Electronic Supplementary Material (ESI) for RSC Advances.

Observation of glucose-6-phosphate anomeric exchange in real-time using dDNP hyperpolarised NMR

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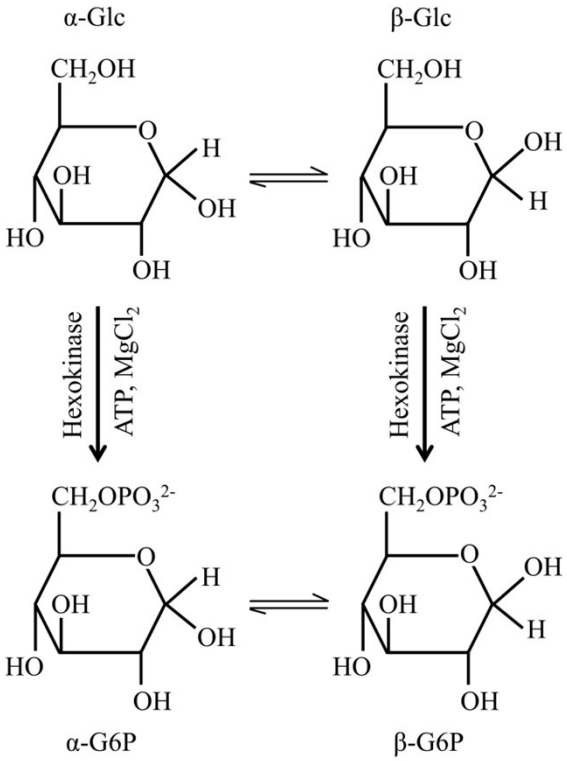
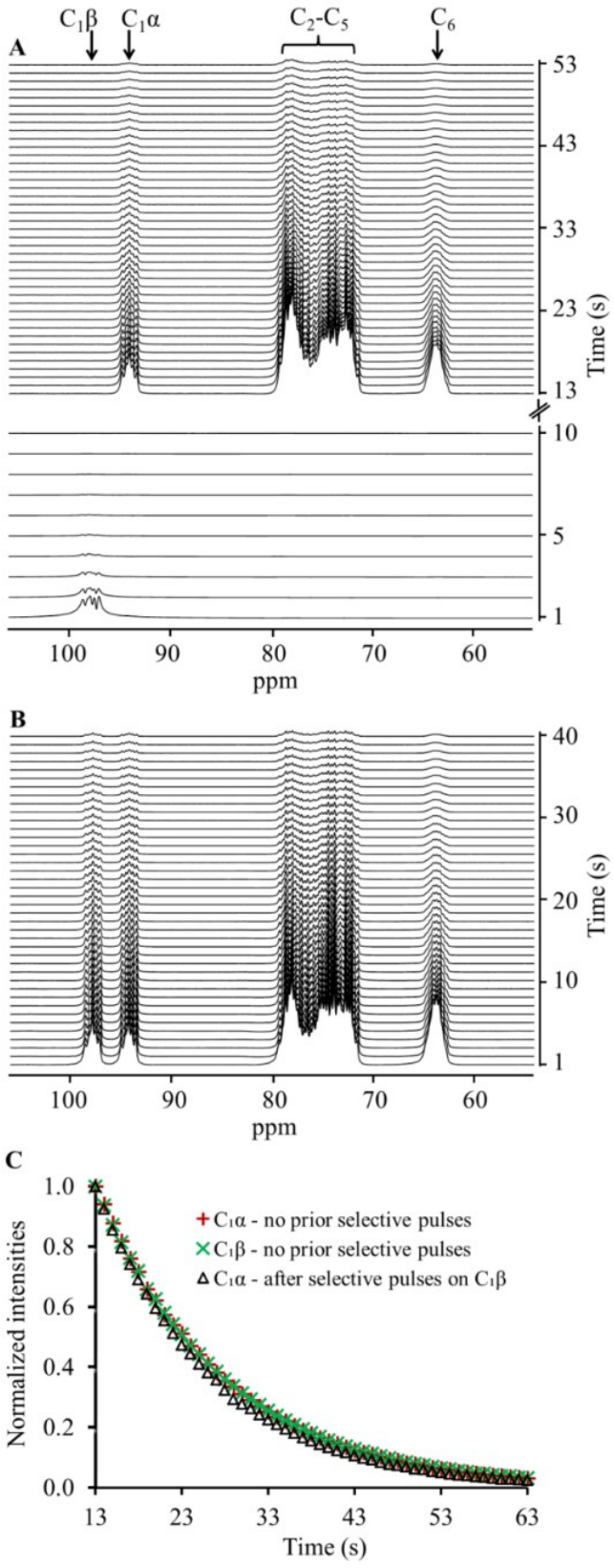
Supporting Information

Supporting Information S1



Supporting Scheme S1. Structures of Glc and G6P anomers and the phosphorylation and anomerization processes.



Supporting Figure S1. ^{13}C-NMR spectra of hyperpolarised [$^{13}C_6$,D_7]Glc acquired with and without selective pulses preceding hard pulses.
A) A series of selective pulses designed to excite the $C_1\beta$ signal was applied (lower panel) and then hard pulses with low flip-angle acquisition followed (upper panel). It can be seen that multiple selective pulses are needed for full depolarization of the desired anomer signal ($C_1\beta$).
B) Only hard pulses (low flip-angle) were applied.
C) A comparison of the decay of the hyperpolarised signals of $C_1\alpha$ and $C_1\beta$ with and without the preceding selective pulses on $C_1\beta$.

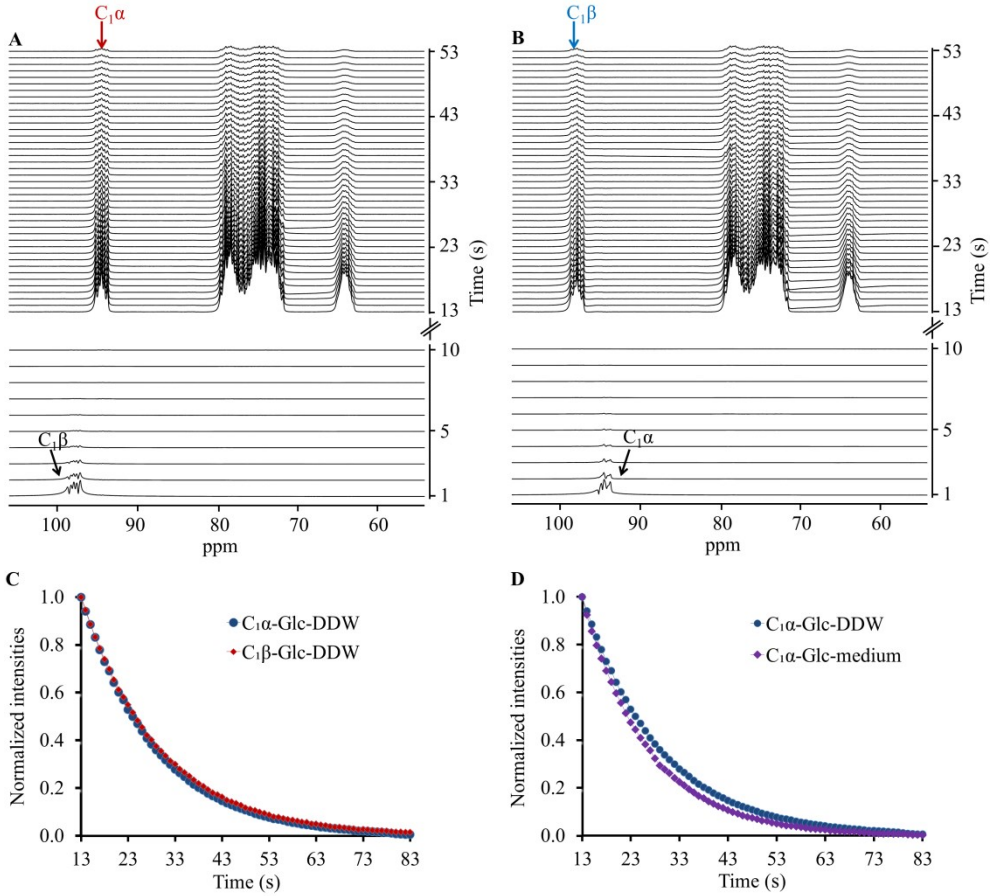
Supporting Table S1. T_1 values of C_1 sites in hyperpolarised [$^{13}C_6$,D_7]Glc, determined with and without selective pulses preparation.

Medium	RF pulsation	T_1 of $C_1\alpha$ (s)	T_1 of $C_1\beta$ (s)
Water	Selective pulses preceding hard pulses	20.5 ± 0.1	22.0 ± 0.1
Reaction medium	Hard pulses only	18.0 ± 0.3	18.2 ± 0.4
Reaction medium	Selective pulses preceding hard pulses	16.9 ± 0.1	Not tested

Each T_1 determination described here was performed once. The error is the 95% confidence interval of the curve fitting. On all of the measurements done with selective pulses preceding hard pulses (three altogether), the depolarisation of the desired anomeric signal was complete. All measurements were carried out at *ca.* 40 °C to prolong the T_1 and the visibility window of the hyperpolarised sites [1]. The reaction medium did not contain the hexokinase enzyme. The reactions carried out in water are further described in the Supporting Figure S2.

Supporting Information S2

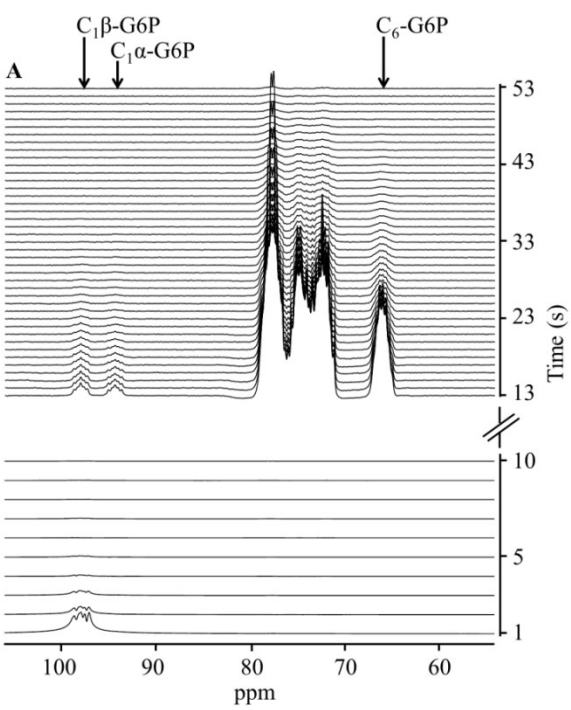
To validate the experimental approach presented in the text and in S1 this experiment was repeated twice in water. On one measurement the $C_1\beta$ signal was selectively depolarised and on the other, the $C_1\alpha$ signal was selectively depolarised (Supporting Figure S2 and Supporting Table S1). In both experiments, a similar behaviour was observed. First, during the selective pulses application, the $C_1\beta$ or the $C_1\alpha$ signal, respectively, was observed and decayed quickly. Then, with the application of the hard pulses, all of the other [$^{13}C_6$,D_7]Glc signals were observed, except for the depolarised signal ($C_1\beta$ or $C_1\alpha$, respectively, Supporting Figure S2A and S2B). This series of studies demonstrated the ability to null a single signal out of a whole hyperpolarised spectrum. The T_1 of the C_1 position in both anomers was longer in water compared to the reaction medium (Supporting Table S1). This is likely due to the presence of minerals including divalent ions and the overall ionic strength of the reaction medium. The T_1 that was determined for the $C_1\alpha$ signal of [$^{13}C_6$,D_7]Glc using this new acquisition strategy was the same as was determined for the same site in the same reaction medium at the same temperature and magnetic field in a previous study [1]. This result served as another validation that the new acquisition strategy does not interfere with the determination of T_1 for the hyperpolarised [$^{13}C_6$,D_7]Glc C_1 sites.

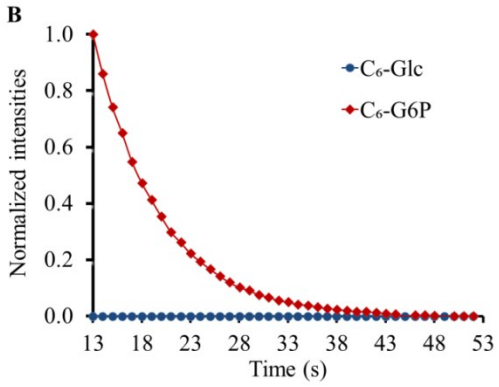


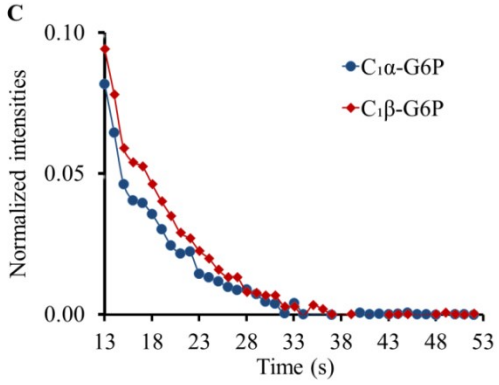
Supporting Figure S2. Hyperpolarised [$^{13}C_6$,D_7]Glc - ^{13}C NMR spectra and hyperpolarisation decay characterisation.
A-B) ^{13}C NMR spectra acquired when the dissolution was performed in 4 mL of double distilled water (DDW).
A) Selective excitation and depolarisation of the $C_1\beta$ signal for 10 s with a TR of 1 s followed by non-selective excitations (from 13th second) with the same TR.
B) Selective excitation and depolarisation of the of $C_1\alpha$ signal for 10 s with TR of 1 s followed by non-selective excitations from 13th second with the same TR.
C-D) Comparison of ^{13}C hyperpolarisation decay curves.
C) Comparison of the decay curves of the $C_1\alpha$ and $C_1\beta$ signals (both obtained from the non-selective spectra presented in panels A and B, respectively). The T_1 of these sites was found to be 20.5 ± 0.1 s and 22.0 ± 0.1 s, see Supporting Table S1.
D) Comparison of the decay curves of the $C_1\alpha$ signal acquired in water and in the reaction medium. Both measurements were carried out using selective excitation and depolarisation of the $C_1\beta$ signal for 10 s with a TR of 1 s followed by non-selective excitations (from 13th second) with the same TR. The respective T_1 values were 20.5 ± 0.1 s (also described in C) and 16.9 ± 0.1 s, see Supporting Table S1.

Supporting information S3

To further test and validate the explanation given to the results presented in Figure 1, the same reaction was carried out with about twice the amount of the enzyme (10.08 mg). In this case, the reaction was much faster and showed a full conversion to [$^{13}C_6$,D_7]G6P already in the first non-selective acquisition (Supporting Figure S3). This was demonstrated by the presence of the C_6-[$^{13}C_6$,D_7]G6P, and the absence of the C_6-[$^{13}C_6$,D_7]Glc signal. Indeed, in this case the $C_1\beta$ signal is observed as well but both the $C_1\beta$ and the $C_1\alpha$ signal are at much lower intensities compared to the reaction with about half the amount of the enzyme (Figure 1). This observation is in agreement with the explanation given in the text, *i.e.* that hyperpolarised [$^{13}C_6$,D_7]Glc underwent phosphorylation during the application of the selective depolarizing pulses on $C_1\beta$. As a result, the $C_1\beta$ of [$^{13}C_6$,D_7]Glc and of the newly formed [$^{13}C_6$,D_7]G6P were continuously depolarised. Due to the anomeric equilibrium of [$^{13}C_6$,D_7]G6P, the signal of the $C_1\alpha$ position of [$^{13}C_6$,D_7]G6P was also affected as it exchanged with a depolarised position, and the signal of the $C_1\beta$ of position of [$^{13}C_6$,D_7]G6P exchanged with a hyperpolarised position ($C_1\alpha$) and therefore appeared in the spectra. However, because the phosphorylation reaction is faster here, the signals of the C_1 position of both anomers are much lower compared to the reaction with less enzyme (Figure 1). Supporting Figure S3B demonstrates the decay of the C_6 position in [$^{13}C_6$,D_7]Glc (virtually non-existent) and [$^{13}C_6$,D_7]G6P. Supporting Figure S3C demonstrates the decay of the $C_1\beta$ and the $C_1\alpha$ signals, which in this experiment can both be attributed solely to [$^{13}C_6$,D_7]G6P from the very first non-selective spectrum.





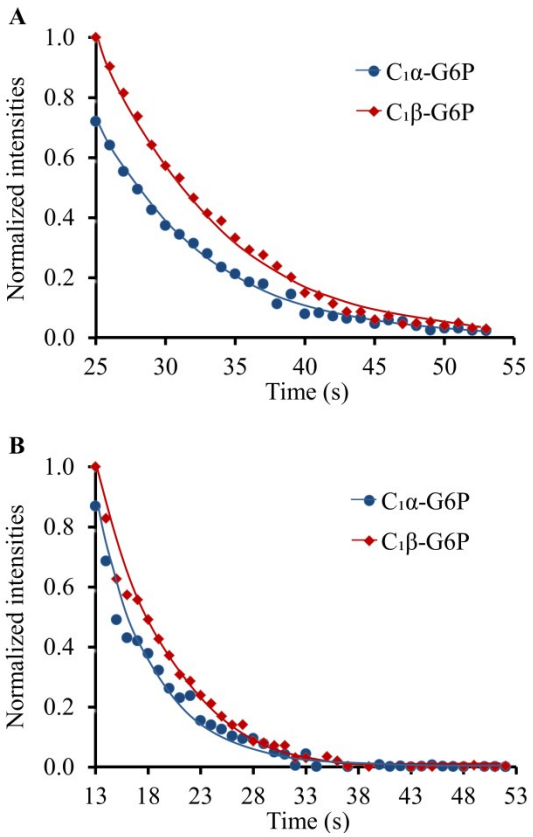


Supporting Figure S3. Hyperpolarised [$^{13}C_6$,D_7]Glc in a reaction mixture with hexokinase (10.08 mg).

A. Lower panel, selective excitation and depolarisation of the $C_1\beta$ position for 10 s with a TR of 1 s. Upper panel, subsequent non-selective (hard pulse) excitations (from the 13th second on) with the same TR.

B. A time course showing the signal decay for the chemical shifts of C_6-[$^{13}C_6$,D_7]Glc and C_6-[$^{13}C_6$,D_7]G6P determined using the non-selective excitations (upper panel in A).

C. A time course showing the signal decay for the chemical shifts of $C_1\alpha$ and $C_1\beta$ positions determined using the non-selective excitations (upper panel in A). Both signals can be considered to be those of [$^{13}C_6$,D_7]G6P and were used as such in the analysis of [$^{13}C_6$,D_7]G6P anomeric exchange.



Supporting Figure S4. Curve fitting of the decay of the $C_1\alpha$ and $C_1\beta$ signals of [$^{13}C_6$,D_7]G6P during the reactions of hyperpolarised [$^{13}C_6$,D_7]Glc with hexokinase.
A) A reaction with 5.43 mg hexokinase.
B) A reaction with 10.08 mg hexokinase.
The data were fitted to a kinetic model that assumed first order reaction rates on both the forward and reverse reaction and allowed for independent T_1s for the substrate ($C_1\alpha$-[$^{13}C_6$,D_7]G6P) and the product ($C_1\beta$-[$^{13}C_6$,D_7]G6P). For each experiment, both curves are fitted simultaneously. The results of the curve fittings are summarised in the Supporting Table S2.

Supporting Table S2. Anomeric exchange rates and T_1s of the C_1 positions of [$^{13}C_6$,D_7]G6P.

Experiment	T_1 of $C_1\alpha$ (s)	T_1 of $C_1\beta$ (s)	$k_{forward}$ α to β (s^{-1})	$k_{reverse}$ β to α (s^{-1})
Reaction with 5.43 mg hexokinase*	10.0	10.0	0.085	0.052
Reaction with 10.08 mg hexokinase**	7.0	7.0	0.090	0.055

Further parameters of the kinetic model fitting results were as follows:
* The R^2s for the $C_1\alpha$ and $C_1\beta$ were 0.9949 and 0.9942, respectively. The initial reaction time was taken as 20 s and the flip angle for non-selective excitation was taken as θ=10°.
** The R^2s for the $C_1\alpha$ and $C_1\beta$ were 0.9923 and 0.9954, respectively. The initial reaction time was taken as 13.5 s and the flip angle for non-selective excitation was taken as θ=11°.

Supporting Note S3. Materials and Methods

Materials

[$^{13}C_6$,D_7]Glc was obtained from Cambridge Isotope laboratories (MA, USA). ATP, $MgCl_2$ and hexokinase type III from Saccharomyces cerevisiae were obtained from Sigma-Aldrich (Rehovot, Israel). The Oxo63 radical (GE Healthcare, UK) was obtained from Oxford Instruments Molecular Biotools (Oxford, UK).

Enzymatic reactions with hexokinase

The reaction of [$^{13}C_6$,D_7]Glc with hexokinase was carried out as previously described by Miclet *et al.* [2] with modifications described by Sapir *et al.*[1]. A solution that contained the enzyme (1 mL) was waiting in the NMR tube, in the spectrometer for the arrival of the dissolution medium containing the hyperpolarised [$^{13}C_6$,D_7]Glc (4 mL). The preparation of this enzyme solution was as follows. ATP disodium was dissolved in 1 mL of TRIS HCl medium with $MgCl_2$. The pH was corrected with 10% NaOH solution to 7.6 [3] and then, ~130 units of hexokinase were added and gently mixed until a clear solution was obtained. The final reaction concentrations, after combining the enzyme solution with the dissolution medium, was 20.0 mM ATP, 10 mM $MgCl_2$, and 11 mM of hyperpolarised substrate.

The hyperpolarised solution containing [$^{13}C_6$,D_7]Glc was injected *via* a Teflon line from the spin-polariser directly into the NMR tube containing the enzyme solution within 3 s of Helium (g) chase. The dissolution of the hyperpolarised substrate was done while the NMR tube was already inside the probe of the NMR spectrometer. The reactions were performed at room temperature (*ca.* 21 °C).

dDNP

Spin polarisation and fast dissolution were carried out in a dDNP spin polarisation device (HyperSense, Oxford Instruments Molecular Biotools, Oxford, UK) operating at 3.35 T. For polarisation, microwave frequency of 94.116 GHz at 100 mW was applied for 2.5 h at *ca.* 1.4 K. For each experiment ~22 mg of a [$^{13}C_6$,D_7]Glc formulation was used. The formulation consisted of 9.6 mg of the [$^{13}C_6$, D_7]Glc mixed with 12.4 mg of an aqueous solution of Oxo63 (20 mM) and Gd^{3+} (Dotarem, 1.7 mM). Prior to insertion to the spin polariser, the formulation was kept at room temperature for at least 2 h to allow for anomeric equilibration in the formulation. Dissolution for the enzymatic reaction or for studies in a reaction medium was carried out with 4 mL of 100 mM TRIS HCl buffer at pH 7.6. Typical 11-13 % polarization was obtained in solution as previously described [1].

^{13}C NMR spectroscopy

^{13}C NMR spectra were acquired using a 5.8 T spectrometer (RS2D, Mundolsheim, France) using a 10 mm broad-band probe. Frequency selective Gaussian pulses (width of 16 ms) were applied with a repetition time (TR) of 1 s to selectively suppress the signals of either the α or the β anomeric C_1 position of [$^{13}C_6$, D_7]Glc. These pulses were applied with a bandwidth of 100 Hz for a 90° flip angle at these frequencies. A single 90° frequency selective pulse did not completely destroy the C_1-beta or alpha position signals even in the samples without the enzyme. There may be several possible explanations to this such as 1) the unsettled sample inside the spectrometer immediately after the ejection from the polarizer, 2) imperfect coverage of the pulse throughout the probe, and 3) flow from regions of the sample above and below the probe. To make sure we do not have contamination from a certain anomer we applied 10 pulses. Indeed, this could be optimized, but, as long as one of the anomers is completely depolarized, it will not affect the results as regards to the anomeric exchange. Full spectral width ^{13}C NMR spectra were acquired using non-selective RF pulses (hard pulses) with a low flip angle (10°) with the same TR. The temperature of the samples during the NMR measurements was determined with an NMR compatible temperature sensor (Osensa, Burnaby, BC, Canada). Spectral processing and calculation of the integrated intensities were performed using MNova (Mestrelab Research, Santiago de Compostela, Spain).

Determination of T_1 for the C_1 position of [$^{13}C_6$, D_7]Glc

Hyperpolarised decays of [$^{13}C_6$, D_7]Glc recorded using hard pulses without hexokinase served to determine the T_1 of the hyperpolarised sites. The data were fit to the equation, $M(t) = M_o \cdot e^{\left(\frac{-t}{T_1}\right)} \cdot \cos\theta^{\left(\frac{t}{TR}\right)}$ where θ is the flip angle of the excitation pulses, and t is the time at the end of each acquisition. Curve fitting was performed using MATLAB (Mathworks, Natick, MA, USA).

Kinetic model

The rate of anomerization and the T_1 times of the α and β anomers during the enzymatic experiments recorded with non-selective excitation pulses were calculated using a previously developed kinetic model [4]. This model uses first order kinetics to determine the forward and backward reaction rate constants. Here the model was used to determine the rate of [$^{13}C_6$, D_7]G6P anomerization and not of the phosphorylation reaction itself. To this end, the integrated signal

intensities of the decaying $C_1\alpha$ and $C_1\beta$ signals of [$^{13}C_6$,D_7]G6P were monitored using non-selective pulses and analysed. In order to use $C_1\alpha$ and $C_1\beta$ signals that were due to [$^{13}C_6$, D_7]G6P, and not [$^{13}C_6$,D_7]Glc, the $C_1\alpha$ and $C_1\beta$ signals were obtained from the time at which there was no [$^{13}C_6$,D_7]Glc left in the reaction mixture. This time was indicated by the lack of C_6-[$^{13}C_6$,D_7]Glc signal, as the C_6 position signals were well resolved for [$^{13}C_6$,D_7]Glc and [$^{13}C_6$,D_7]G6P.

References

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4. Allouche-Arnon, H.; Hovav, Y.; Friesen-Waldner, L.; Sosna, J.; Moshe Gomori, J.; Vega, S.; Katz-Brull, R., Quantification of rate constants for successive enzymatic reactions with DNP hyperpolarized MR. *NMR Biomed.* **2014,** *27* (6), 656.