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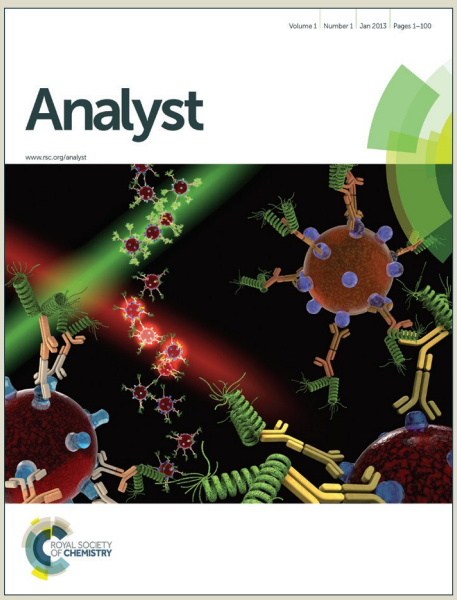

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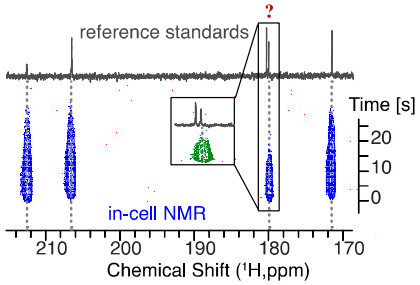
Graphical Abstract



Ambiguities in identifying transient intracellular reaction intermediates are resolved by site-specific isotope labelling, optimised referencing and response to external perturbations.

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COMMUNICATION

Spectroscopic approaches to resolving ambiguities of hyperpolarized NMR signals from different reaction cascades

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Influx of exogenous substrates into cellular reaction cascades on the seconds time scale is directly observable with NMR spectroscopy when using nuclear spin polarization enhancement. Conventional NMR assignment spectra for the identification of reaction intermediates are not applicable in these experiments due to the non-equilibrium nature of the nuclear spin polarization enhancement. We show that ambiguities in the intracellular identification of transient reaction intermediates can be resolved by experimental schemes using site-specific isotope labelling, optimised referencing and response to external perturbations.

The ability to visualize cellular reaction cascades by rapid spectroscopic approaches is significant for defining normal and pathological states of the cell. By virtue of its high spectral resolution, nuclear magnetic resonance (NMR) of ^{13}C isotopes is a well-suited modality for the distinction of chemicals in living cells. The scope of ^{13}C NMR for in cell metabolic studies has been extended by temporary nuclear spin polarization enhancements of exogenous probe molecules. Spin polarization enhancement (hyperpolarization) on the order of 10^4-fold over the equilibrium has permitted real-time observations of probe influx into cellular reaction cascades on the seconds time scale and with sub-second temporal resolution.[1] The temporary non-equilibrium nature of the hyperpolarized signals renders conventional multidimensional assignment spectra for the identification of detected signals impossible. Steady-state cellular metabolite concentrations have been used to rationalize signal identities,[2] but hyperpolarized NMR experiments provide non-steady state information and signal amplitudes cannot easily be assumed to reflect the steady state metabolite pools.[3] In addition, metabolite pools can fluctuate[4] or even oscillate[5] and are thus perilous correlates for hyperpolarized NMR data. The correct interpretation of chemical processes observed in live cells hinges on a correct identification of the chemicals observed. This problem will be further aggravated by improved experimental schemes and instrumentation, rendering more reaction intermediates and products detectable. The tentative identification of transient hyperpolarized signals has hitherto remained a matter of debate especially in distinguishing 3-phosphoglycerate and the 6-phosphogluconate, which are intermediates in the conversion of glucose by degradative and biosynthetic pathways, respectively (Figure 1). Extended cellular reaction cascades spanning more than 13 steps have been described for microbial cell factories such as yeast and bacteria,[6,7] but also for immortalised murine and human cell lines.[8-10] In all of these cell lines, 3-phosphoglycerate or 6-phosphogluconate occur as intermediates, as a corresponding signal near 182 ppm is detected. Genetic knockouts in the biosynthetic pentose phosphate pathway leading to 6-phosphogluconate have aided the identification of transient in vivo NMR signals.[2,7] Such genetic disruptions of biosynthetic pathways are not available for all cells of interest, however, and are not likely to be tolerated by all cells. Here, we therefore evaluate the possibility to distinguish metabolites noninvasively, specifically by (1) probe molecule design, (2) high-field NMR detection and

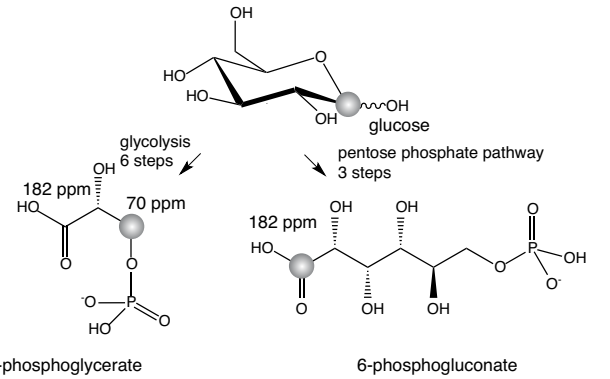

Figure 1. Propagation of the C1-atom from a glucose probe to organic phosphates with ambiguous $^{13}C1$ in vivo NMR signals.

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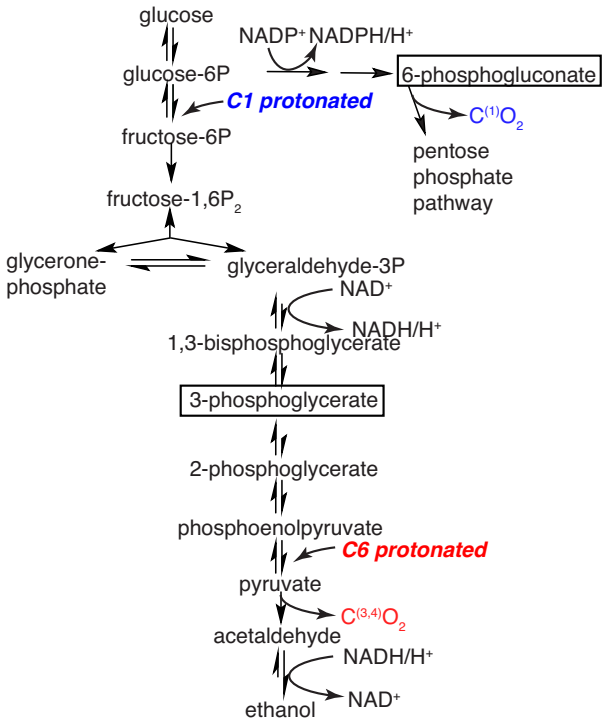

Figure 2. Schematic overview of major glucose conversion routes in yeast. Tautomerisation reactions that result in hydrogen transfer to C1 and C6 are indicated.

accurate chemical shift referencing that considers the effect of intracellular ions on the signal position, and (3) response of signal positions to external perturbations.

Problems in distinguishing 3-phosphoglycerate and the 6-phosphogluconate result from the highly similar ^{13}C chemical shifts of carboxylic acid signals, while the secondary alcohol and phosphate ester ^{13}C signals are congested with signals from the substrate and from other metabolites. However, the carboxylic acid group in both metabolites originates from different positions in the glucose substrate: the C1 atom of glucose turns into C3 of 3-phosphoglycerate with a chemical shift of 68 ppm and into C1 of 6-phoshogluconate with a chemical shift of 182 ppm. Ambiguities arise when using uniformly labelled [U-^{13}C, U-^{2}H] glucose probes. These ambiguities can be addressed by using a [1-^{13}C, 1-^{2}H] glucose probe instead (Figure 1).

If 6-phosphogluconate is detected as the 182 ppm signal when using hyperpolarized [U-^{13}C, U-^{2}H] glucose, then the signal should also be detectable with [1-^{13}C, 1-^{2}H] glucose (Figure 1). 6-phosphogluconate is formed by subsequent oxidation and lactone hydrolysis reactions at the C1 functionality that remains non-protonated throughout. In the degradative pathway towards 3-phosphoglycerate, however, [1-^{13}C, 1-^{2}H] glucose would get protonated at the C1 position, either from the solvent or by direct hydrogen transfer from the C2 position, in the isomerisation of glucose-6phosphate to fructose-6phosphate (Figure 2). Protonation of C1 would then reduce the hyperpolarization lifetime by roughly one order of magnitude and signal would fade within 1-2 seconds for formed sugar phosphates. In addition, [1-^{13}C, 1-^{2}H] glucose directly reports on CO_2 formation from C1 in the pentose phosphate pathway, as CO_2 formation during degradative glycolysis derives from C3 and C4 (Figure 2).

Mechanistic insights from the comparison of hyperpolarized [U-^{13}C, U-^{2}H] and [1-^{13}C, 1-^{2}H] glucose probes are exemplified in Figure 3. Hyperpolarized [U-^{13}C, U-^{2}H] and [1-^{13}C, 1-^{2}H] glucose[11,12] were produced as previously described and fed to 200 mg of compressed yeast (Danish Distillers, Malteser Cross, Copenhagen) as the model system, resuspended in 2 mL of 50 mM MES buffer of pH 5.6 or 50 mM acetate buffer of pH 4.85 and fed with hyperpolarized glucose to a final concentration of 5 mM. Conversion of hyperpolarized glucose isotopomers to reaction intermediates (Figure 3A) and the fading of hyperpolarized ketose phosphate signals (Figure 3B) are shown in red for the [U-^{13}C, U-^{2}H] probe and in blue for the [1-^{13}C, 1-^{2}H] probe. No 6-phosphogluconate signal was detected, while signals of glycolytic degradation evolved as expected: subsequent to protonation in the phosphoglucose isomerisation reaction, hyperpolarised C1 signal was rapidly lost, as evident in the [1-^{13}C, 1-^{2}H] probe. Thus, no 3-phosphoglycerate C3 signal was directly observed here. Instead, the partitioning of glucose into degradative and biosynthetic cascades can be assessed using ketose phosphates and chemicals of the pentose phosphate pathway, including 6-phosphogluconate C1, respectively. The use of [1-^{13}C, 1-^{2}H] glucose lead to a loss of the signal at 182 ppm and did not yield a CO_2 signal, consistent with a low influx of the probe into the pentose phosphate pathway. Instead, the majority of CO_2 released within seconds in the [U-^{13}C, U-^{2}H] experiment must have resulted from pyruvate decarboxylation.

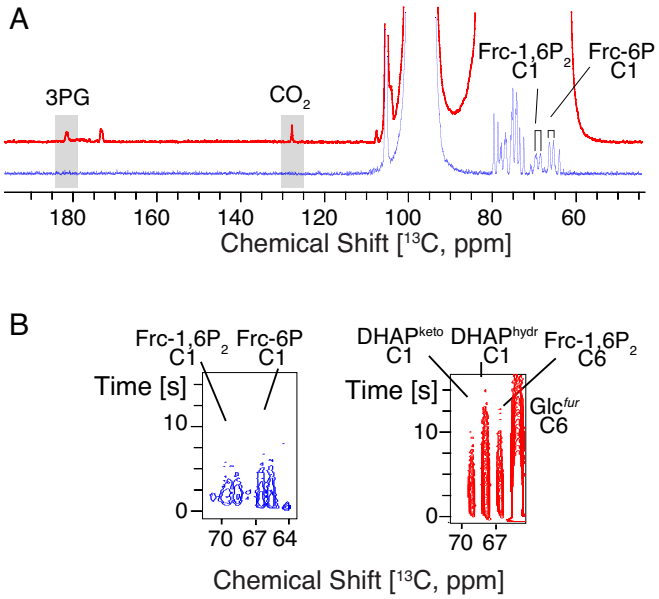

Figure 3. (A) ^{13}C NMR projections of kinetic conversion experiments of hyperpolarized [U-^{13}C, U-^{2}H] glucose (red) and [1-^{13}C, 1-^{2}H] (blue) glucose probes by cellular reaction cascades. Use of [1-^{13}C, 1-^{2}H] glucose abolishes the CO_2 and 182 ppm signals, showing that they derive from degradative conversion. (B) Methoxy-region of the ^{13}C kinetic NMR experiments of (A). Rapid fading of protonated sugar-phosphate signals formed from [1-^{13}C, 1-^{2}H] glucose (blue) in comparison to deuterated methylene groups deriving from C6 of a [U-^{13}C, U-^{2}H] probe (red).

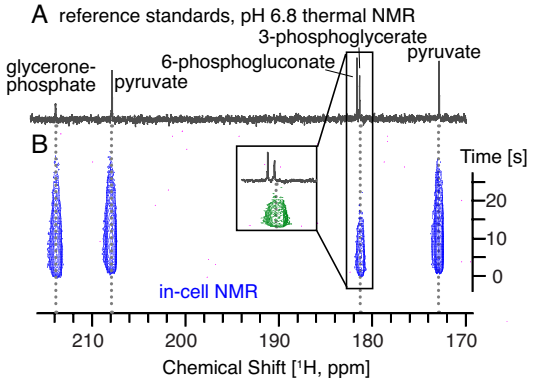

Figure 4. (A) Accurate chemical shift referencing of metabolite mixtues in near-physiological buffer (pH 6.8, 300 mM potassium) at 14.1 T magnetic field. (B) ^{13}C chemical shifts between cellular and reference spectra coincide to within 0.05 ppm.

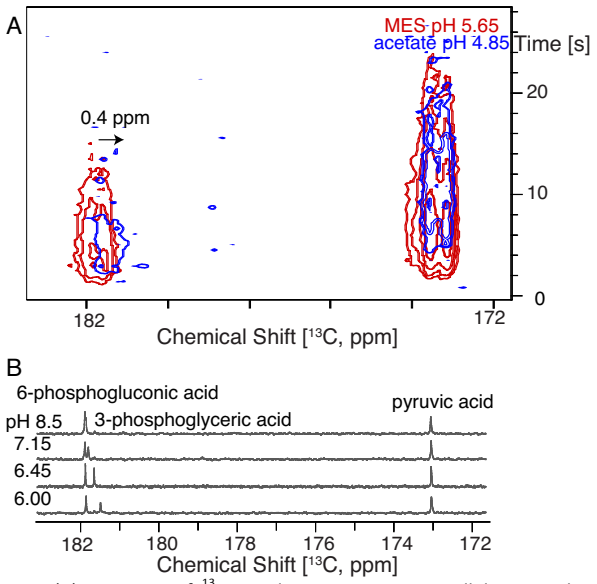

Figure 5. (A) Response of ^{13}C signal positions to extracellular perturbations, resulting in ^{13}C signal shifts of up to 0.4 ppm. (B) ^{13}C signal changes of 0.4 ppm can be observed in the physiological intracellular pH range for 3-phosphglycerate, but not for 6-phosphogluconate.

NMR chemical shifts are generally considered robust and accurately measured parameters. Nevertheless, chemical shift differences on the order of 0.5 ppm in reference compounds have not sufficed to resolve ambiguities of intracellular signals.[2,6,9] In metabolic ex vivo studies, robust chemical shift measurements are warranted by the use of strong buffers to minimise inter-sample variation. Accuracy of chemical shift measurements in vivo hinges on chemical shift calibrations in a milieu that resembles the intracellular milieu. This is especially true for organic phosphates, whose chemical shifts and pKa values depend on the nature and concentration of counter cations.[13,14] Reference spectra were therefore recorded in a buffer resembling the yeast cytosolic milieu with a pH of 6.8 and potassium concentration of 300 mM.[15] Reference spectra were acquired on metabolite mixtures in order to exclude inter-sample variation and provide accurate relative chemical shift values of in vivo metabolites. In addition, accurate reference standard and in vivo chemical shift measurements were conducted using high-field NMR at 14.1 T magnetic field strength.

Chemical shift calibrations using metabolite mixtures at ionic strength and pH resembling the composition of the cytosol were found to accurately reproduce intracellular chemical shifts. ^{13}C chemical shifts determined in this way coincided to within 0.05 ppm between reference and cellular signals. This accuracy was sufficient to distinguish between 6-phosphogluconate and 3-phosphoglycerate, even though the chemical shift difference is only on the order of 0.15 ppm at pH 6.8 in near-physiological buffer (Figure 4). The intracellular signal at 182 ppm matches the 3-phosphoglycerate but not the 6-phosphogluconate reference signal within the error of determination. Accurate chemical shift referencing and high-field NMR detection are thus in agreement with C1-labelling and genetic disruption studies identifying the intracellular metabolite in yeast cells as predominantly 3-phosphoglycerate.

The dependence of some ^{13}C chemical shifts on solution composition indicates that changes of signal positions due to external perturbations could be probed intracellularly and could indicate, which chemical is observed inside the cell. Weak organic acid buffer at a pH near its pKa was used to modulate compartmental composition and thus signal positions. Figure 5 shows that ^{13}C chemical shift changes on the order of 0.4 ppm can be achieved in this manner. Alteration of pH in the reference standard mixture shows that such signal changes at the C1-position only occur in the physiological pH range for 3-phosphoglycerate, while the 6-phosphogluconate ^{13}C1 signal changes by less than 0.1 ppm between pH 8.5 and 6.0.

Conclusion

Ambiguities in the identification of spectroscopic signals in living systems need to be addressed in order to achieve a meaningful interpretation of cellular chemistry. We employ three different approaches that are analogous to methods employed in conventional NMR and that are shown to yield spectroscopic identifications of an ambiguous signal in a yeast model system consistent with identification by gene knockout:[2] (1) Problems in identifying signal positions from transient ^{13}C NMR data can be addressed by accurate referencing. Standard mixtures in solutions resembling the intracellular milieu in ionic strength and pH yield ^{13}C signal positions with accuracies of less than 0.05 ppm when using high-field detection systems. (2) The fact that signal positions depend both on solution composition and on temperature further suggests that response patterns of signals upon disturbances of the extracellular chemical composition can be transduced into the cell to aid signal identification. (3) Finally, ambiguities in the probing of glucose influx into degradative and biosynthetic reaction cascades can be addressed by using glucose with site-specific isotope enrichment. Such probes resolve two ambiguities of experiments using fully labelled probes, as they distinguish the provenance of CO_2 and of the ambiguous signal at 182 ppm from the different reaction cascades.

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