In Vivo NMR at 800 MHz to Monitor Alkaloid Metabolism in Plant Cell Cultures without Tracer Labeling

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Received October 6, 2000
Revised Manuscript Received April 5, 2001

The delineation of biosynthetic pathways is usually achieved by feeding experiments with radioactive isotopes, stable isotope incorporation or enzymatic investigations. These methods require the isolation and identification of metabolic intermediates and end products after destruction of the living cell. The results so obtained do not necessarily reflect the real in vivo situation, because there is the risk of artifact formation during the isolation procedures. Therefore, many attempts have been made in the past to apply NMR spectroscopy in living cells, especially in plant cells,[1−3] as a noninvasive method to elucidate metabolic events. Many of the in vivo experiments used ^{31}P NMR[4−6] and ^{15}N NMR[7] or in some cases ^{1}H NMR.[8] However, ^{13}C was the more commonly applied isotope, taking advantage of ^{13}C labeling[9−12] to circumvent the low sensitivity. To further enhance the sensitivity inverse correlated gradient assisted ^{1}H−^{13}C spectroscopy ((ga)HSQC) was applied.[13] A few investigations have, however, demonstrated that in vivo ^{13}C NMR, even at its low natural abundance, can be used to determine the subcellular distribution of amino acids[14] or to follow the glucosylation of some phenolic compounds in plant cells.[15,16]

Herein we report on the metabolism and biosynthesis of indolic compounds in plant cell suspension cultures followed by ^{1}H and ^{1}H−^{13}C (ga)HSQC in vivo NMR at 800 MHz. The results show that such high-field NMR techniques provide the sensitivity to follow particular metabolic reactions with unlabeled substrates thus coming close to the range of physiological concentrations.

Cell cultures of the Indian medicinal plant *Rauvolfia serpentina* Benth. ex Kurz were grown under standard conditions (LS-Medium;[17] 300 mL Erlenmeyer flasks, 100 rpm, 25 °C, diffuse light ~600 lux). Five days after inoculation 1.5 mL of the cell suspension was transferred to a 5 mm NMR tube and an isatin solution (1 mg in 150 μL of D_2O/MeOH 1:1) was added (4 mM) and immediately measured. Figure 1 depicts the first ^{1}H spectrum, recorded after 7 min of isatin addition (t = 0 min) followed by five additional measurements. Each spectrum was obtained after only 2 min (32 scans) measuring time, demonstrating the excellent sensitivity of the method. Figure 1 also illustrates by the signals in the aromatic region (6.6−7.6 ppm) that a fast reduction of isatin (IS) to dioxindole (D) occurs in vivo and is thus catalyzed by the *Rauvolfia* cells. It should be noted that the reaction in the 5 mm NMR tube is not complete due to a lack of shaking during the experiment. This rapid transformation is followed by a much slower process, which is the glucosylation of the reduction product dioxindole. This conversion could be followed over days, when cells from a larger feeding experiment were taken sequentially and measured by (ga)HSQC. Each measurement lasted 20 min (4 scans) only. After 1 h all the isatin was reduced under these conditions to dioxindole. Only four cross-peaks (**D 7** 6.83/111 ppm; **D 5** 6.96/123 ppm; **D 6** 7.17/130 ppm, **D 4** 7.26/125 ppm) appear in the aromatic region, corresponding to the signals of dioxindole (see also Figure 1). Much later (after 48 h) a new cross-peak **DG 4** appears at 7.32/127 ppm indicating about 50% conversion into the dioxindole-*O*-β-D-glucoside (DG). The other three aromatic cross-peaks of D and DG almost overlap, which leads to a broadening of the signals (data not shown). After 96 h the glucosylation was nearly completed. On one hand this result is additionally supported by the decrease of signal **D 3** (~4.93/70 ppm) for carbon 3 and increase of the appropriate signal of the glucoside **DG 3** (5.13/76 ppm) in the same period of time (data not shown). On the other hand the signal for the anomeric carbon **DG 1′** of the formed glucoside also increased at 4.20/103 ppm (data not shown). These changes in at least four signals can

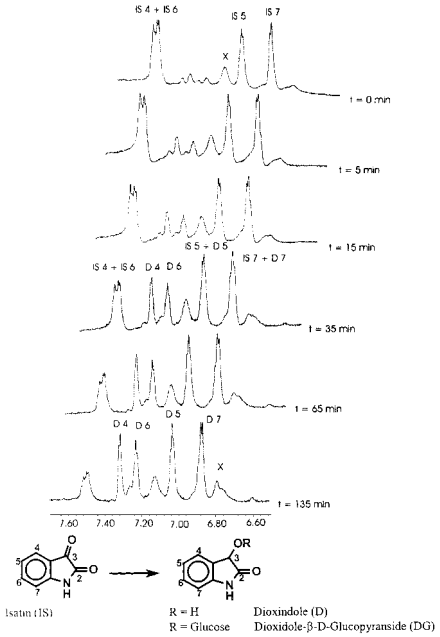

Figure 1. Time course of the reduction of isatin (IS) to dioxindole (D) shown by 800 MHz in vivo ^{1}H NMR spectroscopy. All experiments were recorded on a Bruker AVANCE 800 spectrometer equipped with a 5 mm TXI ^{1}H−^{13}C/^{15}N−D XYZ-GRD probehead. For water suppression the watergate pulse program was applied.[18,19] Acquisition time 1,64 s; recycle time 3,64 s and recovery delay 0.1 ms. X = background signal.

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10.1021/ja003613k CCC: $20.00
Published on Web 05/08/2001

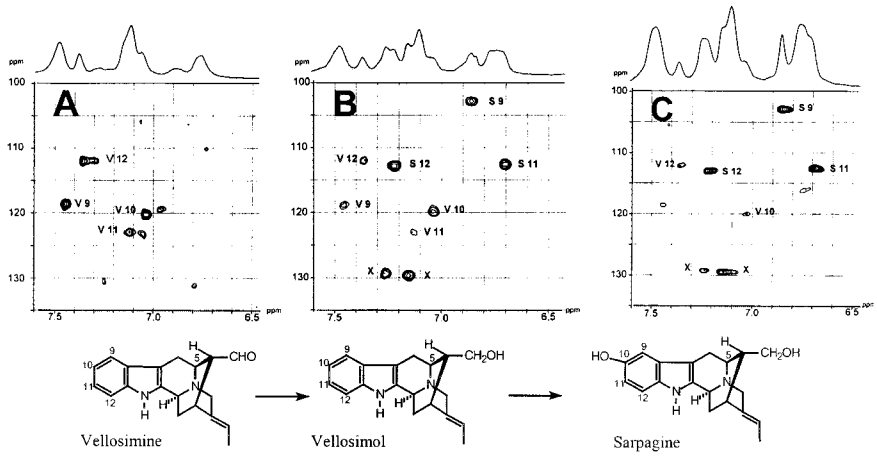

Figure 2. Spectra A, B, and C show the aromatic region 1 h, 48 h, and 4 d after addition of vellosimine to *Rauvolfia* cell suspension recorded by 2D inverse correlated $^1H-^{13}C$ NMR spectroscopy. Pulse sequence was a $^1H-^{13}C$ correlation with a double inept transfer using sensitivity improvement with echo/antiecho processing (invietgpsi) with a QSINE window function of 2 in both dimensions and a linear prediction in f1 of 256 data points.[21−23] GARP ^{13}C decoupling was applied during the acquisition time. The experiments were acquired with 128 increments in f1 and 8 scans (75 min, recovery delay 0.1 ms, acquisition time 0.0512 s, and a recycle time of 2.0512 s). The gradient ratio was 80:20:80. Abbreviations used were V = Vellosimol (=10-desoxysarpagine); S = Sarpagine; X = unknown metabolites.

easily be used to identify the in vivo process as being glucosylation (see Supporting Information). Analysis of the surrounding nutrition medium (by NMR and HPLC) provided clear evidence that glucoside biosynthesis takes place exclusively inside the plant cells.

Whereas this example of in vivo NMR describes the metabolism of an unnatural indolic compound, the subsequent conversion of the monoterpenoid indole alkaloid vellosimine into vellosimol (10-deoxysarpagine) and its further hydroxylation to sarpagine verifies a biosynthetic sequence of alkaloids of the sarpagan class in *Rauvolfia* cells. In the course of this pathway the alkaloid vellosimine is formed by epimerization of epi-vellosimine. The latter is a direct intermediate of the ajmaline biosynthetic pathway.[20] Feeding vellosimine as a CD_3OD/D_2O solution to the *Rauvolfia* cell suspension (final concentration ~1 mM) led to a rapid reduction of the aldehyde group within less than 1 h. In this case the formation of vellosimol could be followed only by disappearance of the aldehyde signal at 9.4 ppm (data not shown), because the aromatic signals of the precursor vellosimine and of its reduction product overlap. After prolonged feeding and sequential analysis of the plant cells by (ga)HSQC (as described above), the intensity of the four aromatic signals of vellosimol decreased whereas only three novel cross-peaks appear (Figure 2 A,B,C). This observation strongly suggested a substitution at the aromatic ring, preferentially a hydroxylation because of the high field shift of H9, H11, and H12. When authentic sarpagine was measured in D_2O, the three aromatic signals showed the identical shift as observed in the in vivo experiment, indicating that *Rauvolfia* cells indeed biosynthesized sarpagine and that deoxysarpagine in fact serves as its immediate biosynthetic precursor.

The findings also demonstrate that ^1H NMR at 800 MHz, which is the highest magnetic field strength currently used, can be applied to monitor fast in vivo metabolic reactions (<135 min) catalyzed by plant cell cultures. When precursor concentrations are in the range of ≤4 mM, only 2 min of measuring time are sufficient to obtain well-resolved spectra. Moreover, (ga)HSQC could successfully be used to observe much slower conversions such as glucosylation and hydroxylation. The relatively high sensitivity of this method allowed us for the first time to follow a short biosynthetic sequence of sarpagine alkaloid biosynthesis at the natural abundance of ^{13}C directly in the living plant cell. Since the final concentration of the precursor alkaloid was 1 mM and at least two compounds can be seen in one spectrum (Figure 2B,C) this method is sensitive enough to noninvasively detect conversions of substances of which the concentration are ~500 μM.

The in vivo NMR method that we describe here is not as sensitive as the routinely used method of applying highly ^{13}C-enriched precursors. However, it is the simplest and most straightforward approach currently available when compared to any other biosynthetic technique. Furthermore, it leads to the immediate identification of products.

In conclusion, in vivo NMR methodologies are ideally suited for biosynthetic pathways that proceed rather slowly, step by step with sufficient accumulation of intermediates, which is frequently the case for the natural products of plant cell cultures. We will be able to significantly increase the sensitivity of the method in the future by the combination of higher fields (900 MHz) and the use of 8 mm probe heads and cryo probes. The disadvantage of signals overlapping especially in the aliphatic region is due to endogenous primary metabolites such as sugars and amino acids. Therefore, further work will concentrate on avoiding these "background signals" by changing the physiological conditions of cell growth. Our aim is to identify biosynthetic intermediates much faster and accurately by revealing a more complete set of signals. Further advances in high-field NMR techniques for in vivo analyses such as we have presented here will undoubtedly soon bring new perspectives with respect to the delineation of cellular biosynthetic pathways and metabolism.

Acknowledgment. Dedicated to Professor Burchard Franck on his 75th birthday. The technical assistance of M. Lucci (Florence, Italy) is highly acknowledged. We also thank the EU (Access to Research Infrastructures Action of Improving Human Potential Programme, Contract No. HPRI-CT-1999-00009), the Deutsche Forschungsgemeinschaft (Bonn, Germany), and the Fonds der Chemischen Industrie (Frankfurt/Main, Germany) for providing financial support. We acknowledge support provided by Profs. Dr. I. Bertini and C. Luchinat (Florence, Italy) and thank Dr. Denis Strand (Mainz) for linguistic advice.

Supporting Information Available: (ga)HSQC spectra of the transformation of D into DG (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

JA003613K

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