

Development of a post-reaction derivatisation and fluorescence assay for 2-OG dependent dioxygenases

L.A. McNeill, K.S. Hewitson, C.J. Schofield, Chemistry Research Laboratory, Mansfield Road, Oxford OX1 3TA, UK
L. Bethge, Humboldt Universität zu Berlin, Institut für Chemie, Brook-Taylor-Str.2, 12489 Berlin, Germany

Application Note 132

Rev. 02/2005



- NOVOstar was used both as incubator and reader for a new assay measuring the consumption of 2-OG by hydroxylase enzymes
- In plates, the calculated kinetic constants were similar to those gathered by other methods
- Assay is amenable to high-throughput screening of molecules that may modulate the activity of this important class of enzyme

Introduction

Members of the family of iron(II) and 2-oxoglutarate (2-OG) dependent dioxygenases fulfil a wide range of functions in natural systems.¹ The most popular activity assay has previously relied on detecting the release of radiolabelled carbon dioxide from an appropriately labelled 2-OG molecule decarboxylated during the reaction. This procedure is time-consuming, tedious and is not easily converted for use in high-throughput or screening assays. The release of radioactive gas is also a point of concern.

This application note describes a novel assay that relies on post-reaction derivatisation of residual 2-OG to determine the extent of enzyme reaction. Use of the NOVOstar both to perform the incubation and to detect the fluorescent product is explained.

The involvement of enzymes of the family in the hypoxic response system in animals has been characterised recently.² Assay of both a prolyl hydroxylase enzyme and an asparaginyl hydroxylase enzyme is described.

Materials and Methods

Enzymes were produced recombinantly by expression in *E.Coli* from the pET28a(+) vector (Novagen). Recombinant substrate was produced from the pGEX6p-1 vector (Amersham Pharmacia Biotech). Synthetic peptide substrates were from Peptide Protein Research Ltd., Fareham, UK. Other materials were from Acros, Sigma, or Melford. Consumables were used as needed from several manufacturers.

The assay of FIH (factor-inhibiting hypoxia-inducible factor (HIF)) activity was carried out by mixing 1 mM DTT (dithiothreitol), 0.6 mg/mL catalase, 2-OG, substrate and 50 mM Tris/HCl pH 7.5 to a final volume of 44 μ L and warming to 37°C for 5 minutes. Concurrently, the enzyme and iron (prepared as 500 mM stock in 20 mM HCl, and diluted with water) were mixed at room temperature for 3 minutes. The reaction was initiated by using the NOVOstar to add 6 μ L of enzyme/iron mix to the substrate/cofactor mix. The reaction was stopped after 5 minutes by addition of 100 μ L 0.5 M HCl, also by the NOVOstar; derivatisation was then achieved by the addition of 50 μ L 10 mg/mL o-phenylene diamine (OPD) in 0.5 M HCl by hand, and heating for 10 minutes at 95°C. After centrifugation for 5 minutes, the supernatant (50 μ L) was made basic by the addition of 30 μ L 1.25 M NaOH and the fluorescence was measured on a NOVOstar with the excitation filter at 340 nm and the emission filter at 420 nm. Assay of prolyl-hydroxylase 2 (PHD2) followed the same procedure but the buffer used was 50 mM HEPES pH 7.0. In assays with inhibitors, the final mix was 1 mM DTT, 0.6 mg/mL catalase, 500 μ M 2-OG, 500 μ M GST HIF1 α (786-826), 1 mM

test compound and 50 mM Tris/HCl pH 7.5 with 4 μ M FIH and 50 μ M iron(II), incubated at 37°C for 15 minutes. Km values were calculated by direct fitting of the Michaelis - Menten equation to the data, and experimental points are quoted as a mean of at least three independent measurements.

For the incubation reaction, all cofactors and substrate were placed in a V-bottomed 96-well plate and equilibrated at 37°C for 2 minutes. The pipettor was used to inject 6 μ L of enzyme mixture into each well (100 μ L/s) after which the plate was shaken and the reaction allowed to proceed for a specified time, where upon 100 μ L 0.5 M HCl was injected through pump 2 (420 μ L/s). The plate was removed and 50 μ L of 10 mg/mL OPD in 0.5 M HCl was added. Plate was incubated at 90°C for 20 minutes and then any precipitate was removed by centrifugation. 50 μ L supernatant was placed into a flat, clear bottomed 96-well plate. The NOVOstar pump 3 was used to inject 30 μ L 1.25 M NaOH into each well, followed by shaking and reading of each well. Default settings (10 flashes, 0.2 s positioning delay, and 2 cycles) were applied for both incubation and reading on the NOVOstar (software version: 1.20-0). Gains were set using the maximum fluorescence on each plate, with standards to ensure uniformity between plates.

Results

Variation of incubation time and substrate concentrations allowed the determination of Km values for 2-OG and all the available peptide substrates for both enzymes.

These compared favourably with those determined previously using the radiolabelled assay, showing that this new assay, while probably unable to give yield detailed kinetic and mechanistic information, may easily be used for high-throughput assays for new substrates and/or inhibitors for these important enzymes.

Table 1: Km values for 2-OG and peptide substrates determined using the fluorescence derivatisation assay. n.d.= not determined. n.r. = not reported. *Activity with this shorter peptide (CAD 19mer) was reported in ref. 5 to be only 9% of that with the longer CAD 35mer. Dashed cells imply a combination of enzyme and substrate that does not productively occur in humans. Values have been corrected for the consumption of 2-OG in the absence of peptide substrate.

Substrate	Enzyme				
	PHD2 Ref. 3	PHD3 Ref. 4	PHD2 (this study)	FIH Ref. 5	FIH (this study)
2-OG	60 μ M	3 μ M (using CODD 19mer)	(25 \pm 6) μ M	25 μ M (using CAD 35mer)	(114 \pm 25) μ M (using CAD 35mer)
CODD 19mer	7 μ M	n.d.	(4 \pm 1) μ M	–	–
CAD 19mer	–	–	–	n.r.*	(420 \pm 50) μ M
CAD 35mer	–	–	–	100 μ M	(205 \pm 30) μ M
GST-HIF1 α (786-826)	–	–	–	n.r.	(285 \pm 35) μ M

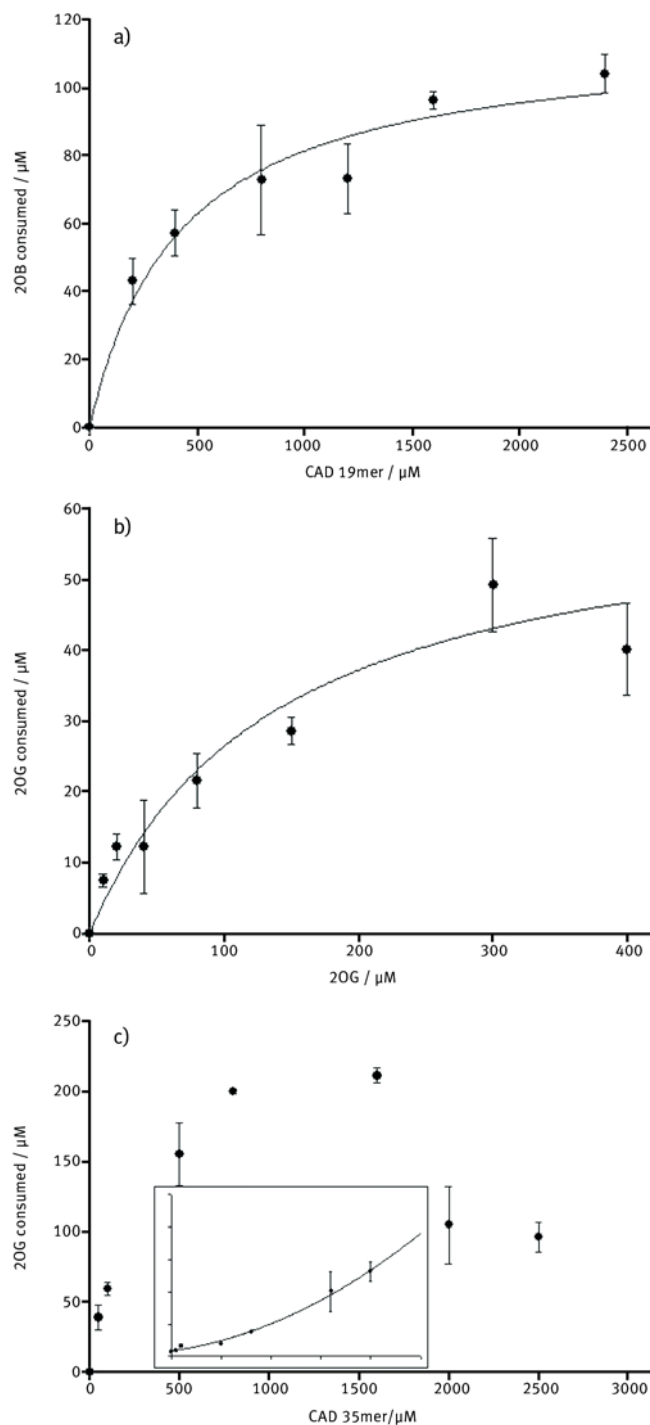


Fig. 1: Determination of the K_m values for (a) the synthetic CAD 19mer substrate in FIH at 500 μM 2-OG, (b) 2-OG in PHD2 at 100 μM CODD 19mer, and (c) the synthetic CAD 35mer substrate in FIH at 500 μM 2-OG. In all cases, the enzyme concentration was 4 μM . In (a) and (b), direct plots are shown of activity vs. substrate concentration. Values were estimated by direct fitting of the Michaelis-Menten equation to these data. In (c), the activity vs. substrate concentration plot is shown, and the Hanes plot of the same data is shown in an inset, showing the characteristic parabolic shape indicative of substrate inhibition. The K_m value was calculated from points up to and including 1.6 mM CAD 35mer, and was similar if calculated from the direct plot or the Hanes plot.

Conclusion

In summary, we have developed an assay for the consumption of 2-OG in 2-OG oxygenase catalysis that is safer and simpler than the existing procedure that is based on the release of radioactive CO_2 . The new procedure does not involve the evolution and capture of gas, or the use of radioactive isotopes. OPD is toxic but is non-volatile and is contained within the assay vessel. This assay procedure also lends itself well to the high-throughput multi-well plate format.

References

1. R. P. Hausinger, Fe(II)/ α -ketoglutarate-dependent hydroxylases and related enzymes, *Crit Rev Biochem Mol Biol*, **39** (2004) 21-68.
2. C. J. Schofield, P. J. Ratcliffe, Oxygen sensing by HIF hydroxylases, *Nat Rev Mol Cell Biol*, **5** (2004) 343-354.
3. M. Hirsila, P. Koivunen, V. Gunzler, K. I. Kivirikko, J. Myllyharju, Characterization of the human prolyl 4-hydroxylases that modify the hypoxia-inducible factor, *J Biol Chem*, **278** (2003) 30772-30780.
4. F. Oehme, W. Jonghaus, L. Narouz-Ott, J. Huetter, I. Flamme, A non-radioactive 96-well plate assay for the detection of hypoxia-inducible factor prolyl hydroxylase activity, *Anal Biochem*, **330** (2004) 74-80.
5. P. Koivunen, M. Hirsila, V. Gunzler, K. I. Kivirikko, J. Myllyharju, Catalytic properties of the asparaginyl hydroxylase (FIH) in the oxygen sensing pathway are distinct from those of its prolyl 4-hydroxylases, *J Biol Chem*, **279** (2004) 9899-9904.

Reprinted from *Analytical Biochemistry*, Vol 336, No 1, 2005, L.A. McNeill et al., "A Fluorescence-based assay for 2-oxoglutarate-dependent oxygenases", pp 125-131, Copyright 2005, with permission from Elsevier.

Headquarters:

Germany:	BMG LABTECH GmbH	Tel: +49 781 96968-0
Australia:	BMG LABTECH Pty. Ltd.	Tel: +61 3 59734744
China:	BMG LABTECH Co. Ltd.	Tel: +86 10 6424063
France:	BMG LABTECH SARL	Tel: +33 1 48862020
UK:	BMG LABTECH Ltd.	Tel: +44 1296 336650
USA:	BMG LABTECH Inc.	Tel: +1 919 806 1735
	www.bmglabtech.com	info@bmglabtech.com