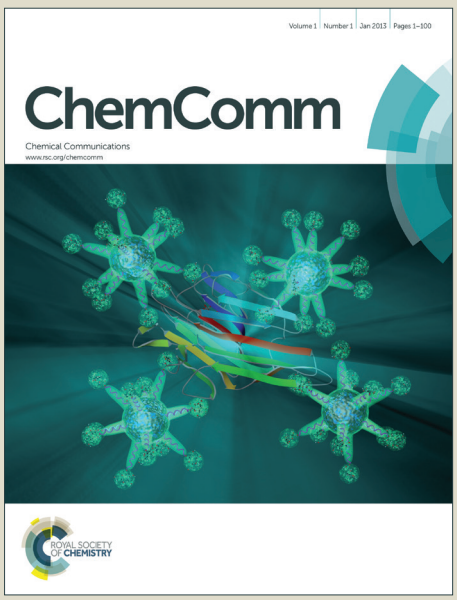
ChemComm

Accepted Manuscript



ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/chemcomm

Journal Name

RSCPublishing

COMMUNICATION

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

Cite this: DOI: 10.1039/x0xx00000x

L. J. Friesen–Waldner,[a] C. N. Wiens,[a] T. P. Wade,[a] K. Thind,[a] K. P. Sinclair,[a] Y. Hovav,[b] J. M. Gomori,[c] J. Sosna,[c] C.A. McKenzie,[¥ a] and R. Katz-Brull[¥ c] *

Received 00th January 2012,
Accepted 00th January 2012

DOI: 10.1039/x0xx00000x

www.rsc.org/

A specialized kinetic analysis of real-time hyperpolarized [1,1,2,2-D$_4$, 1-^{13}C]choline ^{13}C-magnetic resonance spectroscopy, enabled determination of initial rates of metabolic enzyme activity (choline oxidase), enzyme-substrate affinity (K_m), and inhibition. In a clinical MRI scanner, metabolite levels lower than 16 µM were detected at a temporal resolution of 1 s.

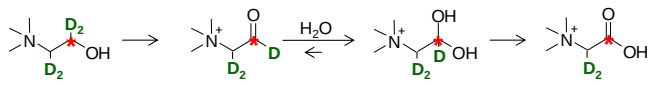
Michaelis-Menten kinetics is a fundamental concept in enzymology. The Michaelis-Menten constant, K_m, is a value that characterizes the actual affinity of an enzyme to the substrate *in situ*. Knowledge of K_m is useful for predicting the enzymatic activity in the presence of a specific substrate concentration and enzyme inhibition. The determination of K_m usually involves running a series of enzyme assays at varying substrate concentrations and measuring the initial reaction rates. However, obtaining these initial rates can be a non-trivial issue for some metabolic products and fast reactions. Initial rates are typically best sampled during the first few seconds of enzyme operation. However, typical biochemical assays have several drawbacks with respect to the direct measurements of these initial rates at these time scales.

1) Assays that utilize radioactive substrates require elaborate biochemical analysis for determination of the chemical identity of the substrate and products, which are rarely carried out at the temporal resolution on the order of a second or in a continuous manner; 2) Assays that rely on changes in spectrophotometric properties of a cofactor or a specific part of the enzyme are typically very quick but determine product formation indirectly; 3) Assays that rely on the release of a by-product that leads to changes that can be sensed in the medium (pH, voltage, *etc.*) can be fairly rapid but are indirect.

Hyperpolarized ^{13}C magnetic resonance spectroscopy (MRS) is a new tool to visualize enzymatic processes in a continuous manner, without sample manipulation [1, 2]. This technology capitalizes on 1) the unique ability of ^{13}C chemical shifts to provide direct and detailed metabolite identification and 2) the extreme signal enhancement of the hyperpolarized technology - more than 10,000 fold compared to thermal equilibrium ^{13}C-MRS [1]. This signal enhancement enables detection of much lower metabolite concentrations in a much shorter time scale (here a temporal resolution of 1 s). We propose that this technology is now also useful for determining initial rates and thereby enzyme-substrate affinity. The initial rates are extracted from the data *via* a newly developed approach for kinetic analysis of real-time hyperpolarized ^{13}C-MRS of consecutive reactions [3].

Several previous studies dealt with kinetic analysis of reactions that were monitored using hyperpolarized nuclei. For instance, Zeng *et al.* [4] interrogated a Diels-Alder reaction, Miéville *et al.* [5] interrogated the exchange between free and bound hyperpolarized ions, Jamin *et al.* [6] monitored the activity of a carboxypeptidase enzyme, and Lerche *et al.* [7] examined ligand binding. Harrison *et al.* [8] compared kinetic models for analysis of pyruvate-to-lactate exchange. All of the above investigated processes did not represent consecutive enzymatic reactions which are relevant for a chain of metabolic events and none has determined the Michaelis-Menten constant for enzyme-substrate affinity.

We have previously shown that the two consecutive reactions of the enzyme choline oxidase (Scheme 1) can be monitored by ^{13}C-MRS and hyperpolarized molecular probes [3, 9]. Here we used one of these probes, namely, [1,1,2,2-D$_4$, 1-^{13}C]choline chloride (CMP1), in a hyperpolarized state, as a substrate for this enzyme. Choline oxidase



[D$_4$,1-^{13}C]Cho (CMP1) [D$_3$,1-^{13}C]BA [D$_3$,1-^{13}C]BAH [D$_2$,1-^{13}C]Bet

Scheme 1. Choline oxidase reactions with CMP1 as a substrate. The red asterisk marks the ^{13}C labelled carbon position. D – deuterium. Cho, choline; BA, betaine aldehyde; BAH, betaine aldehyde hydrate; Bet, betaine. BA is 99% hydrated, i.e. in a BAH form, in aqueous solutions [10].

is the enzyme that catalyses both the oxidation of choline to betaine aldehyde and the oxidation of the latter to betaine in bacteria [11, 12]. In mammals and aquatic invertebrates, this process is catalysed by two distinct enzymes, choline dehydrogenase and betaine aldehyde dehydrogenase, respectively [13, 14]. This metabolic conversion is important to humans and animals [14-16], plants [17], and bacteria [18]. Betaine was found to be an essential nutrient and to have protective

effects from environmental stress, by serving as (1) an organic osmolyte [19]; (2) a chaperone-like stabilizer of protein structure under denaturing conditions [20]; and (3) a supplier of methyl units for the formation of methionine from homocysteine for the benefit of numerous methylation processes. Thus, monitoring of the activity and inhibition of choline oxidative enzymes in bacteria, plants, animals, and humans holds environmental and medical value.

The choline oxidase reactions were performed using CMP1 in a hyperpolarized state at increasing concentrations, from 0.25 mM to 17.5 mM. Examples of the time courses of the hyperpolarized signals of [1,1,2,2-D_4, 1-^{13}C]choline ([D_4, ^{13}C]Cho) and the resulting [1,2,2-D_3, 1-^{13}C]betaine aldehyde hydrate ([D_3, ^{13}C]BAH) and [2,2-D_2, 1-^{13}C]betaine ([D_2, ^{13}C]Bet) are shown in Figure 1. The signal assignments and analysis of the signal split patterns for these compounds (Figure 1A) were previously reported [3]. The rates of the reactions for each dose were calculated using a kinetic model that was described previously [3]. Briefly, based on prior investigations, each of the choline oxidase reactions was assumed to occur with irreversible [21] first order [10] kinetics. The rate of the first reaction, in which the intermediate betaine aldehyde hydrate is formed, is termed here K_1 and the rate of the second reaction, in which betaine is formed, is termed here K_2. The three signal intensity time courses (for [D_4, ^{13}C]Cho, [D_3, ^{13}C]BAH, and [D_2, ^{13}C]Bet) were simultaneously simulated with varying rate constants, relaxation rate constants, and reacting portion of the substrate, taking into account the effects of the detection pulses, given with a constant flip angle at equal time intervals. This simulation was carried out for each experiment, *i.e.* for each substrate concentration, independently. The solid lines in Figure 1B and 1C represent such simulations that best fitted the experimental data, both in terms of R^2 and visually. The R^2 values for all concentrations were above 0.99 for the [D_4, ^{13}C]Cho curves and averaged 0.9 for the [D_3, ^{13}C]BAH curves. For the [D_2, ^{13}C]Bet curves that showed sufficient SNR, the R^2 values ranged between 0.64 and 0.95. Experiments in which the Bet signal SNR was lower than 2 and showed lower R^2 values for the [D_2, ^{13}C]Bet curves were rejected from K_2 analysis.

The rate values that resulted in these best fit simulations are summarized in Figure 2. K_1 increased with increasing CMP1 concentration and plateaued at about 3 mM. The values of K_1 *vs.* CMP1 concentration were then fitted to the Michaelis-Menten equation, resulting in a K_m of 0.9 mM and a V_{max} of 2.0 µmol/min/mg enzyme (R^2 = 0.85). The Michaelis-Menten curve corresponding to these parameters is plotted in Figure 2 (dashed line). A maximal value for K_2 was observed at 1 mM CMP1 concentration, after which, K_2 appeared to decrease with increasing CMP1 concentration. At up to 8 mM of CMP1, [D_2, ^{13}C]Bet synthesis was observed, but, at 17.5 mM, [D_2, ^{13}C]Bet signals could not be detected.

The experiments at 3 mM and 17.5 mM were performed 3 times each for calculations of reproducibility. The standard deviations of K_1 determined in these experiments were 0.94 and 0.80 µmol/min/mg enzyme, respectively, corresponding to 50 % and 41 % of the mean of each group. At 3 mM, the standard deviation of the K_2 values was 0.26 µmol/min/mg enzyme, corresponding to 56 % of the mean of this group. All of the experiments at 17.5 mM did not show [D_2, ^{13}C]Bet synthesis and therefore the corresponding K_2 could not be calculated. Thus it was concluded that the error for each individual determination of K_1 or K_2 is of the order of 50 %. Because the K_m and V_{max} values are derived from a curve fit to all of the K_1 values, a larger number of samples (CMP1 concentrations) or further repetitions of each concentration are likely to result in a better estimation of K_m and V_{max} and reduce the contribution of the error associated with each individual determination of K_1 or K_2 by hyperpolarized ^{13}C-MRS.

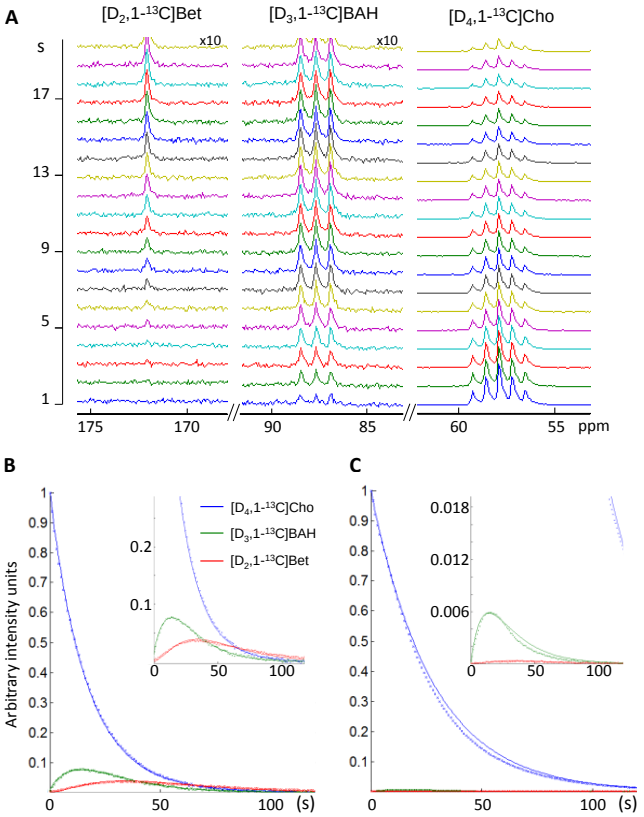


Figure 1. ^{13}C spectra and time courses of the choline oxidase reaction using CMP1 as a substrate.
A – ^{13}C spectra of the reaction in the presence of 0.25 mM of the substrate. The spectra were recorded every 1 s. The first 20 spectra (out of 256 spectra recorded) are shown.
B – Time course of the reaction in the presence of 0.25 mM of the substrate. Individual points represent the experimental data at the acquired temporal resolution. The solid lines represent the simulated time course that was produced by the kinetic model [3] and best fitted the experimental data.
C – Time course of the reaction in the presence of 8 mM of the substrate. The insets in B and C are provided for a close-up inspection of the fit to the experimental data of product formation.

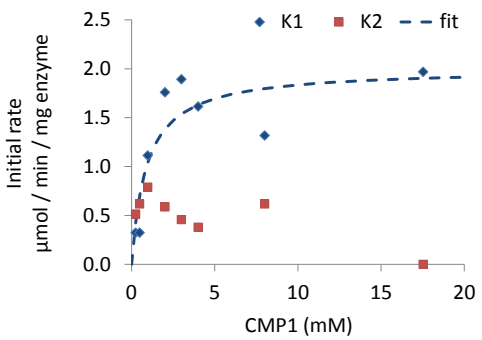


Figure 2. K_1 and K_2 as a function of CMP1 concentration. The dashed line corresponds to the Michaelis-Menten curve that best fitted the K_1 values.

Nevertheless, the K_m that was found here for [D_3, ^{13}C]BAH synthesis (0.9 mM) is in agreement with previous studies. Stemple *et al.* have similarly found that the activity of choline oxidase from Alcaligenes reached saturation at about 3 mM and determined a K_m

of 0.77 mM [22]. Also in Alcaligenes, Ohta-Fukuyama *et al.* found that the K_m for choline oxidase activity was 0.87 mM [23]. In Arthrobacter-globiformis, Ikuta *et al.* have found a K_m of 1.2 mM [11].

Betaine was previously reported to act as a competitive inhibitor for choline oxidase from bacteria [10]. Betaine aldehyde hydrate as well as betaine were previously shown to act as competitive inhibitors of choline dehydrogenase in oysters [14]. In agreement, the trimethylammonium headgroup was previously shown to be a major determinant for substrate binding and specificity in choline oxidase [24]. Thus, the current finding, that [D_2, ^{13}C]Bet synthesis is reduced at increasing CMP1 concentrations most likely reflects inhibition of this synthetic step by [D_4, ^{13}C]Cho, [D_3, ^{13}C]BAH, or [D_2, ^{13}C]Bet itself. Such an inhibition could indicate either substrate inhibition or product inhibition and the nature of the inhibition could be competitive (binding at the active site) or not-competitive (binding at a non-active site with low affinity). A previous report by Fan *et al.* supports the former, showing by spectral changes in the near-UV and visible regions of the flavin absorbance spectrum that betaine binding to the enzyme occurs at the active site [10]. Further studies are required to clarify whether [D_4, ^{13}C]Cho and [D_3, ^{13}C]BAH are also involved in the inhibition mechanism and the nature of this inhibition. Nevertheless, we note that the real-time simultaneous determination of both K_1 and K_2 for these two consecutive reactions opens a new window for investigation of such processes.

We note that the signal enhancement offered by DNP hyperpolarization enabled the detection of very low concentrations of newly-formed metabolites with a temporal resolution of 1 s. To estimate the actual lowest concentration of a newly-formed metabolite detected in the current study we chose to start with the reaction at 17.5 mM of CMP1 and the calculation of [D_3, ^{13}C]BAH level. This is because [D_3, ^{13}C]BAH is not further converted to [D_2, ^{13}C]Bet under this condition and thus the calculation is simplified. The K_1 for the reaction at this CMP1 concentration was 1.97 µmol/min/mg enzyme. Thus, at about 10 s, the amount of [D_3, ^{13}C]BAH is expected to be about 0.6 µmol (1.97 µmol/min/mg enzyme * 1.8 mg enzyme * 10/60 min). Considering the reaction volume of 4.8 ml, the concentration of [D_3, ^{13}C]BAH in this experiment at this time point was therefore, roughly, 0.1 mM. However, metabolites at even lower signal and formation rates were detected. At the lower CMP1 concentrations, K_1 was about 6-fold lower and the consecutive reaction led to a reduction in [D_3, ^{13}C]BAH and yielded [D_2, ^{13}C]Bet at consistently lower amounts than [D_3, ^{13}C]BAH. The metabolite that was detected with the lowest SNR in the current study was [D_2, ^{13}C]Bet. Thus, if we attempt to estimate an upper bound to the level of [D_2, ^{13}C]Bet detected in the current study it can be safely estimated at 16 µM (one sixth of 0.1 mM).

We note that such a low concentration cannot be detected at a 1 s temporal resolution with thermal equilibrium ^{13}C- or ^{1}H-MRS, even in high resolution NMR spectrometers. The fact that such a low concentration was detected at such high temporal resolution on a clinical MRI scanner at a clinically approved magnetic field (3T) raises hope that the approach presented here would enable in the future determination of enzyme-substrate affinity in the live body. This is limited by the concentration of substrate and the T_1 in the specific *in vivo* study. Thus, it remains to be seen if this approach could be beneficial for target validation of drugs that rely on metabolism or inhibition of metabolism, *in vivo*.

Conclusions

Using a well-studied enzymatic process (bacterial choline oxidase) and a comparison between the hyperpolarized ^{13}C-MRS results and the results obtained using classical biochemical techniques, we demonstrate that the kinetic analysis approach for real-time hyperpolarized ^{13}C-MRS data is valid for determination of K_m and enzyme inhibition in complex metabolic reactions. This finding presents a new and unique tool for metabolic research, likely to teach new information on enzyme activity *in situ* (without sample manipulation).

Notes and references

[a] Department of Medical Biophysics, Western University, London, Ontario, Canada.

[b] Weizmann Institute of Science, Rehovot, Israel.

[c] Department of Radiology, Hadassah-Hebrew University Medical Center, Jerusalem, Israel.

[¥] Equal contribution

[*] To whom correspondence should be addressed

† Electronic Supplementary Information (ESI) available. See DOI: 10.1039/c000000x/

The authors thank Shimon Vega for useful discussions in the course of this study. This work was partially funded by the Israel Science Foundation (Grant No. 284/10 to RKB), BrainWatch Ltd., the European Research Council (ERC-2013-StG 338040), and the Ontario Research Fund.

1. J. H. Ardenkjaer-Larsen, B. Fridlund, A. Gram, G. Hansson, L. Hansson, M. H. Lerche, R. Servin, M. Thaning and K. Golman, *Proc. Natl. Acad. Sci. USA*, 2003, **100**, 10158-10163.
2. K. R. Keshari and D. M. Wilson, *Chem. Soc. Rev.*, 2014, **43**, 1627-1659.
3. H. Allouche-Arnon, Y. Hovav, L. Friesen-Waldner, J. Sosna, J. M. Gomori, S. Vega and R. Katz-Brull, *NMR Biomed.*, 2014, **27**, 656-662.
4. H. F. Zeng, Y. Lee and C. Hilty, *Anal. Chem.*, 2010, **82**, 8897-8902.
5. P. Mieville, S. Jannin, L. Helm and G. Bodenhausen, *J. Am. Chem. Soc.*, 2010, **132**, 5006-5007.
6. Y. Jamin, C. Gabellieri, L. Smyth, S. Reynolds, S. P. Robinson, C. J. Springer, M. O. Leach, G. S. Payne and T. R. Eykyn, *Magn. Reson. Med.*, 2009, **62**, 1300-1304.
7. M. H. Lerche, S. Meier, P. R. Jensen, H. Baumann, B. O. Petersen, M. Karlsson, J. O. Duus and J. H. Ardenkjaer-Larsen, *J. Magn. Reson.*, 2010, **203**, 52-56.
8. C. Harrison, C. Yang, A. Jindal, R. J. DeBerardinis, M. A. Hooshyar, M. Merritt, A. D. Sherry and C. R. Malloy, *NMR Biomed.*, 2012, **25**, 1286-1294.
9. H. Allouche-Arnon, A. Gamliel, J. Sosna, J. M. Gomori and R. Katz-Brull, *Chem Commun*, 2013, **49**, 7076-7078.
10. F. Fan, M. W. Germann and G. Gadda, *Biochemistry*, 2006, **45**, 1979-1986.
11. S. Ikuta, S. Imamura, H. Misaki and Y. Horiuti, *J. Biochem.*, 1977, **82**, 1741-1749.
12. G. Gadda, *BBA-Proteins Proteomics*, 2003, **1646**, 112-118.
13. S. A. S. Craig, *Am. J. Clin. Nutr.*, 2004, **80**, 539-549.
14. L. A. Perrino and S. K. Pierce, *J. Exp. Zool.*, 2000, **286**, 250-261.
15. P. M. Ueland, *J. Inherited Metab. Dis.*, 2011, **34**, 3-15.
16. N. O'Donoghue, T. Sweeney, R. Donagh, K. J. Clarke and R. K. Porter, *Biochim. Biophys. Acta-Bioenergetics*, 2009, **1787**, 1135-1139.
17. A. Sakamoto and N. Murata, *Plant, Cell Environ.*, 2002, **25**, 163-171.
18. M. Ghoul, T. Bernard and M. Cormier, *Appl. Environ. Microbiol.*, 1990, **56**, 551-554.
19. M. Lever and S. Slow, *Clin. Biochem.*, 2010, **43**, 732-744.
20. S. A. Kempson and M. H. Montrose, *Pflug. Arch. Eur. J. Phy.*, 2004, **449**, 227-234.
21. G. Gadda, *BBA-Proteins Proteomics*, 2003, **1650**, 4-9.
22. J. Z. Stemple, K. M. Rusin and T. L. Fare, *Anal. Chem.*, 1991, **63**, 1050-1052.
23. M. Ohta-Fukuyama, Y. Miyake, S. Emi and T. Yamano, *J. Biochem.*, 1980, **88**, 197-203.
24. G. Gadda, N. L. N. Powell and P. Menon, *Arch. Biochem. Biophys.*, 2004, **430**, 264-273.