Resolution of H3$_{proS}$ and H3$_{proR}$ deuterations in [2-^{13}C]glutamate

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Introduction. Hydrogen turnover as detected by ^{13}C NMR allows for the investigation of ^{1}H-^{2}H exchange of the different hydrogens of ^{13}C labelled glutamate isotopomers. We develop this approach to be able to investigate trafficking of various [^{13}C,^{2}H] labeled isotopomers, including lactate (lac) and glutamate (glu), between different cellular and subcellular compartments in cell cultures, perfused liver and brain (1-3). By monitoring the exchange of the H3 hydrogens of [2-^{13}C]glu it was possible to observe directly α-kg/glu exchange and its subcellular trafficking through the cytosolic and mitochondrial compartments, a process not previously detectable by ^{13}C NMR . In this respect, the analysis of H3 deuteration of the [2-^{13}C]glu isotopomer is particularly valuable. These H3 deuterations arise from the stereospecific hydration-dehydration reactions catalyzed by cerebral aconitase and isocitrate dehydrogenase on the glu precursors citrate and isocitrate, respectively. Aconitase and isocitrate dehydrogenase originate ultimately the 3R and 3S [2-^{13}C, 3-^{2}H]glu isotopomers, respectively. In our previous investigations on the kinetics of enzyme-catalyzed exchange of the H3 hydrogens of cerebral [2-^{13}C]glu, the discrimination of H3$_{proR}$ or H3$_{proS}$ deuterations was not possible because of the similar isotopic shifts induced in the C2 resonance, implying that the formation of 3R or 3S diastereoisomers could not be adequately resolved experimentally. Here, we provide an experimental approach able to discriminate the vicinal isotopic shifts observed induced by the deuteration in the positions H3$_{proR}$ and H3$_{proS}$ in [2-^{13}C]glu. These results may contribute to the elucidation of α-kg/glu trafficking through the mitochondrial membrane, a vital step in the transfer of reducing equivalents and in the energetics of the adult brain.

Methods. Adult Wistar rats received 50% ^{2}H$_2$O as drinking water ten days before the infusion of [1-^{13}C]glucose (8 μmol.min^{-1}.100g^{-1}). After the infusion (60 min), cerebral metabolism was arrested using focused microwaves (5 kW) and brain extracts prepared (sample 1). We employed an enzymatic coupled assay involving isocitrate dehydrogenase and aspartate aminotransferase in the presence of 50% ^{2}H$_2$O (vol/vol), to produce stereospecifically [3S-^{2}H]glu (sample 2). [2-^{13}C, 3R-^{2}H]glu was custom-made using the Stereo-Array Isotope Labeling (SAIL) method by SAIL technologies Inc. (Kanagawa, Japan). A basic strategy of the SAIL approach, based on chemical and enzymatic synthesis, was applied to prepare [2-^{13}C]glu with a stereo-selective replacement of one ^{1}H by ^{2}H in the methylene group attached to C3. In order to observe the perprotonated singlet and the shifted singlet derived from this custom-made isotopomer, we prepared a solution containing the synthetic compound (0.5 mM) and unlabeled glutamate (50 mM) (sample 3). All these samples were analyzed by ^{13}C NMR spectroscopy (11.7 T and 18.8 T). Spectral deconvolution and multiplet structures were analyzed using NUTS™.

Results. Figure 1A shows the glu C2 resonances obtained at 11.7 T (lower panel) and 18.8 T (upper panel) of brain extracts. In both cases, the C2 resonances of cerebral glu in deuterated animals depicted: i) the highest intensity unshifted singlet resonance from the perprotonated isotopomer; ii) two isotopically shifted resonances from the H3 monodeuterated isotopomers; and iii) the doubly shifted resonance from the H3 bideuterated isotopomers. Interestingly, the existence of two different isotopically shifted peaks derived from the 3R and 3S [2-^{13}C, 3-^{2}H]glu isotopomers, respectively, can be easily observed even at 11.7 T. However, the precise identification of which one of these isotopomers corresponds to the high-field and the low-field shifted singlets was only possible using the non-biological samples 2 and 3 (Figure 1B). Sample 2 corresponds to the natural abundance of glu synthesized in a coupled assay involving isocitrate dehydrogenase and aspartate aminotransferase in the presence of 50% ^{2}H$_2$O (vol/vol). Two peaks were observed: the one corresponding to the perprotonated [2-^{13}C]glu (peak "s") and the shifted singlet corresponding to the [2-^{13}C, 3S-^{2}H]glu (peak "a"). Sample 3 is a solution containing a mixture of the natural abundance of unlabeled glu (preprotonated singlet "s") and the pure custom-made [2-^{13}C, 3R-^{2}H]glu (shifted singlet "b"). The unlabeled glutamate was added to the synthesized product in order to allow identifying the isotopic shift provoked by the monodeuteration in the position H3$_{proR}$ of [2-^{13}C]glu. We observed that the [2-^{13}C, 3R-^{2}H]glu shifted singlet is the high-field resonance (Δ_1= -0.071 ppm), while the [2-^{13}C, 3S-^{2}H]glu has a lower value of vicinal isotopic shift (Δ_1= -0.058 ppm).

Conclusion. The ^{13}C NMR approach outlined here has made it possible to resolve experimentally the relative individual contributions of the deuterated 3R and 3S diastereoisomers of the glu C2 resonance. These findings open the possibility to obtain a quantitative interpretation of the turnover of each of the H3 hydrogens of [2-^{13}C]glu, allowing the investigation of the kinetics of these hydration-dehydration reactions in the brain.

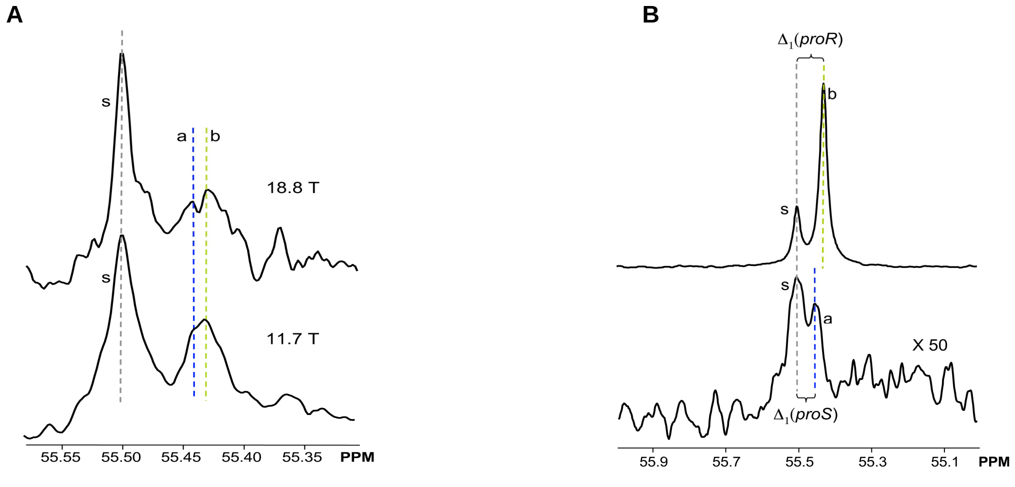

Fig. 1: Panel A. Representative ^{13}C NMR resonances of glu C2 from brain extracts (sample 1) after infusing [1-^{13}C]glucose (60 min), obtained at 11.7 T and 18.8 T). **s**: preprotonated singlet; **a** and **b**: shifted singlets induced by monodeuteration in the position H3$_{proS}$ and H3$_{proR}$. **Panel B:** Representative ^{13}C NMR resonances of glu C2 from samples 2 (lower panel) and 3 (upper panel), obtained at 18.8 T. Sample 2 corresponds to the natural abundance of glu synthesized in a couple enzymatic assay in the presence of 50% ^{2}H$_2$O. Peak "**s**" corresponds to the preprotonated [2-^{13}C]glu and peak "**a**" corresponds to [2-^{13}C, 3S-^{2}H]glu (Δ_1= -0.058 ppm). Sample 3 corresponds to a mix solution containing the natural abundance of unlabeled glu (peak "**s**") and the custom-made [2-^{13}C, 3R-^{2}H]glu (peak "**b**"), with an isotopic shift of Δ_1= -0.071 ppm.

References: 1. Garcia-Martin *et al.* JBC. 2002. 277: 7799. **2.** Rodrigues *et al.* JNR. 2005. 79: 119. **3.** Chapa *et al.* Neurochem Int. 2000. 37: 217.