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

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**Real-time monitoring of betaine aldehyde metabolism at high temporal resolution was accomplished using a hyperpolarized choline analog and $^{13}$C-NMR. This represents the first observation of an aldehyde intermediate on hyperpolarized MR and opens the way for kinetic studies of oxidase/dehydrogenase enzymes *in vitro* and *in vivo*.**

Betaine aldehyde is a key intermediate in the formation of betaine. In bacteria, the choline oxidase enzyme catalyzes both oxidation of choline to betaine aldehyde and oxidation of the latter to betaine.[1] In mammals, this process is catalyzed by two distinct enzymes, choline dehydrogenase (CHDH) and betaine aldehyde dehydrogenase (BADH), respectively.[2] CHDH deletion in mice, *i.e.* a deficiency in the ability to synthesize betaine aldehyde, leads to severe loss of reproductive ability in males and mitochondrial deficiencies.[3] BADH is a member of the aldehyde dehydrogenase (ALDH) superfamily, which plays an important role in the enzymatic detoxification of endogenous and exogenous aldehydes. Mutations in ALDH genes leading to defective aldehyde metabolism are the molecular basis of several diseases.[4] Betaine, the product of betaine aldehyde oxidation, is an essential nutrient.[5] In plants[6] and bacteria,[7] betaine was found to have protective effects from environmental stress. In mammals, betaine serves as: (1) an organic osmolyte;[8] (2) a chaperone-like stabilizer of protein structure under denaturing conditions;[9] and (3) a supplier of methyl units for the formation of methionine from homocysteine.

A direct measure of betaine aldehyde metabolism is thus desired for the study of various conditions. However, the measurement of betaine aldehyde in biological samples is not trivial or common.[10]

Dynamic nuclear polarization (DNP) driven $^{13}$C nuclear magnetic resonance (NMR) spectroscopy has become a promising methodology for monitoring chemical[11] and enzymatic[12] evolution of compounds *in vitro* and *in vivo*, in real time, due to its high signal enhancement (*ca.* 10 000 fold in solution).[13] This is combined with the advantages of conventional $^{13}$C-NMR, which consist of a wide spectral window (200 ppm) and a low background signal of the naturally abundant $^{13}$C nuclei (1.1%).[14] Nevertheless, despite numerous studies that demonstrated the ability to monitor a chemical conversion of substrates in a hyperpolarized state, aldehydes have not yet been observed with this technology.

The use of hyperpolarization in the current NMR spectroscopic investigation was necessary because the expected product level was less than 1 μmol and the desired temporal resolution for monitoring the reaction was 1 s. This product level can be calculated considering the following parameters: (1) the kinetic parameters of the enzyme as previously reported, $V_{max}$ of 5.3 μmol min$^{-1}$ mg$^{-1}$ enzyme and $K_m$ of 1 mM;[15] (2) a time duration of 30 s; (3) a minimum of six enzyme units (0.5 mg of enzyme); and (4) a relatively high choline concentration (2.46 mM) (ESI†). Detection and identification of products in this quantity range with such a high temporal resolution could not have been accomplished without the use of hyperpolarized MR.

Here we present the first demonstration of aldehyde formation and metabolism in real time using DNP hyperpolarized $^{13}$C-NMR.

The hyperpolarized substrate in the current study was a stable isotope-labeled choline analog, namely, [1,1,2,2-D$_4$, 2-$^{13}$C]choline chloride (CMP2), which was chosen based on its ability to serve as a reporting molecule in other metabolic conversions of choline in a DNP-induced hyperpolarized state.[16] The deuteration of the methylene positions in CMP2 prolong significantly the $T_1$ of the $^{13}$C nucleus in this molecule, and thus enables detection in a hyperpolarized state.[17] The label with $^{13}$C provides two orders of magnitude increase in the signal-to-noise ratio compared to the natural abundance of $^{13}$C.

The enzymatic oxidation of native choline (Fig. 1A) was determined using conventional, thermal equilibrium $^{1}$H-NMR. At 11 min past the reaction onset, the signals in the spectrum were assigned to the trimethylamine moieties of choline ($\delta$ = 3.19 ppm), betaine aldehyde hydrate ($\delta$ = 3.23 ppm), and betaine ($\delta$ = 3.25 ppm), see ESI.† The mean ratio of the total oxidation products (betaine aldehyde hydrate and betaine combined) to choline at this reaction time was 0.8 ± 0.3 ($n$ = 3), meaning that 44% of the substrate (choline) was already metabolized. The reaction mixture was then followed longitudinally. After 13 days, choline was fully oxidized to betaine.

To monitor the choline oxidase reaction at its very beginning, *i.e.* in the first seconds of the reaction, we turned to the

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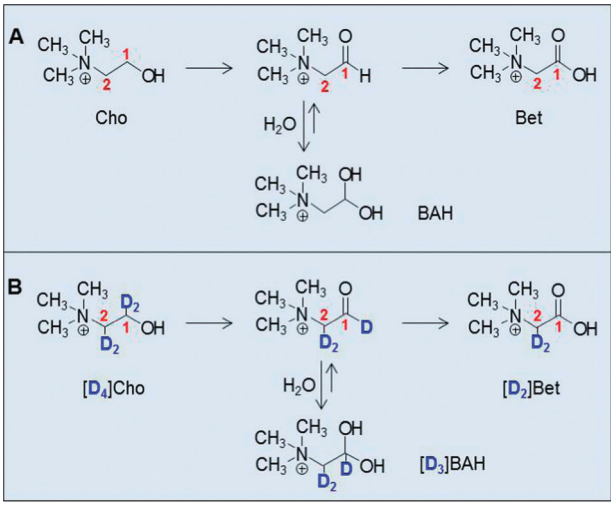


**Fig. 1** Enzymatic oxidation of choline (Cho) to betaine aldehyde hydrate (BAH) and betaine (Bet) catalyzed by choline oxidase (arrows pointing right). Panels A and B show the oxidation of native Cho and [$D_4$]Cho, respectively, and the corresponding molecular structures. Carbon positions are numbered in red.

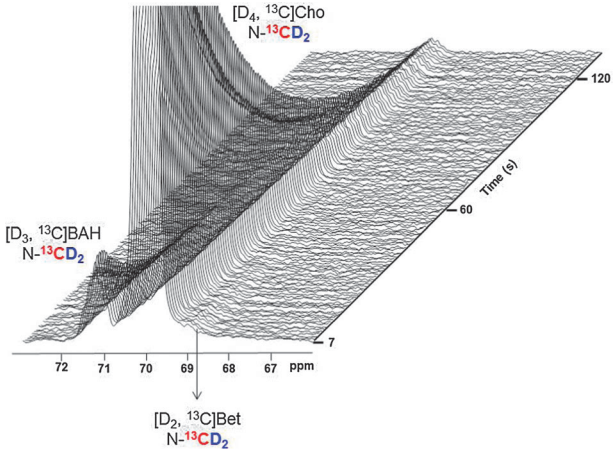


**Fig. 2** Real-time monitoring of the enzymatic conversion of [$D_4$, $^{13}$C]Cho to [$D_3$, $^{13}$C]BAH and [$D_2$, $^{13}$C]Bet using hyperpolarized CMP2 and $^{13}$C-NMR spectroscopy. [$D_4$, $^{13}$C]Cho, [1,1,2,2-$D_4$, 2-$^{13}$C]choline; [$D_3$, $^{13}$C]BAH, [1,2,2-$D_3$, 2-$^{13}$C]betaine aldehyde hydrate; [$D_2$, $^{13}$C]Bet, [2,2-$D_2$, 2-$^{13}$C]betaine. A series of 128 consecutive $^{13}$C spectra was acquired starting at 7 s after reaction onset. The formation of [$D_3$, $^{13}$C]BAH and [$D_2$, $^{13}$C]Bet is demonstrated, as well as the decay of all the signals due to relaxation.

hyperpolarized acquisition using the deuterated choline analog CMP2. However, a previous study found a significant decrease in choline oxidase activity due to deuterium substitution at the methylene positions.[18] A deuterium substitution isotopic effect on enzyme activities or other pharmacokinetic properties was previously reported for other molecular probes as well.[19] For this reason, prior to the hyperpolarized study we explored the extent of this potential isotopic effect in the current experimental conditions. This investigation was also carried out using $^1$H-NMR at thermal equilibrium and additionally using D-NMR. The conversion of a deuterated choline analog, [1,1,2,2-$D_4$]choline, to [1,2,2-$D_3$]betaine aldehyde hydrate ([$D_3$]BAH) and [2,2-$D_2$]betaine ([$D_2$]Bet), (Fig. 1B), was monitored at 12 minutes after the reaction onset. Three singlet signals were detected at 3.19 ppm, 3.23 ppm, and at 3.25 ppm and were assigned to the deuterated choline, deuterated betaine aldehyde hydrate, and deuterated betaine trimethylamine moieties (which were protonated), respectively, in agreement with the above assignment for the non-deuterated compounds (ESI†).

The mean ratio of the total deuterated oxidation products ([$D_3$]BAH and [$D_2$]Bet combined) to deuterated choline at this point in time was 0.50 ± 0.15 ($n$ = 3), meaning that 33% of the substrate (deuterated choline) was oxidized. Thus, the choline oxidation reaction yield was 25% lower in the presence of deuterated choline compared to native choline. However, this decrease was not significant ($p$ = 0.25, two tailed $t$-test). Therefore, we could not point to a clear isotopic effect of deuteration in the current specific reaction conditions.

The reaction mixture was further monitored longitudinally: at 8 days, the reaction still progressed but choline was not fully oxidized. After two months, a single trimethylamine moiety signal was visible on the $^1$H spectra. This signal was unequivocally identified as [$D_2$]Bet using a D spectrum, where only one singlet peak at 3.88 ppm appeared and was attributed to the [$D_2$]Bet deuterated methylene group. This result indicated a full conversion of deuterated choline at this time point.

These results suggested that choline oxidase activity can last for many days. This was surprising considering the dependence of the enzyme activity on the availability of oxygen. It appears that the oxygen bubbled through the medium prior to the reaction and the partial pressure of oxygen in room air (locked in the NMR tube) were sufficient for carrying out the reaction to completion, even if on a long time scale.

Nevertheless, the aim of the present study was to monitor the initial phase of the reaction in a hyperpolarized state. Despite the possible slight reduction in choline oxidase activity due to deuteration, the CMP2 probe offered an important feature for hyperpolarized magnetic resonance (MR), namely, prolongation of the $T_1$ relaxation time of the reporting $^{13}$C-labeled nucleus.[16] A typical experiment of choline oxidase reaction on hyperpolarized CMP2 is shown in Fig. 2.

Indeed, a signal of [1,2,2-$D_3$, 2-$^{13}$C]betaine aldehyde hydrate ([$D_3$, $^{13}$C]BAH) which was produced from hyperpolarized CMP2 was unequivocally visible at 71.13 ppm (Fig. 2, signal assignment is provided in the ESI†). The signal of [2,2-$D_2$, 2-$^{13}$C]betaine ([$D_2$, $^{13}$C]Bet) was visible as well at 68.87 ppm. The spectra in Fig. 2 demonstrate the decay of the hyperpolarized CMP2 signal at 69.89 ppm due to both the product synthesis and the spin lattice relaxation. In contrast, the signals of hyperpolarized [$D_3$, $^{13}$C]BAH and [$D_2$, $^{13}$C]Bet are first building up and then decaying. This behavior is a result of the synthesis of the respective compounds on one hand and the decay of their signals due to spin–lattice relaxation on the other. Owing to a high polarization level, we were able to detect also the naturally abundant $^{13}$C of the [1,1,2,2-$D_4$, 2-$^{13}$C]choline methylene group (at position 1) which resonates at 58.23 ppm (ESI†).

The time course for the signal intensities of hyperpolarized CMP2 (blue), [$D_3$, $^{13}$C]BAH (red), and [$D_2$, $^{13}$C]Bet (green) in an oxidation reaction is shown in Fig. 3. The apparent exponential decay of the substrate signal can be appreciated together with the synthesis and the decay of [$D_3$, $^{13}$C]BAH and [$D_2$, $^{13}$C]Bet, all within a temporal window of about 30 s. A kinetic analysis of

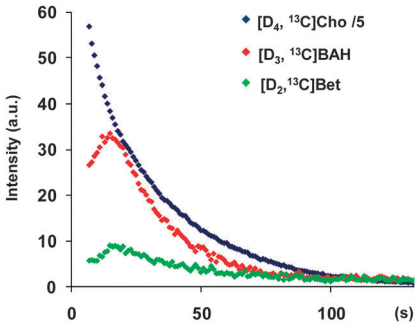


**Fig. 3** Time course of a [$D_4$, $^{13}$C]Cho oxidation reaction as observed by hyperpolarized $^{13}$C-NMR spectroscopy. The data shown here were obtained from the experiment shown in Fig. 2. The [$D_4$, $^{13}$C]Cho signal intensity was reduced 5 fold for the purpose of presentation.

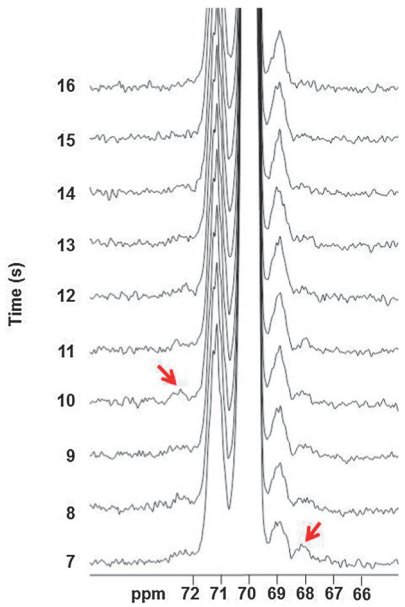


**Fig. 4** Real-time detection of choline oxidase activity as observed by hyperpolarized $^{13}$C-NMR spectroscopy at 7–16 s from reaction onset. Red arrows point to the signals of possible intermediate enzyme–product complexes.

these results yielded choline oxidase rate constants that were in agreement with the literature (ESI†).

Interestingly, in addition to the main products of this reaction, betaine aldehyde hydrate and betaine, fast acquisition in the hyperpolarized state also enabled visualization of N-$^{13}CD_2$-signals of unidentified origin at 68.0 ppm and 72.55 ppm (Fig. 4). These signals may represent hyperpolarized signals of unknown impurities containing $^{13}$C nuclei in natural abundance or in excess. However, because these signals were observed in all of the four studies described herein and not in others (see ESI†) another explanation is suggested.

A previous investigation of the kinetic mechanism of this enzyme concluded that the release of betaine aldehyde and betaine is preceded by the formation of intermediate complexes that consist of the enzyme in its reduced or oxygenated state with the product (*i.e.* betaine aldehyde or betaine).[1] In the current study, the two unidentified signals likely represented compounds that originated from hyperpolarized CMP2 (based on their line-width and chemical shift) and were detected at about 7–12 s following the reaction onset. Based on the short time scale in which these signals were detectable and the proposed action mechanism of choline oxidase, we speculate that these signals represent the enzyme–product complexes that were formed before the release of [$D_3$, $^{13}$C]BAH and/or [$D_2$, $^{13}$C]Bet.

The characterization of enzyme kinetics is an important tool in the exploration of enzymatic mechanisms and activities. Here we focused on a metabolic pathway which involves the oxidation of choline to betaine aldehyde and then to betaine and showed the feasibility of monitoring this enzymatic reaction at a high temporal resolution (1 s) using hyperpolarized MR. To the best of our knowledge, this is the first demonstration of an aldehyde intermediate in a hyperpolarized state. Thus, this finding opens the way for further direct investigation and characterization of these important groups of enzymes – aldehyde synthesizing enzymes and aldehyde dehydrogenases. Because the DNP hyperpolarized magnetic resonance technology has already proven useful for *in vivo* investigations,[20] it is likely that the investigation of aldehyde metabolism could also be carried out *in vivo*, pending an appropriate model system.

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