Available online at www.sciencedirect.com
ScienceDirect
Metabolic Engineering 9 (2007) 252–257
METABOLIC ENGINEERING
www.elsevier.com/locate/ymben

Application of 2D-TOCSY NMR to the measurement of specific ^{13}C-enrichments in complex mixtures of ^{13}C-labeled metabolites

Stéphane Massou[a,b,1], Cécile Nicolas[a,1], Fabien Letisse[a,b], Jean-Charles Portais[a,b,*]

[a]*MetaSys, Laboratoire d'Ingénierie des Systèmes Biologiques et Procédés, UMR INSA/CNRS 5504 INRA 792, LBB/INSA, 135 Avenue de Rangueil, 31077 Toulouse Cedex 4, France*
[b]*Université Paul Sabatier, 118 Route de Narbonne, 31062 Toulouse Cedex, France*

Received 1 February 2007; received in revised form 2 March 2007; accepted 5 March 2007
Available online 16 March 2007

Abstract

A 2D-NMR method based on zero-quantum filtered (ZQF-) TOtal Correlation SpectroscopY (TOCSY) was applied to measure ^{13}C-enrichments in complex mixtures of ^{13}C-labeled metabolites generated in carbon-labeling experiments. Using ZQF-TOCSY, more than 30 ^{13}C-enrichments could be potentially measured from the analysis of a biomass hydrolyzate prepared from *Escherichia coli* cells grown on a mixture of 20% [U-^{13}C]-glucose and 80% [1-^{13}C]-glucose, without need for separation of metabolites. The method is applicable to biomass hydrolyzates, cell extracts, and other complex biological samples. It is also applicable to any combination of labeled substrates and provides a basis for examining non-steady-state conditions.

Keywords: Fluxomics; Quantitative 2D-NMR; ^{13}C-labeling experiments; Complex biological mixtures

1. Introduction

NMR combined with ^{13}C-labeling strategies has been increasingly used over the past decade to measure metabolic fluxes in living systems (Kelleher, 2001; Ratcliffe and Shachar-Hill, 2005; Shulman and Rothman, 2001; Wiechert et al., 2001a, b). Metabolic fluxes are estimated from the distribution of label within metabolites extracted from biological material (cells, tissues or organisms) incubated with ^{13}C-labeled substrate(s). NMR is unique in providing direct access to the amount of ^{13}C atom incorporated in individual carbon positions of metabolites. This makes NMR a reliable method for monitoring the fate of specifically labeled substrates—e.g. [1–^{13}C]-glucose—in biological systems. In such experiments, the carbon positions of metabolites that receive the label provide information on the structure of the metabolic network, and the flux distribution can be obtained from the amounts of ^{13}C incorporated therein. This approach can be potentially applied to a wide range of conditions and biological systems, but despite some interesting attempts (Carvalho et al., 1998; de Graaf et al., 2000; Schmidt et al., 1999), its application suffers from the lack of reliable 2D-NMR method for measuring ^{13}C-specific enrichments in complex mixtures. The accurate determination of ^{13}C enrichments in individual carbon positions usually requires the purification of metabolites, from which 1D ^{13}C- and ^{1}H NMR spectra can be recorded properly (Eisenreich et al., 2006; Portais et al., 1993; Rontein et al., 2002), a tedious process that has limited so far a wider application of this approach.

TOtal Correlation SpectroscopY (TOCSY) is a widespread 2D-NMR experiment that potentially enables the measurement of specific enrichments in complex mixtures. But standard TOCSY experiments contain signal distortions originating from zero-quantum coherences that prevent the accurate quantification of isotopic enrichments. Here, we have applied a zero-quantum filtered (ZQF-) TOCSY experiment to measure ^{13}C enrichments in complex mixtures, i.e. without need for separation of metabolites. It is based on the experiment developed by

*Corresponding author. MetaSys, Laboratoire d'Ingénierie des Systèmes Biologiques et Procédés, UMR INSA/CNRS 5504 INRA 792, LBB/INSA, 135 Avenue de Rangueil, 31077 Toulouse Cedex 4, France.
E-mail address: jean-charles.portais@insa-toulouse.fr (J.-C. Portais).
[1]Equal contribution of both authors.

1096-7176/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.ymben.2007.03.001

Thrippleton and Keeler (2003) to suppress zero-quantum coherences. The reliability of ^{13}C-enrichment determination by ZQF-TOCSY was demonstrated from the analysis of standard solutions of labeled glucose. It was further applied to a complex mixture of proteinogenic amino-acids prepared from *Escherichia coli* grown on labeled glucose.

2. Materials and methods

2.1. NMR spectroscopy

All 1D and 2D-NMR spectra were recorded on an Avance 500 MHz spectrometer (Bruker) using a 5 mm *z*-gradient TBI probe. The data were acquired and processed using XWINNMR 3.5 software. The temperature was 298 K.

Proton spectra were acquired by using a 30° pulse, 2500 Hz (glucose samples) or 5000 Hz (all other samples) sweep width and 3.3 s acquisition time. A total of 64 scans were recorded and the relaxation delay between scans was 30 s to have full signal recovery.

The 2D ZQF-TOCSY pulse sequence used in this study (Fig. 1) was derived from the sequence developed by Thrippleton and Keeler (2003) to suppress zero-quantum interferences. It was slightly modified to measure ^{13}C enrichment by introducing a ^{13}C 180° refocusing pulse during t1 evolution to remove heteronuclear scalar coupling in the indirect dimension. DIPSI-2 TOCSY mixing sequence was applied during 25 or 50 ms (8.3 kHz). The adiabatic pulses were 180° CHIRPs with 10% smoothing and an adiabatic factor of 3. The frequency was swept through 20 kHz in 50 and 30 ms for the first and the second pulses, respectively. A squared 0.2 G/mm gradient pulse was applied during the total length of the pulses. At the end of the mixing period, in-plane magnetization was defocused by a 2.6 G/mm squared purge gradient pulse. Two-dimensional spectra were obtained with quadrature phase detection in both dimensions using TPPI in the indirect dimension. For each 512 increments in the F1 dimension, eight transients were accumulated with the same sweep width, relaxation delay (5 s), and acquisition time than the 1D spectra. The total acquisition time was 16 h. The conventional 2D-TOCSY was recorded with the same parameters except for the zero-quantum filters that were suppressed.

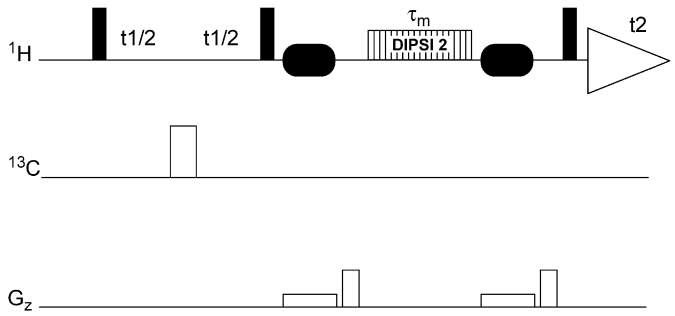

Fig. 1. Two-dimensional-TOCSY pulse sequence. Quantitative TOCSY experiment was obtained by application of a DIPSI-2 mixing sequence in combination with a refocusing 180° ^{13}C pulse during t1 evolution. See text for details.

Before Fourier transformation, 1D FIDs were not weighed but were zero filled to 32k complex points. Spectrum baseline correction was applied before 1D integration was carried out. For the 2D spectra, a $\pi/2$ shifted square sinebell function was used in the indirect dimension. The final spectrum matrix size was 32k × 512 complex points. To perform 2D quantification, a 1D pseudo-spectrum was created by summation of rows containing the cross-peaks of interest and was integrated like a normal 1D spectrum.

2.2. Standard sample preparation

Ten samples containing various combinations of unlabeled—i.e. ^{13}C at natural abundance—and [1-^{13}C]-glucose (Eurisotop, France) ratio were prepared. The total amount of glucose was 20 mg and the enrichment percentages were 1.1% (natural abundance), 2.5%, 3.5%, 5%, 6%, 10%, 25%, 50%, 75% and 90%, respectively. To avoid systematic errors due to solution stock dilution, each sample was weighted independently. Labile protons were exchanged with deuterium by lyophilizing twice in 2 ml D_2O 99.9% (Eurisotop, France) and the final sample was suspended in 600 µl of D_2O.

2.3. Biomass sample preparation

E. coli MG1655 was grown on a minimal medium containing 80% of [1-^{13}C]-glucose and 20% of [U-^{13}C]-glucose at a total concentration of 2 g/l. Batch cultures were performed at 37 °C in a 2 l stirred tank with a total partial pressure of oxygen maintained over 20% and pH was kept constant at 6.9 by addition of NaOH. Steady-state cells ($OD_{600\,nm} = 1$, total biomass content: 200 mg DCW) were harvested by centrifugation and hydrolyzed in 6 M HCl at 105 °C for 24 h. The hydrolyzate was vacuum-dried and resuspended in 600 µl of 20 mM DCl before the NMR measurements were carried out.

3. Results and discussion

In this paper, the term 'specific ^{13}C-enrichment' will refer further to the amount of ^{13}C-label incorporated in a specific carbon position of a metabolite.

3.1. 2D-TOCSY NMR

TOCSY is a widespread NMR experiment where the magnetization of a spin A can be transferred to a spin B linked to A by—mainly—scalar coupling (Bax and Davis, 1985). In 2D ^{1}H–^{1}H TOCSY experiments, correlation peaks—or cross-peaks—between two protons A and B are observed outside the diagonal, where the resonance of A is