Double-quantum ^1H NMR studies of nitrogen metabolism in yeast

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Using double-quantum ^1H NMR spectroscopy, the nitrogen metabolism of yeast cells pulse-labelled with ^{15}N-enriched ammonium sulfate has been detected. The sensitivity of this indirect method is far higher than direct observation of the ^{15}N signals by ^{15}N NMR. Hence, a spectrum with an excellent signal-to-noise ratio was obtained in 25.6 s. The observed proton signals were assigned to specific metabolites using two-dimensional reverse shift correlation spectra.

^1H-NMR *In vivo NMR* *Nitrogen metabolism* *Yeast metabolism* *2D NMR*

1. INTRODUCTION

In the last years, there has been an increasing interest in studying the in vivo nitrogen metabolism in bacteria [1], fungi [2] and yeast [3] using high resolution ^{15}N NMR spectroscopy. The use of ^{15}N NMR spectroscopy for in vivo studies is difficult due to the low NMR sensitivity and natural abundance of the ^{15}N nucleus. The experimental difficulty arising from the low sensitivity may be overcome by using double quantum NMR spectroscopic techniques. From heteronuclear shift correlation experiments it is known that in principle, the detection of the nucleus with the highest magnetogyric ratio offers the highest sensitivity [4,5]. Recently, several schemes for selectively detecting protons bound to ^{13}C [6] or ^{15}N [5] have been suggested. The selective detection is generally achieved by alternate addition and subtraction of spectra which differ only in the phase of the heteronucleus-bound protons.

Here we present a modification of the double-quantum NMR technique to study the nitrogen metabolism in yeast and the application of two-dimensional reverse-correlated ^1H-^{15}N spectroscopy to identify the resonances observed in the ^1H NMR spectrum.

2. METHODS

Ammonium sulfate (95% ^{15}N-enriched) was obtained from Rohstoff Einfuhr GmbH (Düsseldorf). All other chemicals were AR grade.

Commercially available baking yeast (*Saccharomyces cerevisiae*, Deutsche Hefe Werke, Hamburg) was cultured at 30°C in the medium described in [9].

The cells were harvested by low-speed centrifugation at 5°C and were resuspended in the same medium after addition of 50 mg $(^{15}NH_4)_2SO_4$ per 100 ml medium. At this time the cell density was about 10^9 cells/ml. After 90 min incubation at 30°C in a rotating shaker, the cells were collected by centrifugation and washed once with chilled water. The cells were pelleted by centrifugation and transferred to a 5 mm o.d. NMR tube; 0.05 ml D_2O were added to provide a lock signal. The viability of the cells before and after the NMR experiments was checked by cell counting and plating, no significant loss of viability being detected.

The NMR measurements were conducted at 500 MHz on a Bruker AM 500 spectrometer. A sweep-width of 15 151 Hz was used, transients were collected into 2 K data points with an acquisi-

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tion time of 0.068 s and a relaxation delay of 0.032 s. Usually, 256 scans were collected, the total accumulation time being 25.6 s. Cold nitrogen gas was blown through the probe continuously to keep the temperature close to 20°C. Chemical shifts are referenced to external TSP (3-trimethylsilyl-2,2,3,3-tetradeutero sodium propionate).

The pulse-sequence suggested in [5] was modified so as to include the ^1H pulse scheme developed in [7] to reduce the intensity of the water proton signal. The following pulse sequence was used:

$$90° (^1H,x)\text{–}D1\text{–}90° (^1H,y)\text{–}D2\text{–}90°$$

$$(^{15}N, PH1)\text{–}90° (^{15}N, PH2)\text{–}D2\text{–AQ (PH3)}$$

where $D1$ is a suitable delay for the water signal suppression (about 0.00014 s), and $D2$ is a fixed delay of 0.0056 s corresponding to $(2J)^{-1}$ for the N–H bonds concerned. The delay $(2J)^{-1}$ allows the selection of compounds according to the specific $^1J_{N-H}$ coupling constant; x and y define the axes along which the corresponding pulse is being applied. The phase cycling used to cancel all non-^{15}N-coupled proton signals is the following [5]:

PH1	PH2	PH3
x	x	x
x	$-x$	$-x$
x	y	y
x	$-y$	$-y$

For the two-dimensional reverse-correlated experiment, this sequence was modified to include in between the two ^{15}N pulses two incrementable delays τ and a 180° ^1H refocusing pulse:

$$\cdots{}^{15}N\text{–}\tau\text{–}180° (^1H)\text{–}\tau\text{–}{}^{15}N\text{–}\cdots$$

The spectral width along the ^{15}N axis was 500 Hz; 40 experiments with 256 scans each were recorded in 17 min total accumulation time. During this time period and under the conditions described no gross changes in the ^{15}N NMR spectrum were observed.

3. RESULTS

^1H NMR spectra of a *S. cerevisiae* cell suspension pulse-labelled with ^{15}N-enriched ammonium sulfate are shown in fig.1. Only the spectral region

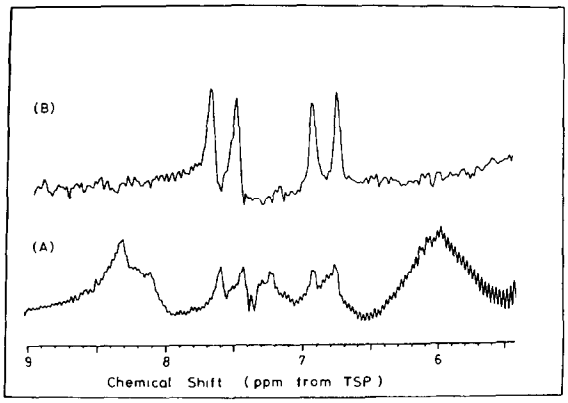

Fig.1. 500 MHz ^1H NMR spectra of a *Saccharomyces cerevisiae* suspension pulse-labelled with $(^{15}NH_4)_2SO_4$, (A) using a water signal suppression scheme, (B) using additionally a double-quantum filter. See text for experimental details.

downfield of the water signal is depicted. Spectrum (A) was measured using only the water signal attenuation scheme presented in [7]. Spectrum (B) was obtained with the double-quantum pulse sequence described in section 2. Spectrum (A) exhibits some broad components, a few sharper lines being better resolved. In spectrum (B), only two doublets with a $^1J_{N-H}$ coupling constant of 87 Hz and centered at 7.63 and 6.90 ppm are observed.

To assign these resonances, a reverse-correlated two-dimensional spectrum was measured, a section of which is shown in fig.2. Evidently, both doublets are correlated with one nitrogen nucleus only, which has a chemical shift value of 92.7 ppm downfield of 4 M NH_4Cl in 2 M HCl. The amide nitrogen of glutamine has been observed to resonate at this position [1–3]. Hence, the doublets in the ^1H NMR spectrum are due to the two amide protons of glutamine. Due to the strong double-bond character of the C–N link, the free rotation of the $-NH_2$ group is severely hindered and, therefore, the magnetic effect of the neighbouring carbonyl group renders both amide protons anisotropic. This assignment is additionally supported by ^{15}N NMR spectra of the same cell suspension obtained with selective ^1H-decoupling at 7.63 and 6.90 ppm. In both experiments, instead of the usual glutamine amide ^{15}N triplet signal, a doublet is observed [3].

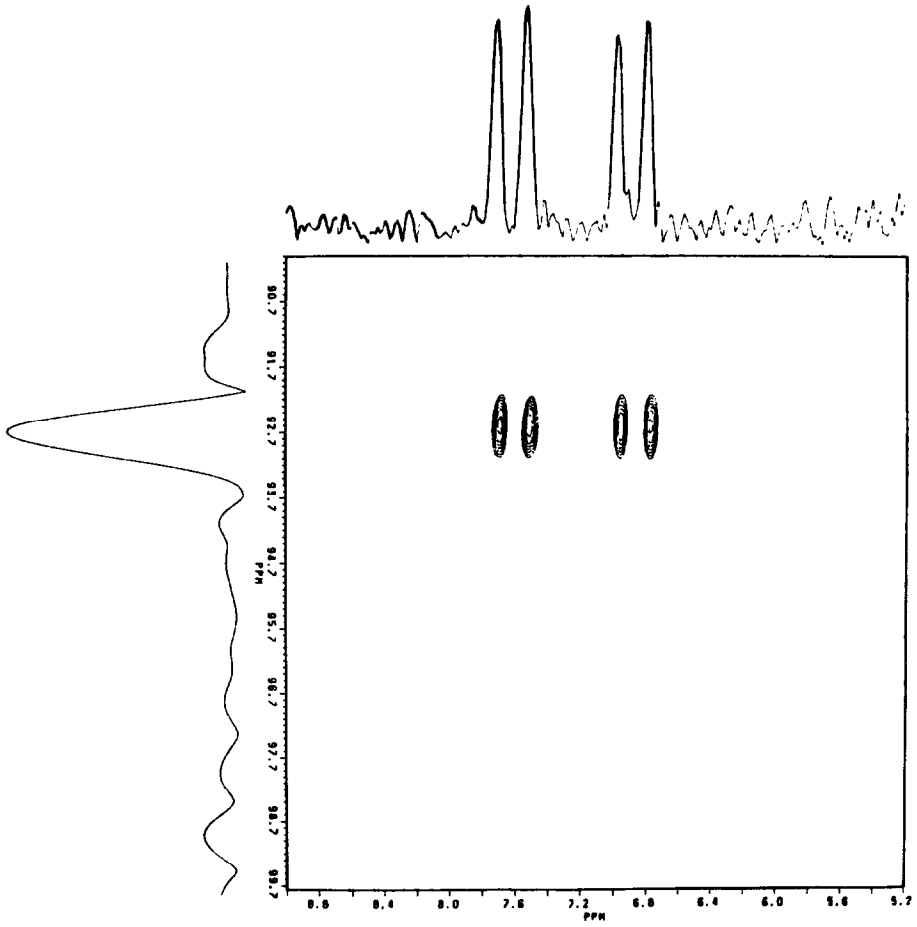

Fig.2. Contour plot of a 2D-reverse-correlated spectrum of a *S. cerevisiae* suspension to assign the observed ^{1}H signals through the ^{15}N NMR spectrum. Horizontal and vertical slices through the contour plot at the position of the signals are also shown. Experimental details, see text.

4. DISCUSSION

The detection of protons attached to ^{15}N in water solution relies on the reduction of the water signal intensity by a suitable pulse sequence and by a double-quantum filter. Suppression of the water signal by saturation is not advisable, as due to chemical exchange the saturation will be transferred to the exchangeable protons bound to nitrogen.

In fact, the characteristic exchange of nitrogen-bound protons with water protons limits the applicability of this method to those molecules in which this exchange is slow enough so as not to reduce the size of the $^{1}J_{N-H}$ coupling constant drastically. Thus, no amino group proton of the several amino acids formed under the experimental conditions was detected [3].

Glutamine plays, together with glutamic acid, a crucial role in assimilating ammonia and in acting as a nitrogen donor for the synthesis of other metabolites. Additionally, glutamine is possibly involved in the regulation of other nitrogen-related enzymes [8].

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