Electronic Supplementary Material (ESI) for Chemical Communications.

Supporting Information

Direct Enzyme-Substrate Affinity Determination by Real-Time Hyperpolarized ^{13}C-MRS

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Materials and Methods

Materials

[1,1,2,2-D_4, 1-^{13}C]choline chloride (CMP1) was donated by BrainWatch Ltd. (Tel-Aviv, Israel). Choline oxidase from *Alcaligenes* (E.C. 1.1.3.17) was obtained from Sigma-Aldrich (Canada). This enzyme converts choline to both betaine aldehyde and betaine in the presence of oxygen and flavin adenine dinucleotide. The Trityl radical OX063 was obtained from GE Healthcare, (London, UK). ProHance was purchased from Bracco Diagnostics Inc. (Italy).

Sample preparation, hyperpolarization, and reaction conditions

The samples on which MRS measurements were made contained a mixture of an enzyme solution and a dissolved polarization preparation, with the latter containing the CMP1 reactant in a hyperpolarized state. The enzyme solution was prepared prior to the measurement and contained 1.8 mg enzyme dissolved in 4 ml of 100 mM Tris buffer. Oxygen was directly bubbled into this solution for about 15 min prior to the reaction onset.

The polarization preparation consisted of CMP1 (0.5 to 18.3 mg) combined with an aqueous solution of OX063 (62 mM) and ProHance (2.9 mM) at a 1:2.7 weight ratio between the choline molecular probe and this aqueous solution, respectively.

The polarization preparation was hyperpolarized in a 3.35 T dissolution DNP polarizer (HyperSense, Oxford Instruments, UK). Polarizations were carried out at 1.4 K, using a MW frequency of 94.1 GHz and an output power of 50 mW. The polarization build-up time constant was 1614 ± 275 s (n=11). Dissolution of the polarization preparation was performed with 3.5 ml of 100 mM Tris buffer (pH = 8) containing 2 mM EDTA. This volume was used in all of the experiments except for the experiment with the lowest CMP1 concentration (0.25 mM) which was performed with 14 ml of this solution. The level of polarization in liquid state was 36 ± 1 %, as previously reported [1].

The reactor consisted of 4 ml of enzyme solution in a 10 ml syringe. This was placed in a solenoid radio-frequency coil at the center of the magnetic field immediately before the injection of the dissolved polarization preparation. A picture of the radio-frequency coil with the reactor placed within is shown in Figure 1. Eight hundred µl of the dissolved polarization preparation were manually injected (1-3 s injection time) using a syringe connected to a line into the reactor situated in the magnet. The time between the ejection of the dissolved polarization preparation from the DNP polarizer to the injection into the reactor was between 15 and 20 s. The resulting final CMP1 concentrations in the reactor were 0.25, 0.5, 1, 2, 3, 4, 8, and 17.5 mM. The 3 mM and 17.5 mM concentrations were performed three times for reproducibility measures. Monitoring by ^{13}C-MRS started immediately upon the start of injection. The points recorded prior to the end of the injection (1-3 s) were removed from presentation and further analysis and were used only as an independent monitor to the progression of the injection process (not shown in figures).

The temperature was maintained at 37 °C before the addition of the dissolved polarization preparation and during the enzymatic reaction. The enzyme solution was kept in a water bath with a temperature of 37 °C during oxygenation and was placed in the magnet immediately prior to the choline injection. While in the magnet, a warm water blanket was wrapped around the coil to maintain heat within the coil and the reactor within.

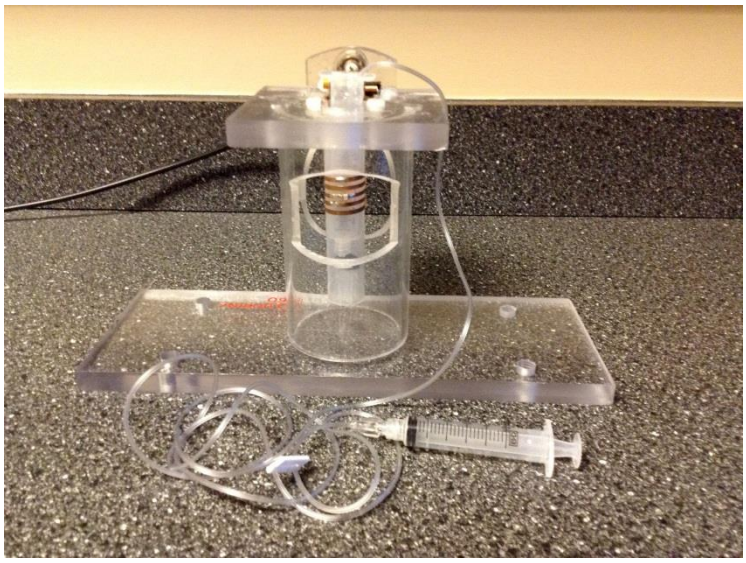

Figure 1. The radio-frequency solenoid coil, the reactor, and the injection line.

The reactor is situated within the radio-frequency coil. The radio-frequency coil consists of 5 turns. An injection line is connecting the reactor and the injection syringe shown at the bottom. A water blanket was wrapped around the coil during the experiment. This water blanket is not shown in the picture because it would block visibility of the coil.

Signal measurements and processing

MRS measurements of the hyperpolarized samples were performed on a 3 T clinical scanner (GE Healthcare, UK) equipped with a home-built ^{13}C solenoid coil (22 mm in diameter and a length of 20 mm, 5 turns, as shown in Figure 1). Prior to insertion of the reactor into the solenoid coil, calibration of the flip angle was performed using a 5 ml cylinder of water. Calibration was performed at the proton frequency using the water resonance and then frequency shifted to ^{13}C. Linear order shimming was performed at the proton frequency using this water cylinder. Higher order shims were not available at this clinical scanner. The ^{13}C signal of the reaction mixture was acquired using a series of 256 consecutive repetitions of a detection pulse and an FID signal acquisition, with a repetition time (*i.e.* beginning of one acquisition to beginning of next acquisition) of 1 s. The acquisition time was 410 ms and the post-pulse delay was 590 ms. The detection pulse was calibrated to a flip angle of 10°. A readout filter of 10,000 Hz was applied with 4096 points.

The FID signals were Fourier transformed to yield ^{13}C spectra. The integrated signal intensities were determined and stored for analysis. Spectral post processing (phase and baseline correction) as well as signal integration were carried out using Matlab (MathWorks, Natick, MA, USA).

Application of the kinetic simulation

A kinetic model previously developed by our group was used in order to determine the enzymatic reaction rates K_1 and K_2 [1]. Briefly, the implementation of this model in the current study consisted of an iterative fitting process, aimed at achieving a maximal agreement between the experimental data points and three simulated time dependent curves (for each metabolite signal). The reaction rates K_1 and K_2 are two of the parameters that were determined in this procedure. The other

parameters include the relaxation rate ($1/T_1$) for each of the three signals and the reacting portion of the substrate, defined as the fraction of the hyperpolarized substrate involved in the oxidation reaction and reflecting an inhomogeneous mixing of the reaction solution at the short time scales of the observation [1]. In practice, since a change in any single parameter affected the shape of all three curves, the three experimental curves were simulated and fitted simultaneously. This was achieved by manually modifying the various parameters of kinetic rates and relaxation time parameters until the R-square values of all three curves were maximal (and a best visual match was obtained as well). This kinetic model analysis was carried out using Matlab (MathWorks, Natick, MA, USA).

The entire parameter set determined using this kinetic model in the entire set of experiments described in the current manuscript is summarized in Table 1.

Summary of data

Table 1. The experimental data and fitted parameters* in of all of the experiments described in the paper.

Choline chloride in the reactor (mM)	$1/T_1$ cho	$1/T_1$ BAH	$1/T_1$ bet	S_1/S_0 reacting substrate / non-reacting substrate	K_1 (s^{-1})	K_2 (s^{-1})	K_1 ($\mu mol_{*}min^{-1}{}_{*}$ mg $enzyme^{-1}$)	K_2 ($\mu mol_{*}min^{-1}{}_{*}$ mg $enzyme^{-1}$) using choline concentration	t0	flip angle (°)
0.25	0.0330	0.0130	0.0270	0.60	0.022	0.035	0.32	0.51	0.8	10
0.49	0.0300	0.0130	0.0290	0.60	0.011	0.021	0.32	0.62	0	10
0.98	0.0300	0.0130	0.0290	0.42	0.024	0.017	1.11	0.79	2	10
2.02	0.0300	0.0125	0.0330	0.10	0.060	0.020	1.76	0.59	0	10
3.00	0.0195	0.0650	0.0330	0.10	0.029	0.0003	1.27	0.013**	0.0	10
3.00	0.0230	0.0210	0.0330	0.10	0.033	0.010	1.44	0.44	0	10
3.00	0.0220	0.0190	0.0400	0.09	0.075	0.012	2.97	0.48	0	10
4.00	0.0250	0.0125	0.0330	0.08	0.034	0.008	1.61	0.38	1	10
8.00	0.0200	0.0850	0.0330	0.12	0.0096	0.0045	1.32	0.62	0	10
17.47	0.0303	0.0125	0.0294	0.0045	0.12	0	1.50	0.00	0.0	9.8
17.58	0.0303	0.0125	0.0294	0.0045	0.12	0	1.51	0.00	0.0	8.5
17.54	0.0303	0.0125	0.0294	0.0045	0.23	0	2.89	0.00	0.0	8.0

*The parameters of the fit correspond to the parameters of the kinetic model as was previously described [1], with two minor modification: $k_{1\rightarrow2}$ and $k_{2\rightarrow3}$ were replaced here by K_1 and K_2, respectively.

**rejected from K_2 analysis due to low signal-to-noise ratio of the [D_2, ^{13}C]Bet signal, as described in the paper.

Reference

1 Allouche-Arnon, H. et al. Quantification of rate constants for successive enzymatic reactions with DNP hyperpolarized MR. NMR Biomed. 27, 656-662 (2014).