N*-methyl-*N*-(*tert*-butyldimethylsilyl)trifluoroacetamide (MTBSTFA, Pierce Chemical Co., Rockford, Ill.) as described by Fortier et al. (1986). Fifty microlitres of derivatising mixture, MTBSTFA:pyridine:triethylamine (15:15:1 by volume) was added to dry samples and the vials were heated at 75° C for 30 min. ^{15}N incorporation into each amino acid (including glutamine and asparagine) was carried out by GC/MS analysis of t.BDMS derivatives using a VG7070 H mass spectrometer linked to a Finnigan Incos data system. Samples of 0.2–0.4 µl were applied to a Pye-Unicam 204 gas chromatograph with an all glass dropping-needle solid injector and fitted with a 25-m polydimethylsiloxane, 0.25-µm film thickness, fused silica capillary column. Carrier gas, helium, was at a column head pressure of 9.5 psi and the oven was temperature programmed from 120° C for 1 min, +4° C/min to 280° C. Mass spectra were acquired using an electron energy 70 eV; mass range was scanned from m/z 750 to 35 every 2 s with a total cycle time of 3 s. ^{15}N incorporation (atom % excess) was calculated after integrating the areas obtained for (M−57)+ and (M−56)+ over each amino acid peak. Corrections were made for natural abundance of isotopic elements present (^{13}C, ^{29}Si, ^{15}N).

t.BDMS derivatives allow the determination of total ^{15}N incorporated into glutamine and asparagine and the proportion of amides that are singly or doubly labelled. To determine ^{15}N label in both amide-N and amino-N a second derivative was prepared. The remaining 0.5 ml amino acid extract was separated into neutral/basic and acidic amino acids by Dowex 1-acetate ion exchange chromatography (Rhodes et al. 1981). The neutral and basic amino acid fractions were lyophilised, redissolved in 50% methanol and transferred to a silanised glass vial. Amino acids were dried under N_2 and derivatised as their heptafluorbutyryl isobutyl (HFIB) esters (Rhodes et al. 1981). On preparation of these derivatives, amide groups of glutamine and asparagine are lost to form glutamate and aspartate, respectively. GC/MS of HFIB derivatives was performed as for t.BDMS derivatives, but the mass range scanned was m/z 200–400 and the GC was temperature programmed from 120° C for 2 min, +6° C/min to 280° C. ^{15}N in amino-N of glutamine was calculated after integration of ions m/z 298, 299. ^{15}N-label in amide-N of glutamine was then calculated by difference.

Results

Following transfer of nitrogen-limited mycelia to an ammonium-containing medium, the glutamine concentration increased from 0.1 to 4.5 µmol/g fresh wt., and glutamate from 0.3 to 1.4 µmol/g fresh wt. (Fig. 1). There was little change in the concentrations of ammonium, aspartate, alanine and serine, the only other amino compounds present in readily measurable amounts. Addition of methionine sulphoximine (MSX), an inhibitor of GS, resulted in a very substantial inhibition of glutamine accumulation after 2 h; thereafter glutamine concentrations decreased (Fig. 1). Accumulation of glutamate was reduced by around 70% and there was a marked increase in ammonium concentration in mycelium treated with MSX.

Azaserine (AZA), an inhibitor of amide-transfer reactions completely inhibited accumulation of glutamate (Fig. 1) and, while glutamine accumulation was similar to that in control mycelium over the first 4 h, concentrations subsequently decreased. AZA increased serine levels approximately 10-fold over concentrations observed in control mycelium. There was an initial build up of ammonium over the first 4 h followed by a substantial decline.

Addition of the aminotransferase inhibitor, aminooxyacetate (AOA), slowed down the rate of glutamine and glutamate accumulation, although concentrations at 8 h were similar to those in control mycelium (Fig. 1). Ammonium increased to concentrations comparable with those in MSX-treated mycelium.

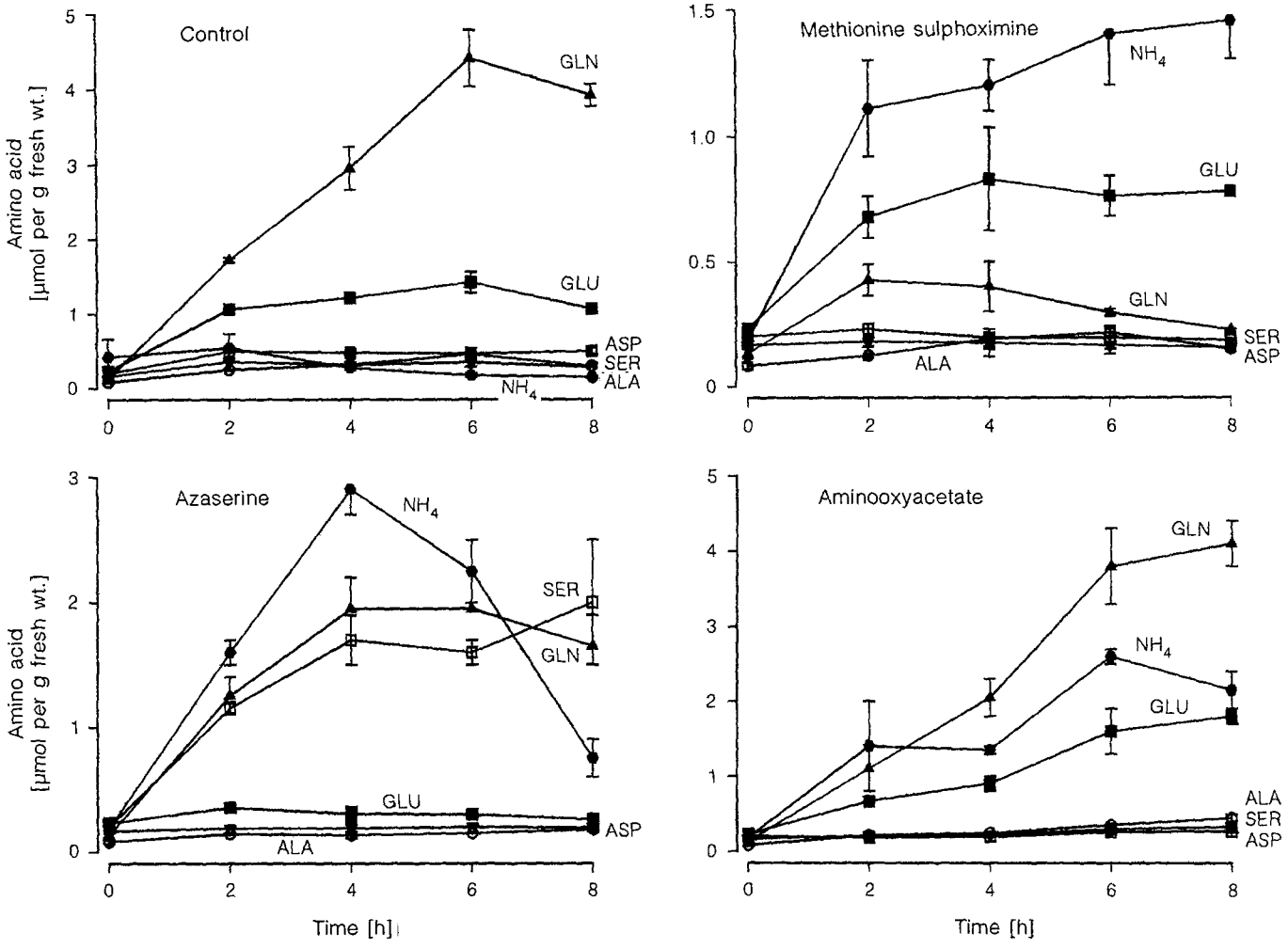

Fig. 1. Effect of inhibitors of the glutamate synthase cycle on assimilation of ammonium and amino acid levels in N-limited *Pisolithus tinctorius*

It is clear that glutamine is the major product of ammonium assimilation when nitrogen-starved cultures of *P. tinctorius* are supplied with ammonium. The marked inhibition of glutamate accumulation by AZA, together with a similar, albeit less pronounced inhibition by MSX, provide evidence that ammonium is assimilated through the glutamate synthase cycle.

Incorporation of ^{15}N into glutamine was inhibited 80–90% by MSX, 40–45% by AZA and 30–35% by AOA (Fig. 2). With MSX, no further ^{15}N-labelling of glutamine occurred after 2 h. Incorporation of label into glutamate was inhibited 75–80% by AZA, over 50% by MSX but less than 20% by AOA (Fig. 2). Further increase in labelling of glutamate ceased after 2 h in AZA. This suggests that GDH plays only a minor role in ammonium assimilation.

Serine labelling was similar with all three inhibitors and was reduced by about 80% after 2 h (Fig. 2). Although AZA markedly stimulated serine accumulation, it actually inhibited its ^{15}N-labelling. These results suggest that AZA may inhibit some serine-dependent reaction.

AOA was a potent inhibitor of ^{15}N-labelling of alanine, after 2 h treatment no label could be detected in this amino acid and at 4 h labelling was inhibited 70% compared with its labelling in control mycelium (Fig. 2). AZA and MSX reduced the labelling of alanine by 80% at 2 h and 67% at 4 h. The three inhibitors reduced incorporation of ^{15}N into aspartate by 60–70% (Fig. 2). AOA appeared to be less effective in inhibiting aspartate synthesis compared with alanine.

MSX reduced total ^{15}N incorporation by 76% at 2 h and 85% at 4 h, while the initial rate of ammonium assimilation was 8 nmol m^{-1} per g fresh wt. compared with 32 nmol m^{-1} per g fresh wt. in control mycelium. AZA inhibited total ^{15}N incorporation by 58% and 55% at 2 and 4 h, respectively, and the initial rate of ammonium assimilation was 13 nmol m^{-1} per g fresh wt. AOA had less effect than both AZA and MSX, incorporation was inhibited by 44% and 37% at 2 and 4 h, respectively, and ammonium was assimilated at 21 nmol m^{-1} per g fresh wt. These results are again consistent with ammonium assimilation into glutamine amide and its subsequent transfer to 2-oxoglutarate to synthesize glutamate. Cessation of glutamate synthesis after 2 h in AZA and its marked inhibition by MSX provide little indication of a direct incorporation of ammonium into glutamate via GDH. Inhibition of ^{15}N transfer to alanine,

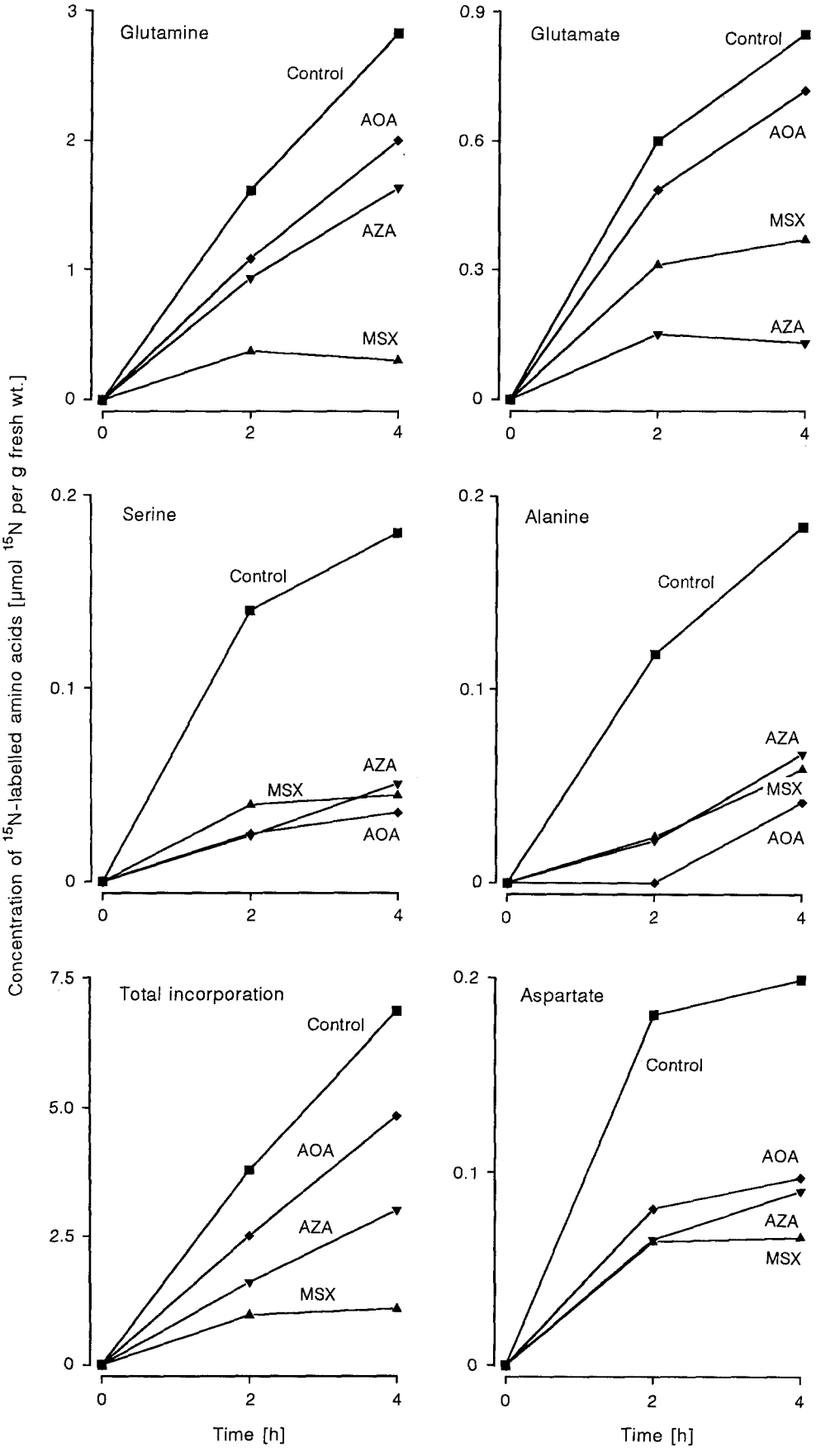

Fig. 2. Time course of changes in ^{15}N-labelled amino acids and amide in *P. tinctorius* supplied with 2 mM [^{15}N]H_4^+ in the presence or absence of inhibitors of the glutamate synthase cycle

Table 1. [^{15}N] abundance of glutamate and glutamine nitrogen in the presence and absence of glutamate synthase cycle inhibitors. MSX, Methionine sulphoximine; AZA, azaserine; AOA, aminooxyacetate

Time (h)		Glutamate atom % excess	Glutamine					
			Single (%)	Double (%)	Amino atom (%) excess	Amide atom (%) excess	Single amino (%)	Single amide (%)
Control	2	60	36	58	63	87	6	30
	4	67	27	69	75	90	6	21
MSX	2	45	45	40	33	76	9	36
	4	43	42	47	45	81	8	34
AZA	2	42	40	36	47	66	11	30
	4	56	38	45	35	71	13	34
AOA	2	74	24	74	76	96	2	22
	4	79	12	86	81	95	1	11

aspartate and serine in MSX and AZA can be explained if their synthesis occurs via transamination of glutamate; certainly the pronounced inhibition of ^{15}N-labelling into alanine seen with AOA is consistent with this hypothesis.

The derivitisation procedures employed here allow the labelling of glutamine amino- and amide nitrogen together with the proportion of singly and doubly labelled glutamine molecules to be determined (Table 1). Glutamine-amide N is always more highly enriched than glutamine-amino N, although in the AZA treatment the ratio of amide to amino enrichment was 1.2 compared with values above 2 in MSX and AZA treatments. If it is assumed that the mycelial ammonium pool has the same ^{15}N abundance as that in the medium (i.e. 99%), then the expected ratios of double:single:unlabelled glutamine species can be calculated using the actual ^{15}N abundances determined for its precursor, glutamate. Thus for control mycelium (2 h) the expected values are 58:41:1, while the actual values are 58:36:6. The underestimation of unlabelled glutamine is even more pronounced in MSX and AZA treated mycelium, 1.7% compared with 15% and 1.3% compared with 24%. It is striking that calculated values for glutamine species of AOA-treated mycelium come very close to the actual values, particularly with respect to unlabelled glutamine species, 1% compared with 2%. One possible explanation for this is that the synthesis of unlabelled glutamate is inhibited by AOA. Calculations suggest that transamination could contribute up to 20% of glutamate in this fungus.

If a lower enrichment of the ammonium pool is assumed, then the proportion of unlabelled glutamine approaches that observed experimentally but the predicted proportions of single- and double-labelled glutamine are markedly different from observed values. These results imply a compartmentation of glutamate and glutamine metabolism. Moreover, the predicted ratios of double- to single-labelled glutamine species, calculated for a range of ammonium enrichments, are always lower than observed values. This suggests that there is a precursor glutamate pool for glutamine synthesis which is more heavily labelled than the bulk pool.

Glutamine, labelled only in amide nitrogen, comprises around 30–35% of the bulk pool except of mycelium treated with AOA, where the proportion of single, amide-labelled glutamine falls to below 20%. Both MSX and AZA greatly reduce the proportion of amino-labelled glutamine, while this increases in AOA-treated mycelium. An interesting feature of the results is the occurrence of glutamine labelled only in the α-amino nitrogen. The presence of this single amino-labelled glutamine implies glutamine was synthesised from an unlabelled ammonium pool. Moreover, the precursor glutamate must have been synthesised via aminotransferases or GDH rather than the glutamate synthase cycle. After 2 h of labelling the single, amino-labelled species comprised 6% of the glutamine in control mycelium, going up to 11% in AZA and down to 2% in AOA. Inhibition of single amino-labelled glutamine by AOA is suggestive of glutamate synthesis by transamination from a labelled amino donor.

Plots (Fig. 3) of the pool sizes of unlabelled, single-labelled and double-labelled glutamine species indicate that there is a relatively stable and apparently metabolically inactive pool of glutamine which remains at a constant level (150–200 nmol per g fresh wt.). This unlabelled pool of glutamine depletes rapidly in the presence of AOA, whereas accumulation of metabolically active, double-labelled glutamine species is similar to that in control mycelium. AOA reduces the pool of singly labelled glutamine and the ratio of double:single labelled glutamine increases to a value of 7.8. In the absence of AOA, the ratio was only 2.1. This is again consistent with a substantial input of glutamate from transamination reactions.

Discussion

It is apparent that both the accumulation and ^{15}N-labelling of glutamate in nitrogen-deficient mycelia of *P. tinctorius* are sensitive to inhibition by the amide-transfer inhibitor, AZA. This strongly suggests that glutamate synthesis in this species occurs largely via the activity of GOGAT. After 2 h of labelling with ^{15}N ammonium, 40% of the label is recovered as glutamine am-

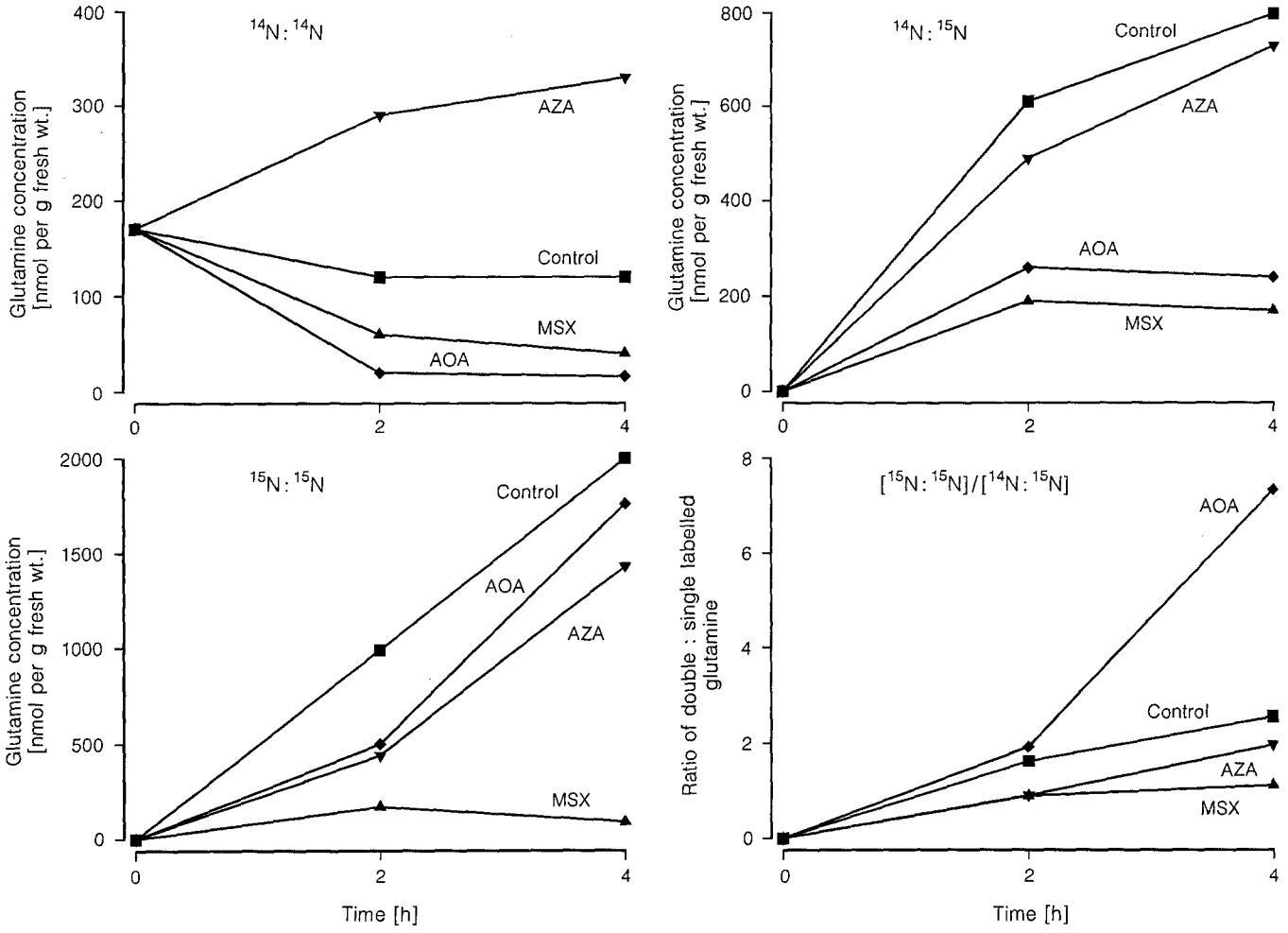

Fig. 3. Time course of changes in levels of unlabelled, single-labelled and double-labelled glutamine and ratio of double:single labelled glutamine following addition of $[^{15}N]H_4^+$ to *P. tinctorius* grown in the presence and absence of inhibitors of the glutamate synthase cycle

ide in control mycelium while for mycelium treated with MSX, AZA and AOA the proportions are 34%, 50% and 42%, respectively. Increased labelling of glutamine-amide in the presence of AZA is consistent with its utilisation in the synthesis of glutamate by GOGAT. Seventeen per cent of ^{15}N-label is recovered as glutamate-amino in control mycelium, 32% in MSX-, 9% in AZA- and 20% in AOA-treated mycelium. The greatly reduced proportion of amino-labelled glutamine in the AZA treatment is again consistent with the synthesis of glutamate via GOGAT. The partial inhibition of glutamate accumulation and ^{15}N-labelling in MSX treatment would be expected if glutamate was synthesised from glutamine. It is somewhat surprising then that enzymological studies of *P. tinctorius* indicated the absence of GOGAT, although GS was present (Vezina et al. 1988). The metabolic studies described in the present paper are clearly indicative of ammonium assimilation via the glutamate synthase cycle. There is no evidence that GDH is active in ammonium assimilation.

The lack of any pronounced effect of AOA on the accumulation of glutamine and glutamate suggests that the w-amidase pathway of glutamine degradation described for *N. crassa* (Mora 1990) may not be operative in *P. tinctorius*.

Compared with the control, the AOA treatment has a low proportion of unlabelled glutamine molecules and a high proportion of double-labelled molecules, indicating aminotransferase activity may be an important source of glutamate for glutamine synthesis. This suggestion is reinforced by labelling of glutamine in AZA-treated mycelium. Here at 4 h the proportion of unlabelled glutamine molecules has risen to 31%, but the concentration of singly labelled glutamine is little affected. These results would be expected if there were reactions other than GOGAT contributing glutamate to the bulk pool.

The present results are in marked contrast to those obtained for *Cenococcum geophilum*, where inhibition of GS by MSX had no effect on glutamate accumulation and stimulated incorporation of ^{15}N-label into this amino acid and its derivatives (Genetet et al. 1984; Martin et al. 1988). Moreover, the amidetransferase inhibitor albizzine was without effect on glutamate synthesis. Clearly these two species of ectomycorrhizal fungi exhibit different ammonium assimilatory pathways.

Studies of beech ectomycorrhizas showed that they assimilated ammonium exclusively via the glutamate synthase cycle and it was suggested that the fungal assimilatory pathway (then assumed to be GDH) was repressed. The present results indicating that some ectomycorrhizal fungi employ the glutamate synthase pathway of ammonium assimilation invalidates a model for mycorrhizal assimilation based on suppression of the fungal assimilatory route. It is interesting that studies with *Picea/Hebeloma* association indicated only the fungal GDH route was active in ammonium assimilation and the higher plant glutamate synthase cycle inoperative (Chalot et al. 1991). It is tempting to conclude that in ectomycorrhizas it is only the fungal microsymbiont which participates in ammonium assimilation, using either the GDH route as in *Hebeloma* or, as we have shown here for *Pisolithus*, the glutamate synthase cycle.

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