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Communication

In Situ Nuclear Magnetic Resonance of ^{15}N Pulse Labels Monitors Different Routes for Nitrogen Assimilation[1]

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ABSTRACT

Nuclear magnetic resonance offers the possibility of noninvasive in situ observation of ^{15}N pulse labeling in the presence of light. In vivo, exclusively the δ-nitrogen of Gln is labeled in the cyanobacterium *Microcystis firma* when glutamate synthase is inhibited by azaserine. In contrast, the green alga *Chlorella fusca* is additionally capable of incorporating nitrogen into Glu, thus providing evidence for an anabolic function of glutamate dehydrogenase in this organism.

In 1974, Lea and Miflin (7) suggested that the GS/GOGAT[3] cycle was the main route of entry for ammonia in higher plants when they described a ferredoxin-dependent GOGAT (EC 1.4.7.1). Thereby, Gln, the initial organic N compound synthesized, should serve as the amino donor for the reductive amination of 2-oxoglutarate to yield two molecules of Glu. Although this proposition has been generally accepted, the function of the enzyme GDH (EC 1.4.1.2–4), that is known to be present in most living organisms (15) and that catalyzes the reversible conversion of 2-oxoglutarate, ammonia, and Glu still remains an open question. GDH may serve primarily as a catabolic enzyme (12); however, there is still controversy over whether it may have an anabolic function (11, 13).

Evidence for the routes of N assimilation with respect to photoautotrophic conditions has been based on the analysis of cell extracts for enzymes, amino acid labeling patterns, and inhibition and mutant studies (10). In vivo NMR studies of pulse labels under aerobic, dark conditions were reported for spruce buds (16) and cell suspension cultures of carrots (12). The aim of the present work was to develop further the in vivo ^{15}N NMR studies by following the assimilation of ammonia in photoautotrophic microorganisms under photosynthetic conditions.

MATERIALS AND METHODS

Plant Material

This study investigated the prokaryotic cyanobacterium *Microcystis firma* (Breb. and Lenorm.) Schmidle, strain Gromov/Len. 398, from the culture collection of the State University "A. A. Shdanow," St. Petersburg, Russia, and the eukaryotic green alga *Chlorella fusca* var *vacuolata* (Shih. et Krauss) strain 211-15 from the Pringsheim culture collection, Göttingen, FRG. Both organisms were cultivated as described elsewhere (*M. firma*, ref. 2; *C. fusca*, ref. 4).

^{15}N NMR Spectroscopy

Prior to the NMR measurements, the cells were harvested by centrifugation (5000*g*) and washed twice in buffer and once in the presence of 2.5 mM EDTA. Sodium phosphate buffer was used in the final resuspension (18 mM, pH 7.2, for *M. firma* and 20 mM, pH 6.8, for *C. fusca*). The cell densities used were between 5×10^8 and 6×10^{10} cells mL^{-1}. The in situ ^{15}N pulse labeling was performed by adding 20 mM $^{15}NH_4Cl$ to the medium of the cells in the NMR tube. Inhibitors were added 30 min prior to measurements.

Spectra were obtained using a 20-mm broadband probe on a Bruker AM 360 spectrometer at a frequency of 36.5 MHz with ^1H broadband decoupling. Free induction decay values were recorded in blocks of 900 transients over periods of several hours using a flip angle of 60° and an overall repetition time of 2 s. The spectra shown represent accumulation times between 0.5 and 9.5 h. An exponential line broadening of 10 Hz was applied prior to Fourier transformation. Further conditions were as described previously (2). Chemical shifts were referenced to 1 M $H^{15}NO_3$ in D_2O using urea as an external standard, which varied in concentration among the experiments. During the measurement, between

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[3] Abbreviations: GS, glutamine synthetase; GOGAT, glutamate synthase; GDH, glutamate dehydrogenase; GABA, γ-aminobutyric acid; MSO, methionine-S-sulfoximine; MSX, methionine-*R,S*-sulfoximine.

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acquisition times, the cells were sparged with 95% N_2/5% CO_2 and were constantly supplied with light sufficient to support photosynthesis from an illumination system (500 W m^{-2}) as described before (4).

RESULTS

At first, an in situ ^{15}N pulse-labeling experiment was performed under standard conditions, i.e. without application of inhibitors, by adding 20 mM $^{15}NH_4Cl$ to the medium of the cells in the NMR tube. The high level of ammonia has been shown to affect adversely neither the intracellular pH nor the energy status of cells of *M. firma* (2) and *C. fusca* (18) for several hours. Figure 1a shows a spectrum of *M. firma* as a summation of a recorded time course (9.5 h) monitoring the ^{15}N incorporation by the cells at a time resolution of 30 min (spectra not shown). Ala, Arg, Gln, acid amides, and possibly the nonprotein amino acid GABA were detected.

GABA occurrence in plant tissues has been interpreted as a stress response (14). However, the signal at −342 ppm assigned as GABA may in fact contain contributions from ornithine, lysine, and possibly polyamines such as putrescine (2, 16). Moreover, there is no reason to believe that this signal is due to a physiologically unfavorable status, because it has been detected under various conditions in several photoautotrophic microorganisms (2, 6, 19). In addition, experiments with prolonged N deficiency did not give rise to changes in this signal (R. Altenburger, unpublished results).

The ammonium ion signal, resonating at −354 ppm, is not presented in these spectra because it underwent zero transition in intensity due to loss in nuclear Overhauser effect enhancement. This effect has already been described (2, 5),

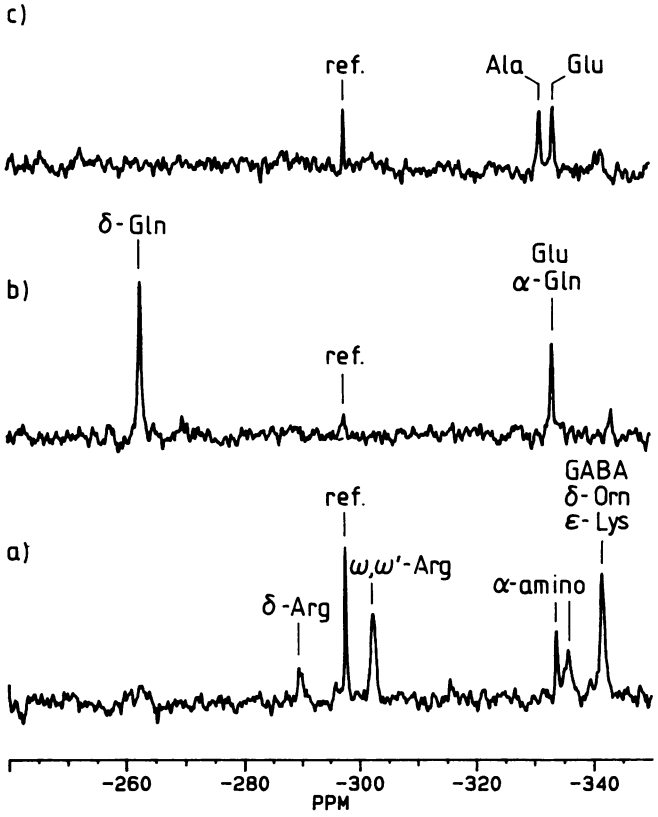

Figure 2. ^{15}N NMR spectra of the green alga *C. fusca*. a and b, Conditions as in Figure 1, a and b, respectively; c, application of 20 mM $^{15}NH_4Cl$, 5 mM MSO, and 0.2 mM azaserine.

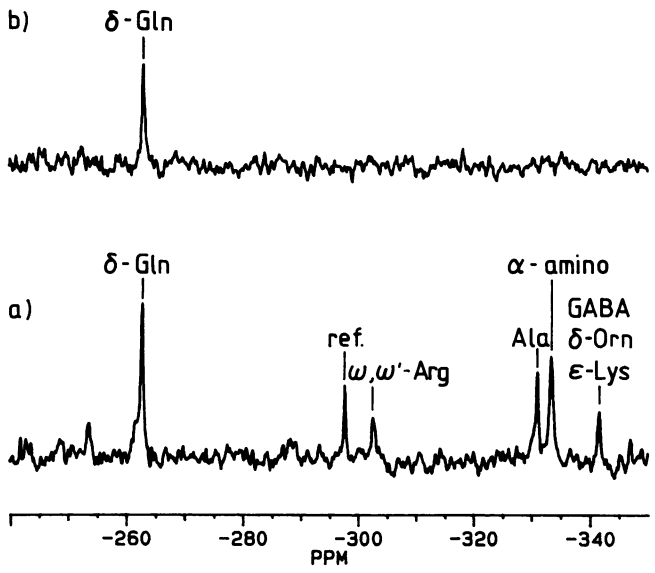

Figure 1. ^{15}N NMR spectra of the cyanobacterium *M. firma*. a, Labeling was performed in the NMR tube (in situ) by application of 20 mM $^{15}NH_4Cl$; b, application of 0.2 mM azaserine and 20 mM $^{15}NH_4Cl$ in situ. During the measurement, cells were sparged with 95% N_2/5% CO_2 and illuminated. The chemical shifts are referred to 1 M $H^{15}NO_3$ in D_2O as 0 ppm using urea as an external standard.

and its consequences for measurements of uptake and assimilation will be discussed separately (L. Walter et al., manuscript in preparation). However, in the experiments presented here, we detected no signal from intracellular ammonia, as has been observed for other conditions by Walter et al. (manuscript in preparation). Thus, we may assume, in accordance with Altenburger et al. (2), that the amount of intracellular ammonia does not even reach the level of steady state, which for these conditions and a 20 mM ammonia pulse would be around 3 mM. To test ^{15}N incorporation through pathways other than the GS/GOGAT cycle, cells were exposed to the transaminase inhibitor azaserine at 0.2 mM 30 min prior to the addition of ammonia to evoke inhibition of GOGAT. Under these conditions (see Fig. 1b), *M. firma* is able to incorporate ^{15}N from ammonia only into the δ-nitrogen position of Gln. This is as expected if the GS/GOGAT cycle is the exclusive assimilation route. The corresponding experiments were also performed with *C. fusca*. Figure 2a shows the in situ ^{15}N pulse-labeling spectrum without enzyme inhibition. After exposure to azaserine (Fig. 2b), *C. fusca* shows an additional capability for incorporating $^{15}NH_4^+$ compared with *M. firma*, revealed by peaks at −333.5 and −341.7 ppm in the spectrum.

The signal at −333.5 ppm may originate from the amino nitrogen of either Glu or Gln, which cannot be resolved in the in vivo ^{15}N NMR spectra. To distinguish between these

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possibilities, NMR spectra of *C. fusca* were recorded under combined exposure to MSO, an inhibitor of GS (8), and to azaserine during ammonia assimilation. With an MSO concentration of 5 mM, growth and reproduction of the organism are reduced by 50% (1). The resulting spectrum, shown in Figure 2c, contains no signal from the δ-nitrogen of Gln, thus demonstrating that GS was inhibited. The experiment with MSO alone, applied at its EC_{50}, also showed no signal from the δ-nitrogen of Gln, but did from two α-amino signals at −334.2 and −333.5 ppm (L. Walter, unpublished results). Hence, the signal at −333.5 ppm does not derive from the α-nitrogen of Gln, but from Glu, demonstrating the presence of an alternative route of assimilation for ammonia in *C. fusca*. The enzyme most likely to be responsible for the production of Glu from ammonia and 2-oxoglutarate is GDH.

DISCUSSION

Apparently, the main route for ammonia assimilation is the GS/GOGAT pathway in cyanobacteria (3, 9). However, substantial activities of other enzymes of ammonia incorporation, like GDH and alanine dehydrogenase, cannot be excluded, as the short-term studies by Meeks et al. (9) showed using the radioactive isotope ^{13}N. Our results for *M. firma* are in agreement with the suggestion that the GS/GOGAT cycle is the major route of ammonia assimilation. Moreover, they demonstrate that no ammonia incorporation through GDH takes place over an extended period of time at a high extracellular ammonia level, even when GS is inhibited.

For *Chlorella sorokiniana*, Tischner (17) gave evidence for a possible participation of a NADP-dependent GDH in ammonia assimilation when ammonia is added into cultures growing in nitrate, or when GS has been inhibited by MSX. Both treatments induced de novo synthesis of two isoenzymes of GDH. Values of K_M for ammonia were found to depend on the NADPH concentration, with the lowest K_M value being nearly 10^3-fold lower than usually reported. Considering that obtaining these data required assaying the enzymes in vitro, our results from *C. fusca* in vivo provide an important contribution with respect to the role of GDH for ammonia incorporation. The data presented here show unambiguously that GDH may have an anabolic role in ammonia assimilation in the green alga *C. fusca* under appropriate circumstances. This is true in the case of GS inhibition by MSO, and also when GS is active but GOGAT is inhibited. Because a signal from Glu was recorded between 0.5 and 1 h after application of $^{15}NH_4Cl$ (data not shown), there is no evidence for a lag phase of GDH activity, as has been seen for *C. sorokiniana* (17).

CONCLUSIONS

The main conclusions drawn from this work are: (a) NMR of ^{15}N pulse labels in situ under photosynthetic conditions is possible for plant systems, opening new possibilities for monitoring physiological processes by ^{15}N NMR in vivo, and (b) the first applications of this technique demonstrate that pathways of nitrogen assimilation differ between the green alga *C. fusca* and the cyanobacterium *M. firma*.

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