Electronic Supplementary Material (ESI) for Chemical Communications


*Supporting information*

*for*

# *In vitro* visualization of betaine aldehyde synthesis and oxidation using hyperpolarized magnetic resonance spectroscopy

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# Methods

## *Materials*

Choline chloride, betaine hydrochloride, [1,1,2,2-$D_4$]choline chloride, and choline oxidase from Alcaligenes (E.C. 1.1.3.17) were obtained from Sigma-Aldrich (Israel). The latter is an enzyme that converts choline to both betaine aldehyde and betaine in the presence of oxygen. [1,1,2,2-$D_4$, 2-$^{13}$C]choline chloride (CMP2) was donated by BrainWatch Ltd. (BW-42, BrainWatch Ltd., Tel-Aviv, Israel). Betaine aldehyde was synthesized in house (see below). Trityl radical OX063, was obtained from GE Healthcare, (London, UK). ProHance was purchased from Bracco Diagnostics Inc. (Italy).

## *High resolution spectrometers*

Studies on enzymatic reactions and standard solutions that were performed at thermal equilibrium condition were carried out on an 11.8 T high-resolution spectrometer (Varian, Palo Alto, CA, USA) with a 5 mm direct multinuclear probe. The spectrometer is located at Hadassah-Hebrew University Medical Center.

Studies in a hyperpolarized state were carried out on a 14.1 T high-resolution spectrometer (Bruker, Germany), with a 5 mm inverse detection probe. The system is located at Albeda Research Laboratory (Copenhagen, Denmark).

## *Enzymatic reactions at thermal equilibrium*

Choline oxidase, 12–14 units, was dissolved in 100 mM phosphate buffer in purified water and placed in a 5 mm NMR tube. Oxygen was bubbled into the NMR tube for ca. 3 min to saturate the medium. Choline chloride (3 mM) or [1,1,2,2-$D_4$]choline (3 mM) were added to the reaction mixture, and the tube was gently mixed and quickly placed in the spectrometer for monitoring by $^1$H-NMR. The saturated buffer was maintained at 37 °C until mixing. Mixing and transfer to the spectrometer were carried out at room temperature. The temperature in the spectrometer was maintained at 37 °C. Proton spectra in water were acquired using a standard pre-saturation pulse sequence for water suppression with 4 transients, 10 s repetition time, and a 90 degree flip angle.

Deuterium spectra were recorded 2 months after the start of [1,1,2,2-$D_4$]choline oxidation using 220 transients, 2 s repetition time, and a 90 degree flip angle.

## *Hyperpolarization*

The polarization solution consisted of CMP2 (10 mg, 69 µmol) and 2.7 mg of an aqueous solution of OX063 (62 mM) and ProHance (2.9 mM). The DNP samples were hyperpolarized in a 3.35 T home-built dissolution DNP polarizer located at Albeda Research Laboratory (Copenhagen, Denmark) at ca. 1.3 K, 93.9 GHz, and 100 mW.

## *Dissolution*

Dissolution of the above polarization solution was performed with 4 ml of 100 mM Tris buffer (pH 8) containing 2 mM EDTA.

## *Enzymatic reactions in a hyperpolarized state*

Six to 24 units of choline oxidase (0.5 to 2 mg enzyme, respectively) were dissolved in 100 mM Tris buffer (600 µl) which was bubbled for about 15 min with oxygen, placed in a 5 mm NMR tube, and maintained at 37 °C. One hundred microliters of the hyperpolarized media containing CMP2 were added to the reaction mixture in the NMR tube, resulting in a final CMP2 concentration of 2.46 mM. The sample was then gently mixed before it was placed at 37 °C in the spectrometer for enzymatic monitoring by $^{13}$C-NMR. The transfer time was ca. 7 s, measured as the time between mixing of the hyperpolarized medium and the enzyme

solution and the first pulse taken in the spectrometer. A series of 128 consecutive $^{13}C$ spectra were acquired with a repetition time of 1 s, and a 10 degree flip angle.
Two experiments were carried out with 6 units of choline oxidase, one with 12 units, and one with 24 units.
Shimming was performed on a sample tube containing the same buffer at the same volume of solution (same sample height) prior to the hyperpolarized measurement. Additional shimming on the sample containing the hyperpolarized medium was not performed, to facilitate recording the very early time points of the reaction.
Antimicrobial compounds were not added to the hyperpolarized reaction mixture to prevent possible unknown effects of these compounds on enzyme activity.

## *Control studies*

### Hyperpolarized $^{13}C$

As a control for the possible metabolism by environmental bacteria, the same protocol was performed without the addition of choline oxidase enzyme. These control studies were published earlier. Briefly, none of the metabolic conversions that produced the chemical shifts shown in the current study were detected during three separate types of studies, all carried out in the same laboratory as the current study, using the same instrumentation and same overall environmental bacterial flora, at 37 °C, as summarized in the table below.

| Number of repetitions | Medium * | Reference |
|---|---|---|
| n = 5 | Phosphate buffer | a |
| n = 3 | Human whole blood | a |
| n = 2 | Tris buffer containing carnitine acetyltransferase | b |

* Medium in which hyperpolarized CMP2 was observed.
a Allouche-Arnon, et al. Contrast Media Mol Imaging, 2011; 6:499-506
b Allouche-Arnon, et al. Contrast Media Mol Imaging, 2011; 6:139-147

### Thermal equilibrium $^{1}H$

Choline chloride is a very stable compound in water-based media, showing no degradation over years of storage. Numerous tests in our laboratory using choline chloride as a standard for $^{1}H$-NMR in non-sterile buffers and body fluids under various temperatures never showed any sign of metabolism over the course of an hour and more. Choline metabolism in general, and oxidative metabolism specifically, have only been observed when the appropriate biological agent was added (cells or enzymes).

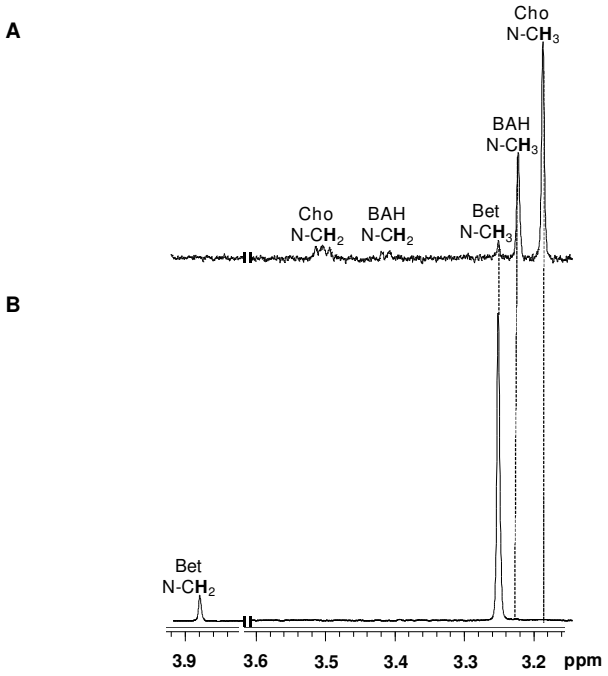
# Monitoring of choline oxidase reaction

## *$^1$H-NMR at thermal equilibrium*

Proton spectra of the enzymatic reaction mixture were recorded at 11 minutes (Figure 1A, below) and 13 days (Figure 1B, below) after reaction onset. The 11 min time lag between the start of the reaction and acquisition of the first spectrum was used to adjust the water suppression on our spectrometer. The second spectrum was acquired 13 days after reaction onset to observe the composition of products after reaction completion. The samples were kept at 37 °C between the two measurements.

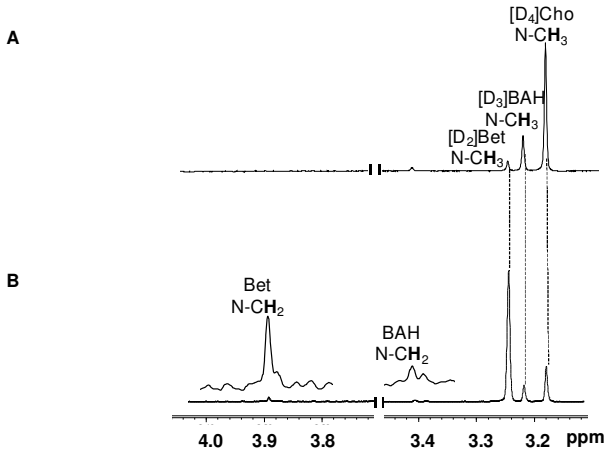
The signals in 1B were both assigned to betaine, with the singlet at 3.25 ppm assigned to the trimethylamine group and the singlet at 3.88 ppm assigned to the methylene moiety. At this late reaction time, 100 % of the substrate had been fully oxidized to betaine. For each spectrum, four transients were acquired within 40 s. The spectra are not to scale, since spectrum 1A was recorded with a higher gain. The assignment of choline (Cho), betaine aldehyde hydrate (BAH) and betaine (Bet) is further described below in the section on "Signal assignment".

**Figure 1**



Proton spectra were also used to monitor the enzymatic oxidation of a deuterated Cho analog ([$D_4$]Cho). Spectrum 2A (below) was recorded at 12 min demonstrating the formation of [1,2,2,-$D_3$]betaine aldehyde hydrate ([$D_3$]BAH) and [2,2-$D_2$]betaine ([$D_2$]Bet). Spectrum 2B (below) was recorded at 8 days after reaction onset. The samples were kept at 37 °C between the two measurements.
The inserts in 2B show 10-fold enlargements of the corresponding spectral regions. These signals are due to the isotopic impurity of protonated methylene moieties of the [$D_4$]Cho (98% enriched with D atoms), which were converted to BAH and Bet. Spectra 2A and 2B were recorded with four transients each and a repetition time of 10 s.

**Figure 2**




The difference in the number of days (8 and 13 days, Figures 1B and 2B, respectively) used for this end-point determination of the native and deuterated substrates, respectively, was only due to spectrometer availability. Both time points represent times in which the reaction was expected to have long been completed. Spectrum 2B shows the presence of both the substrate and products signals, indicating that the reaction had progressed but deuterated choline was not fully oxidized at this time point.

## *Kinetic analysis of the thermal equilibrium studies*

Because thermal equilibrium studies consist of only one point acquired late in the reaction, only a limited kinetic analysis could be performed.
Using the native choline, at 11 min past the reaction onset, the mean ratio of the oxidation products (betaine aldehyde hydrate and betaine combined) to choline was 0.8 ± 0.3 ($n$ = 3), meaning that 44 % of the substrate (choline) was already metabolized. Taking into account an initial 3 mM choline concentration in 0.7 ml of solution and 12 enzyme units, an overall reaction rate can be calculated to be 0.92 µmol/11 min/12 units or 0.084 µmol/min/mg enzyme. Using the deuterated choline, at 12 min past the reaction onset, the calculated mean ratio of the deuterated oxidation products to deuterated choline was 0.50 ± 0.15 ($n$ = 3), meaning that 33 % of the substrate was oxidized. Taking into account the same choline concentration, sample volume, and enzyme units, an overall reaction rate can be calculated to be 0.69 µmol/12 min/12 units or 0.058 µmol/min/mg enzyme.

## *Kinetic analysis of the hyperpolarized studies*

To obtain kinetic rate constants for the consecutive first order enzymatic studies that were monitored in the current study, we developed a general model for absolute quantification and kinetic analysis of enzymatic reactions involving the formation of more than a single product in a hyperpolarized state. This model takes into account, in addition to the reaction processes, the effects of the longitudinal relaxation of the hyperpolarized nuclei and the detection pulses. The effects of mixing and transportation time to the spectrometer are also taken into account. The details of the model will be described elsewhere (Hyla Allouche-Arnon, Yonatan Hovav, Jacob Sosna, J. Moshe Gomori, Shimon Vega, and Rachel Katz-Brull, unpublished results). Using this model, the kinetic rate constants were found to increase linearly with the amount of enzyme used, as expected. Thus it was found justified to report the average rate constants (per unit enzyme). The average kinetic rate constants per unit enzyme were found to be 9.1 ± 1.0 x $10^{-3}$ $s^{-1}unit^{-1}$ for [$D_3$,$^{13}$C]BAH formation, (n = 4), and 3.2 ± 1.2 x $10^{-3}$ $s^{-1}unit^{-1}$ for ([$D_2$,$^{13}$C]Bet formation, (n = 3, one fit excluded due to low SNR).

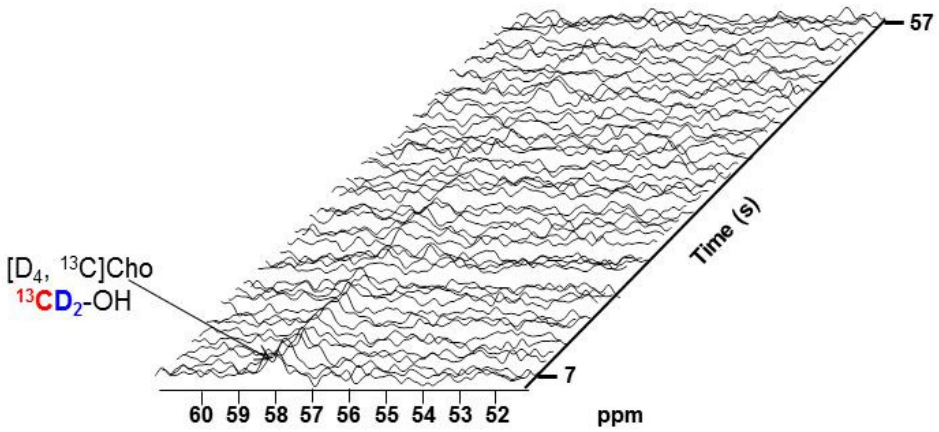
Comparison to literature data:
A previous analysis of betaine synthesis by the same enzyme (without consideration of the intermediate kinetics) found a $K_m$ of 1 mM and a $V_{max}$ of 5.3 µmol/min/mg enzyme at 23 °C [1]. Another study found a $V_{max}$ of 13.5 µmol/min/mg enzyme at 37 °C [2]. The current choline oxidase product specifications indicate a $K_m$ of 2.84 mM for choline and 5.33 mM for betaine aldehyde. The mean rate constant for the formation of [$D_3$,$^{13}$C]BAH formation that was found, (9.1 x $10^{-3}$ $s^{-1}unit^{-1}$), is equivalent to 6.6 $min^{-1}mg\ enzyme^{-1}$ and thus predicts that the rate of betaine aldehyde hydrate formation in the current experimental conditions (2.46 mM in 0.7 ml) is approximately 11.3 µmol/min/mg enzyme. This value is similar to the $V_{max}$ for betaine synthesis which was previously determined at 37 °C. We note that this value is ca. 200 fold higher than the reaction rate that was determined using a single measurement at 12 min of reaction at thermal equilibrium. This emphasizes the importance of measuring reaction rates using early data points and the role for hyperpolarization in this context, for reactions in which the initial rates cannot be determined at thermal equilibrium.

We note that the rate of betaine formation was lower, 3.2 ± 1.2 x $10^{-3}$ $s^{-1}unit^{-1}$. There could be several reasons for this: 1) The $K_m$ for betaine aldehyde hydrate is higher (5.33 mM) meaning that the reaction for betaine formation occurred at a rate lower than its $V_{max}$; 2) An isotopic effect owing to the fact that a deuterated choline analog was used instead of the native choline[3]; and 3) Possible insufficient oxygen supply in the experimental conditions used here.

1. J. Z. Stemple, K. M. Rusin and T. L. Fare, *Anal. Chem.*, 1991, **63**, 1050-1052.
2. M. Ohtafukuyama, Y. Miyake, S. Emi and T. Yamano, *J. Biochem.*, 1980, **88**, 197-203.
3. G. Gadda, *Biochim. Biophys. Acta. Proteins Proteomics*, 2003, **1650**, 4-9.

## *Observation of naturally abundant $^{13}C$ in hyperpolarized spectra*

The hyperpolarized spectra acquired during choline oxidase reaction on hyperpolarized CMP2 also showed the signal of the naturally abundant $^{13}C$ of [$D_4$, $^{13}C$]Cho methylene group (position 1). Decay of this signal at 7–57 s is shown in the spectra below.



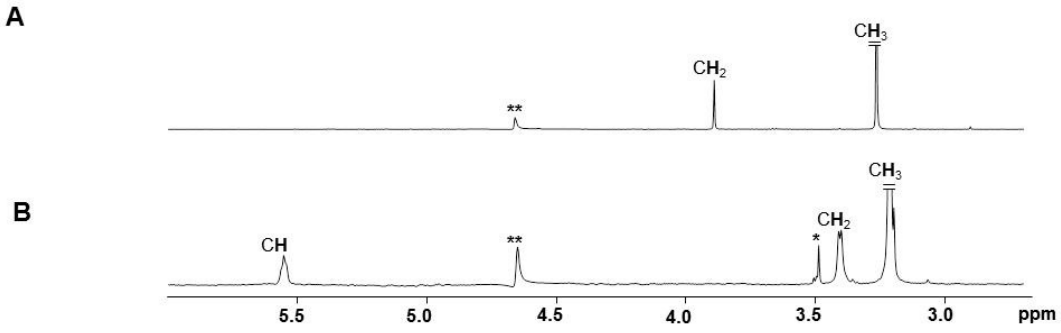
## Signal assignment

Betaine aldehyde hydrate and betaine signals were assigned according to $^{1}H$ and $^{13}C$ NMR spectra of aqueous standard solutions (10% $D_2O$, pH 7.0, 37 °C at 11.8 T). The spectra of these standard solutions are presented below. The resonances observed in these spectra were as follows:

**Betaine**
$^{1}H$ spectrum: methyls (singlet) at 3.25 ppm; N-$CH_2$ (singlet) at 3.88 ppm
$^{13}C$ spectrum: methyls (singlet) at 56.54 ppm; N-$CH_2$ at 68.87 ppm; COOH at 171.68 ppm

**Betaine aldehyde hydrate (betaine aldehyde in water)**
$^{1}H$ spectrum: methyls (singlet) at 3.23 ppm; N-$CH_2$ (doublet) at 3.42 ppm, $J_{H-H}$= 4.75 Hz; $CH(OH)_2$(triplet) at 5.56 ppm
$^{13}C$ spectrum: methyls (singlet) at 56.97 ppm; N-$CH_2$ at 71.31 ppm; $CH(OH)_2$ at 87.79 ppm

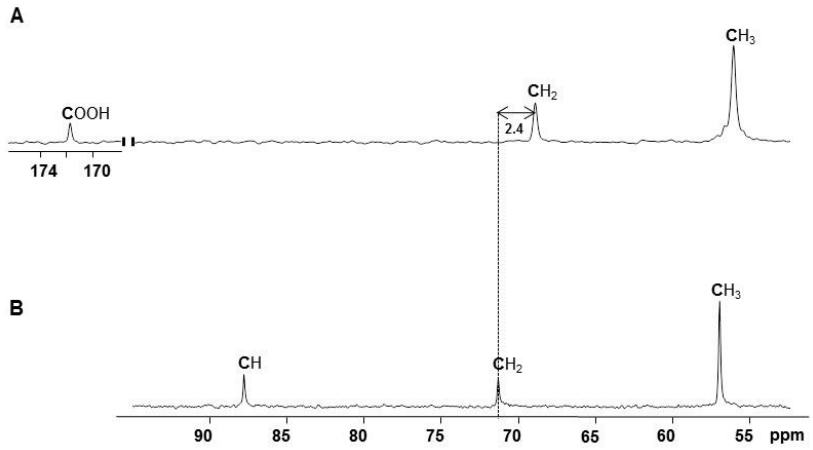
### *Proton NMR spectra of chemical standards*




A) Betaine chloride (300 mM) in aqueous solution
B) Betaine aldehyde hydrate (439 mM) in aqueous solution

Both spectra were acquired at 11.8 T with eight transients, repetition time of 5 s, and a 90 degree flip angle. TSP-$D_4$ was used as chemical shift reference, calibrated at -0.015 ppm for both spectra. * Impurity, ** $H_2O$ signal.

### *$^{13}$C NMR spectra of chemical standards*




A) Betaine chloride (300 mM) in aqueous solution (842 transients)
B) Betaine aldehyde (439 mM) in aqueous solution (205 transients)

Both spectra were acquired with a repetition time of 5 s and 90 degree flip angle. NOE and proton decoupling were applied. TSP-$D_4$ was used as chemical shift reference, which was calibrated at -0.12 ppm for both spectra.

## Synthesis of betaine aldehyde

Betaine aldehyde was synthesized in house using the reaction scheme described below: