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# MAGIC-ANGLE N-15 NMR STUDY OF NITRATE METABOLISM OF NEUROSPORA CRASSA

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SUMMARY: Magic-angle cross-polarization $^{15}N$ nmr spectra of intact lyophilized mycelia from N. crassa cultured on media containing [$^{15}N$] nitrate have been obtained at 9.12 MHz. The time development of the uptake and distribution of label into protein and amino-acid metabolites can be observed directly. Nitrate metabolism is delayed about one hour if the cells innoculating the culture are grown on nitrate-free medium.

INTRODUCTION: In addition to its ability to grow on various amino acids, urea, and ammonia, Neurospora crassa contains an inducible assimilatory nitrate reductase (1) which permits the utilization of nitrate as a source of inorganic nitrogen. The pathway for assimilation of nitrate involves the reduction of nitrate to nitrite and then to ammonia which is directly incorporated into amino acids and, ultimately, more complex biomolecules. Nitrate reductase is maximally induced 2 to 3 hours after mycelia are transferred into a nitrate medium (1, 2), after which specific activity drops slightly over the next 7-8 hours.

In this paper we report the results of an investigation of the metabolism of [$^{15}N$] nitrate by N. crassa as determined by solid-state magic-angle cross-polarization $^{15}N$ nmr (3). The nmr experiments, performed on intact (lyophilized) quenched samples harvested as a function of time from a large fermenter, lead to the direct measurement of total nitrogen flux into both solid (protein) and soluble (amino acid) metabolites.

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ABBREVIATIONS: nmr, nuclear magnetic resonance; EDTA, ethylenediaminetetraacetic acid.

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MATERIALS AND METHODS: Spores of N. crassa STA4 were obtained from the Fungal Genetics Stock Center, Humboldt State University Foundation, Arcata, CA. Growth and induction was carried out at 30°C in modified Fries basal media. Ammonia medium contained sodium tartrate (4.28 g/L), $NH_4Cl$ (3.6 g/L), and $KH_2PO_4$ (1 g/L). Nitrate medium contained $KH_2PO_4$ (3 g/L), $NaNO_3$ (2 g/L), and sodium tartrate (1 g/L). In addition, both media contained (in g/L) the following: sucrose, 20; $MgSO_4$, 0.27; $CaCl_2{:}2H_2O$, 0.13; NaCl, 0.1; sodium tetraborate, $8.8 \times 10^{-5}$; sodium molybdate, $8.8 \times 10^{-5}$; $FeCl_3 \cdot 6H_2O$, $9.6 \times 10^{-4}$; $ZnSO_4 \cdot 7H_2O$, $8.8 \times 10^{-3}$; $CuCl_2 \cdot 2H_2O$, $3.4 \times 10^{-4}$; $MnCl_2 \cdot 4H_2O$, $7.2 \times 10^{-5}$; and biotin, $5 \times 10^{-6}$. Neurospora was maintained by transfer and growth of conidia in 125-mL Erlenmeyer flasks containing 50 ml of 2% Bacto-agar ammonia medium.

Culture Methods. The conidia from one flask grown for at least 7 days were suspended in 50 mL of sterile distilled water and used to innoculate 3 L of either ammonia or nitrate medium (depending on whether cells were to be pretreated with nitrate before growth in [$^{15}$N] nitrate medium). After 27 hours of growth with aeration and stirring, the cells were further grown in 8 L of the corresponding ammonia or nitrate medium contained in a 20-L fermenter. After 12 hours of growth with aeration and agitation the Neurospora was harvested by filtration of the culture through several layers of cheesecloth and washed with distilled water. A portion of the harvest (designated "0 hr mycelia") was frozen in liquid nitrogen; the remaining material was used to innoculate 7.5 liters of medium containing 2 g/L 95% $^{15}$N-enriched sodium nitrate as the sole nitrogen source. Every hour 400 mL of liquid culture was removed from the 20-L fermenter and filtered. The harvested mycelia were washed, frozen in liquid nitrogen, and lyophilized.

Extraction Procedures. Extraction of the soluble components present in a lyophilized sample of N. crassa was performed with 80% ethanol, repeated 6 times or until a ninhydrin spot test for amino acid was negative. Crude extracts of N. crassa for enzyme assay were prepared as described previously (4).

Analytical Methods. Protein was determined by the microbiuret method with bovine serum albumin as the standard (5). Assays of nitrate reductase activity were performed by measuring the formation of nitrite (6) at 25°C. Activity is reported in terms of nanomoles nitrate reduced per 10 minutes.

Nitrogen-15 NMR. Lyophilized samples of N. crassa were examined as powders contained in a Beams-Andrew 420-µl hollow rotor spinning at 1.3 kHz at the magic angle. Typical sample weights ranged from 300 to 400 mgs. Cross-polarization nmr spectra were obtained at 9.12 MHz using matched spin-lock transfers (7) with 1-msec single contact times and 32-kHz radiofrequency fields. The data acquisition time was typically 18 hours, although usable spectra could be obtained in 1-2 hours. Fast cross-polarization transfer rates, relatively long proton rotating-frame lifetimes, and high concentrations of protons in these biological samples ensure representative nmr intensities for all nitrogens (8). The one exception is the $^{15}$N signal from ammonium ion, where rapid internal molecular motion in the solid state reduces the dipolar coupling between nitrogens and protons. This reduces the proton-nitrogen cross-polarization transfer rate, thereby producing about a two-fold discrimination in detection under the conditions of our experiments. Technical details of the spinning and cross-polarization procedures have been presented elsewhere (9).

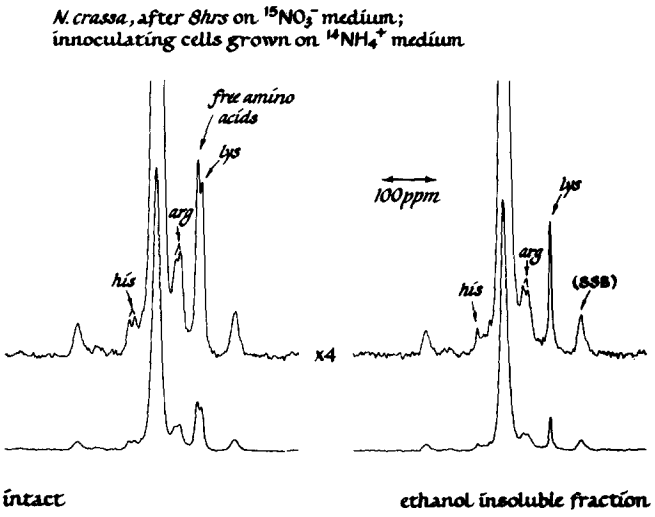


Figure 1. Magic-angle cross-polarization $^{15}N$ nmr spectra of lyophilized N. crassa mycelia, harvested after 8 hours in [$^{15}N$] nitrate medium, intact (left) and ethanol-extracted (right). Innoculating cells were grown in natural-abundance ammonia medium. The peaks at the extremes of the spectra are spinning side bands (SSB) arising from the 1.3-kHz mechanical sample spinning.

RESULTS: N. crassa harvested after 8 hours in a [$^{15}N$] nitrate induction medium gives rise to the solid-state cross-polarization $^{15}N$ nmr spectrum shown in Figure 1 (left). The major signal (100 ppm downfield from solid ammonium sulfate), is due to amide groups, predominantly in peptide linkages of proteins, with minor signals due mostly to lysine, arginine, and histidine side-chain nitrogens, as well as nitrogens in various free amino acids. Following extraction of the soluble components from this sample with 80% ethanol, over 90% of the protein amide signal is recovered, as well as substantial fractions of the amino-acid side-chain signals (Figure 1, right). The most obvious result of the extraction is the elimination of the resonance due to free amino acids. An ethanol extraction performed on a sample of N. crassa that had been induced for 2 hours in a [$^{15}N$] nitrate medium produced a similar result. Amino acid analysis of the ethanol-soluble extract of this 2-hr induced sample indicated that almost 90% of the amino

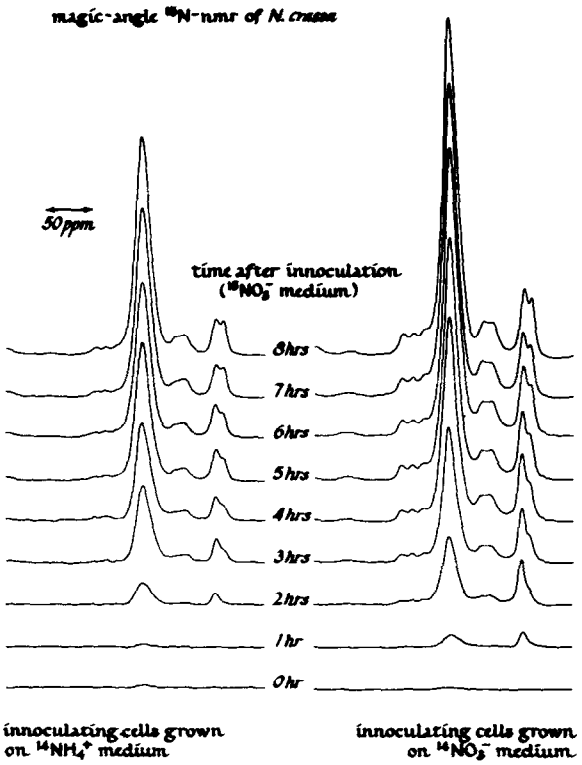


Figure 2. Magic-angle cross-polarization $^{15}$N nmr spectra of intact lyophilized N. crassa mycelia grown in [$^{15}$N] nitrate medium for various times after innoculation with cells grown in a natural-abundance ammonia medium (left) or nitrate medium (right).

acids present were comprised of alanine, serine, glutamic acid (or glutamine), and aspartic acid (or asparagine).

Figure 2 shows the cross-polarization $^{15}$N nmr spectra for N. crassa grown on either ammonia or nitrate media, and then transferred to [$^{15}$N] nitrate media and harvested on the hour for 8 hours. The spectra have all been normalized for differences in sample weight so that direct comparisons can be made. At zero time only a small natural-abundance $^{15}$N signal is observed. At later times, various peaks identified in Figure 1 begin to appear. The time development of these signals is different in the two experiments. In the case of N. crassa grown continuously on nitrate before being switched to [$^{15}$N] nitrate medium, rapid uptake of nitrogen is evident by the first hour. The partitioning of the nitrogen at that time is almost

equal between free amino acids and protein amide. By contrast, N. crassa initially grown on ammonia medium exhibits a spectrum 1 hour after innoculatio of the [$^{15}$N] nitrate medium that is identical to the natural abundance spectrum, indicating no appreciable uptake of nitrogen during this period. Other comparisons between the two sets of spectra are also possible but will not be detailed here.

A measure of the nitrate reductase specific activity of crude extracts made from the mycelia harvested in this time-course study showed that nitrate reductase was initially absent from mycelia grown on ammonia medium, grew to 360 units/mg after 2 hours, and was maximally induced (450 units/mg) by the third hour after innoculation of the [$^{15}$N] nitrate medium. Activity subsequently declined to a value at 8 hours of 230 units/mg. Mycelia grown continuously for 39 hours on nitrate retained 85 units/mg of activity when used for the innoculation of the [$^{15}$N] nitrate medium at zero time; specific activity then increased to a value of 320 units/mg two hours after innoculation, and remained near this level for the remainder of the experiment.

DISCUSSION: We have demonstrated the use of solid-state cross-polarization $^{15}$N nmr to follow directly and quantitatively the uptake and metabolism of [$^{15}$N] nitrate in N. crassa. Massive amounts of label appeared in newly synthesized protein and amino-acid components, but at no time did we observe signals for nitrate, nitrite, or ammonia in any of our samples, indicating that the in vivo concentrations of these species were below the detectability level of this experiment (100 µg $^{15}$N per g of cellular dry weight). As suggested by the results of conventional enzyme assays, we find that appreciable uptake and metabolism of nitrate, in fact, do not occur until high levels of nitrate reductase are reached. Since the amount of nitrate reductase which can be extracted from various tissues of higher plants varies with plant species, age, and cultural techniques (10),

accurate standard determinations of reductase activity are generally difficult to make. An alternate method using solid-state magic-angle N-15 nmr would be to observe directly the metabolism of [$^{15}$N] nitrate supplied to incubated or cultured intact tissue.

These solid-state cross-polarization $^{15}$N nmr experiments on lyophilized biological samples have the desirable feature that minimal biochemical manipulations are required for analysis. This eliminates the possibility of any loss or alteration of the materials being examined. Consequently, the technique may prove useful for those cases where extraction or isolation is particularly difficult, such as, for example, in studies of membrane-bound proteins.

Magic-angle spinning cross-polarization nmr experiments on *in vivo* functioning biological systems may also prove practical, at least for those organisms which metabolize relatively slowly, such as bacterial spores or plant tissue in cell culture. Cross-polarization signals will arise from those components of the cell which are completely, or partially immobilized. The technique therefore complements the use of Fourier transform $^{13}$C or $^{15}$N nmr to measure the flux of stable isotopes through the soluble pools of *in vivo* cellular systems (11, 12, 13).

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