^{15}N-NMR studies of nitrogen assimilation and amino acid biosynthesis in the ectomycorrhizal fungus *Cenococcum graniforme*

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^{15}N-NMR spectroscopy was used to follow nitrogen assimilation and amino acid biosynthesis in the intact mycelium and extracts of the symbiotic fungus *Cenococcum graniforme*. The primary incorporation of $^{15}NH_4^+$ appears to proceed via the glutamine synthetase activity. Glutamine, γ-aminobutyrate, alanine and arginine were the main labelled nitrogenous pools. The quenching of arginine in intact mycelium suggest that the amino acid is associated with a cellular component which restricts its rotational freedom.

^{15}N-NMR *In vivo NMR* *Nitrogen metabolism* *Amino acid* *Ectomycorrhizal fungus*

1. INTRODUCTION

To date, most techniques used to study nitrogen metabolism in plant tissues have involved destructive chemical analyses or use of radiolabelled isotopes. NMR spectroscopy is a useful technique for observing the flow of metabolites through pathways in vivo, as has been demonstrated by ^{13}C-NMR studies of glycolysis and Krebs cycle in bacteria, fungi, and mammalian tissues [1]. The feasibility of observing intracellular [^{15}N]amino acids in intact cells of *Brevibacterium lactofermentum* [2] and mycelia of *Neurospora crassa* [3] has recently been demonstrated by using high-resolution ^{15}N-NMR. Despite these promising pioneering works, ^{15}N has not been used extensively as an NMR probe.

The purpose here was to obtain kinetic labelling data by NMR from experiments in which *Cenococcum graniforme*, an ectomycorrhizal ascomycete, growing on a nitrogen-rich medium, was fed ^{15}N-labelled ammonium. ^{15}N was also tracked in free amino acids to determine the fate of absorbed nitrogen. Low sensitivity of natural abundance ^{15}N-NMR spectroscopy is overcome by using enriched ^{15}N-precursor and improved instrumental sensitivity (40.5 MHz ^{15}N-NMR spectrometer).

2. EXPERIMENTAL

Mycelium of the ectomycorrhizal ascomycete *C. graniforme* Ferd. and Wing., strain Sivrite, was grown in a medium containing 2.5 mM diammonium tartrate as described previously [4]. Mycelial growth was performed, with aeration provided by shaking, at 25°C for 16–18 days (balanced phase of growth). Growing mycelium was transferred to a fresh medium for 3 days before the ^{15}N labelling to avoid depletion of any components. The mycelium was collected by filtration and transferred to an N-free medium supplemented with 2.5 mM [^{15}N]diammonium sulfate (50% ^{15}N, CEA, France) for specified periods.

For NMR measurements, samples were harvested by filtration and used immediately. After spectra had been recorded, the mycelium was collected and extracted as described in [4]. Packed living mycelium (~1.0 g wet wt) and fungal extracts were studied in 10 mm o.d. spinning tubes at 4°C. ^{15}N-NMR spectra, in the Fourier transform

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mode with proton broad band decoupling, were measured at 40.51 MHz with a Bruker AM 400 spectrometer. The operating conditions were: 17-μs pulse width with a 2 s delay time and 8 K data storage array. Spectra of mycelial extracts were recorded in 20% D_2O to provide a lock signal. Chemical shifts are reported relative to $^{15}NH_4^+$ at 0 ppm. Assignments of ^{15}N resonances were made by comparison with known spectra of amino acids and published data [2,3]. The assignments are additionally supported by proton-coupled ^{15}N-NMR spectra of the samples.

3. RESULTS

3.1. *In vivo spectra*

^{15}N-NMR spectra of intact mycelia of *C. graniforme* resuspended in a medium containing [^{15}N]ammonium sulfate are shown as a function of time after feeding ^{15}N in fig.1. Spectra taken over a period of 2 days show the gradual appearance of clearly resolved signals corresponding to intracellular [^{15}N]amino acids. For each amino acid, the intensity of its ^{15}N resonance is proportional to its ^{15}N population because all of the spectra were taken under identical operating conditions. To estimate the relative intracellular concentrations of different ^{15}N-labelled amino acids from their resonance intensities, it is necessary to take into account the respective spin-lattice relaxation times and contributions of nuclear Overhauser effect to the resonance intensities. Values of these NMR parameters determined in the present experimental conditions (not shown) indicate that the ^{15}N resonance intensities correspond, within 20%, to the respective intracellular concentrations. The first spectrum was obtained 1 h after ^{15}N labelling was started. Three peaks were observed: the strong signal at 90.4 ppm is due to the glutamine amide-N and the weaker resonances detected at 19.7 and 0.3 ppm are assigned to glutamine amino-N and ammonium ion, respectively. Glutamine amide-N is the most highly labelled component over the first hours of the time course experiment, while glutamine amino-N shows lower ^{15}N incorporation. This pattern is at least qualitatively consistent with a primary incorporation of ammonium via the glutamine synthetase activity. The signals of

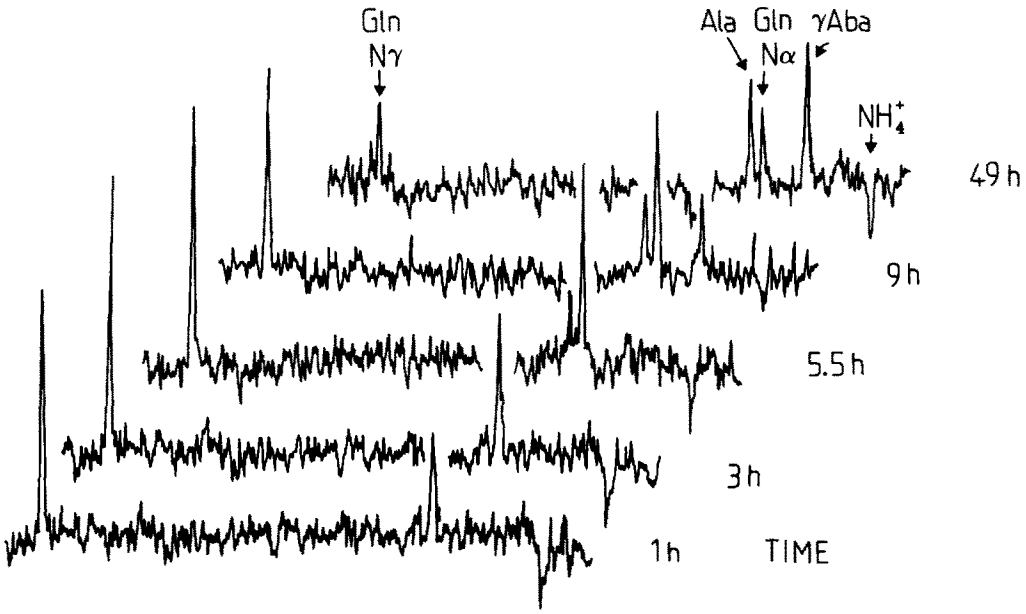

Fig.1. ^{15}N-NMR spectra (40.5 MHz) of intracellular [^{15}N]amino acids in intact mycelia of *C. graniforme* obtained after feeding [^{15}N]ammonium sulfate (50% ^{15}N) at time zero. Spectral conditions were as given in section 2 (1000 scans). The spectral phase has been multiplied by 180° for convenience. Resonance frequencies (ppm): NH_4^+, 0; γ-aminobutyrate, 11.6; Gln Nα, 19.7; Ala, 21.9; Gln Nγ, 90.4.

glutamine Nα increase with time and become almost as intense after 9 h of ^{15}N feeding. With time, other peaks appear at 11.6 and 21.9 ppm; these have been assigned to γ-aminobutyrate and alanine. After 49 h of ^{15}N incorporation, γ-aminobutyrate, glutamine and alanine account for about 41, 34, and 24% of the total observed ^{15}N, respectively.

3.2. *In vitro spectra*

In these spectra, the additional resonances identified arose from arginine nuclei (Nα, 19.5 ppm; Nω,ω′, 50.8 ppm; Nδ, 63.7 ppm) (fig.2). In the corresponding whole mycelium spectra, the absence of arginine resonances is noteworthy, because in the extract spectra they are rather intense and contain a high proportion of observed ^{15}N after 9 h of isotopic labelling.

The ratio of glutamine amide-^{15}N/glutamine amino-^{15}N in labelling experiments is presented in fig.3. It shows that the label was primarily incorporated into the amide group of glutamine and

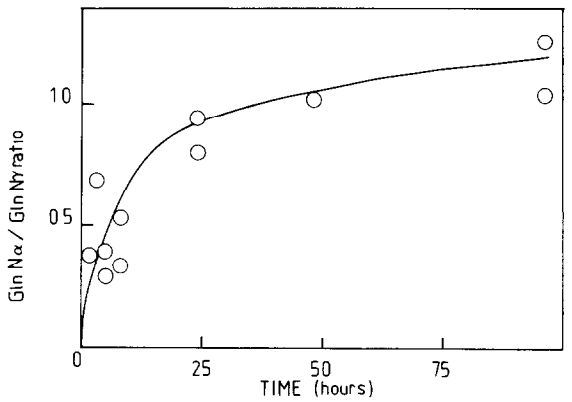

Fig.3. Nα/Nγ ratios for resonances of glutamine during the course of [^{15}N]ammonium feeding.

that the relative enrichments of the amido- and amino-N of the amide were similar after about 1 day of labelling. The proportions of labelled amino acids were compared (fig.4). The ^{15}N in the glutamine pool is considerably higher than in other

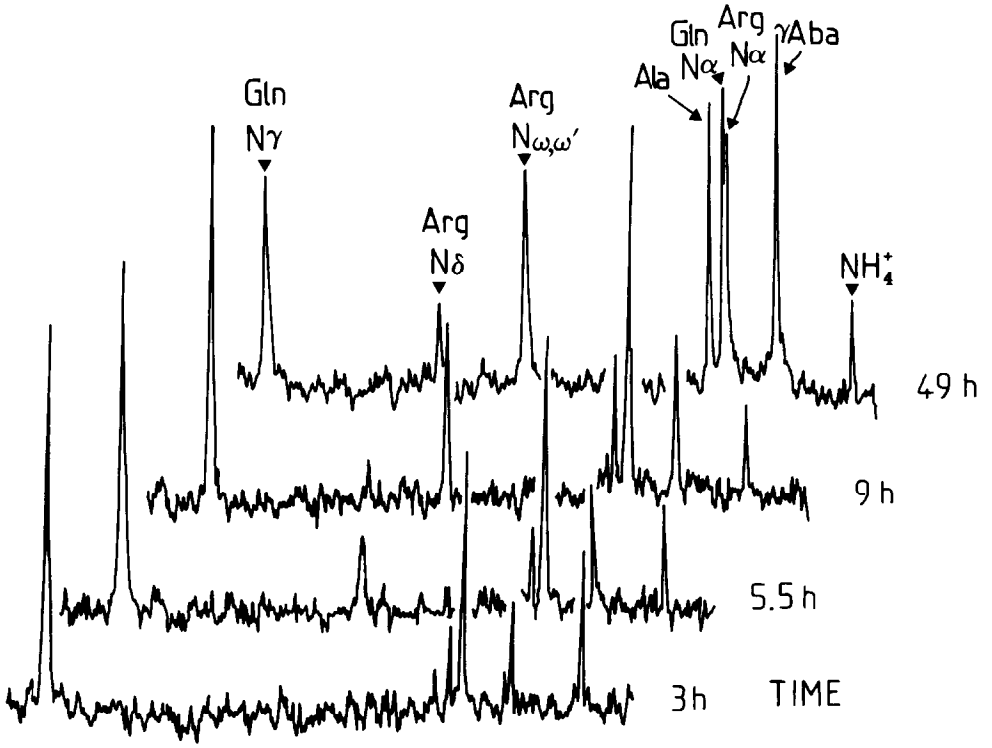

Fig.2. ^{15}N-NMR spectra (40.5 MHz) of extracts of *C. graniforme*. Immediately after the spectra shown in fig.1 were recorded, mycelia were frozen and extracted. Spectral conditions were as given in section 2 (2500 scans). Additional resonance frequencies (ppm): Arg Nα, 19.5; Arg Nω,ω′, 50.8; Arg Nδ, 63.7.

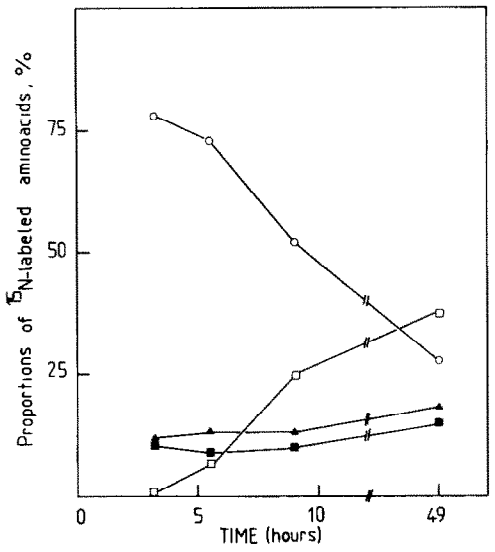

Fig.4. Proportions of ^{15}N-labelled amino acids in extracts of *C. graniforme* as a function of time.

amino acids during the first 9 h. Alanine and γ-aminobutyrate followed and after 5.5 h ^{15}N could be observed in amino- and guanidino-nitrogens of arginine. After 2 days, the glutamine peaks decreased and the arginine signals strongly increased and became the most prominent resonances observed.

4. DISCUSSION

Two different pathways are involved in the primary incorporation of ammonium into organic compounds by higher fungi, namely the glutamate dehydrogenase pathway and the glutamine synthetase pathway [5]. Direct incorporation of newly absorbed ammonium nitrogen via glutamine synthetase first introduces the label into the amide-N position of glutamine, the amino-N of this amino acid being derived from originally unlabelled glutamate. The flow of ^{15}N in the present experiments is consistent with this pathway. The progression of the relative intensities of the amide- and amino-N of glutamine (fig.3) suggests that there is only a small proportion of glutamine singly labelled in the amino-N position, and therefore that most of the amino-labelled glutamine is in fact doubly labelled material which has cycled through a larger and poorly enriched pool(s) of glutamate. These results contrast with our previous findings [4] obtained with N-starved *C. graniforme*, in which the ammonium assimilation appears to proceed via the glutamate dehydrogenase pathway. Biosynthesis of glutamine is rapid and is presumably used by the mycelium to store nitrogen and detoxification. Another feature is the significant formation of alanine, γ-aminobutyrate and arginine. Labelling of arginine at Nα, Nδ, and Nω,ω' positions is consistent with the operation of the ornithine cycle and the large ^{15}N enrichment of this amino acid indicates that this cycle is a major sink of the newly assimilated nitrogen.

The failure to observe the arginine Nω,ω' resonance in the intact mycelia is unexpected since this particular arginine nitrogen has a large nuclear Overhauser effect of -3.6 and a slightly shorter spin-lattice relaxation time than glutamine amide-N in aqueous solution [6]. This failure could be due either to field inhomogeneities within the sample or to an increased rotational correlation time for the enriched amino acid in the intracellular environment. A field inhomogeneity would similarly decrease the resonances of other amino acids. Thus, the arginine broadness is presumably the result of an interaction of the amino acid with cellular components which restrict its rotational freedom. It has been suggested that the positively charged guanidino groups of arginine are associated with the negatively charged polyphosphates in the fungal vacuole [7] and Kanamori et al. [6] demonstrated that polyphosphates decreased the spin-lattice relaxation time of arginine ^{15}Nω,ω' resonance. The presence of a significant amount of polyphosphates in the mycelium of *C. graniforme* [8] supports this interpretation.

In conclusion, ^{15}N-NMR allows the rapid examination of nitrogen assimilation, amino acid biosynthesis and observation of the intracellular environment.

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