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Studies on the activity of the hypoxia-inducible-factor hydroxylases using an oxygen consumption assay

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The activity and levels of the metazoan HIF (hypoxia-inducible factor) are regulated by its hydroxylation, catalysed by 2OG (2-oxoglutarate)- and Fe(II)-dependent dioxygenases. An oxygen consumption assay was developed and used to study the relation-ship between HIF hydroxylase activity and oxygen concentration for recombinant forms of two human HIF hydroxylases, PHD2 (prolyl hydroxylase domain-containing protein 2) and FIH (factor inhibiting HIF), and compared with two other 2OG-dependent dioxygenases. Although there are caveats on the absolute values, the apparent K_m (oxygen) values for PHD2 and FIH were within the range observed for other 2OG oxygenases. Recombinant protein substrates were found to have lower apparent K_m (oxygen)

INTRODUCTION

The α/β -heterodimeric protein HIF (hypoxia-inducible factor) is a central regulator in the response of metazoans to hypoxia [1,2]. Human HIF regulates the transcription of genes encoding proteins involved in glycolysis, erythropoiesis and angiogenesis that counteract the effects of hypoxia [3,4]. Manipulating the hypoxic response is of therapeutic interest both from the perspectives of cancer (inhibiting HIF activity) and ischaemic disease (promoting HIF activity). In contrast with the levels of the β -subunit, which is constitutively expressed [5], both the activity and levels of the HIF- α subunits are regulated by oxygen.

In both the HIF-1 α and HIF-2 α forms of HIF- α , the N-terminal domain contains subdomains that enable dimerization with HIF-1 β and binding to response elements linked to hypoxic response genes [6]. The central HIF-1 α region contains the structural elements involved in its oxygen-dependent proteolytic degradation. In the presence of sufficient oxygen, HIF- α undergoes trans-4-hydroxylation of two proline residues within LXXLAP motifs (Pro⁴⁰² and Pro⁵⁶⁴ in human HIF-1 α) [7,8]. This modification enables binding of the HIF- α subunits to the pVHL (von Hippel–Lindau protein), which targets HIF- α for ubiquitination and proteasomal degradation. Human HIF-1 α and HIF-2 α also possess a conserved site of asparagine hydroxylation (Asn⁸⁰³ of HIF-1 α) in their CADs (C-terminal transcriptional activation domains) [9]. Hydroxylation at the *pro-S* β -position of Asn⁸⁰³ [10] ablates the interaction between HIF and the transcriptional coactivator p300, thus blocking HIF-mediated transcription [11,12].

In humans, HIF- α proline hydroxylation is catalysed by three Fe(II)-dependent dioxygenases PHD1–3 (proline hydroxylase domain-containing enzymes 1–3; or EGLN2/1/3 or HPH3/2/1),

values compared with shorter synthetic peptides of HIF. The analyses also suggest that human PHD2 is selective for fragments of the C-terminal over the N-terminal oxygen-dependent degradation domain of HIF-1 α . The present results, albeit obtained under non-physiological conditions, imply that the apparent $K_{\rm m}$ (oxygen) values of the HIF hydroxylases enable them to act as oxygen sensors providing their *in vivo* capacity is appropriately matched to a hydroxylation-sensitive signalling pathway.

Key words: dioxygenase, glucose oxidase (GOX), hydroxylase, hypoxia-inducible factor (HIF), 2-oxoglutarate (2OG), oxygen consumption assay.

with closely related catalytic domains [13,14]. Asparagine hydroxylation of the HIF- α CAD is also catalysed by a dioxygenase, FIH (factor inhibiting HIF) [15,16]. The four identified human HIF hydroxylases belong to the non-haem Fe(II) and 2OG (2oxoglutarate) dioxygenase superfamily [3,17,18]. Like most other family members they catalyse the incorporation of one oxygen atom from dioxygen into their alcohol product and one into the succinate co-product [15,19,20].

Using baculovirus-produced PHDs, Hirsilä et al. [21] reported K_m (oxygen) values for the PHDs (230–250 μ M) that were higher than that of human procollagen proline 4-hydroxylase (40 μ M), an observation interpreted as being significant in terms of the role of the PHDs as oxygen sensors. Koivunen et al. [22] reported a lower K_m (oxygen) value for FIH (90 μ M) and argued that a larger decrease in oxygen concentration was required for a significant decrease in FIH activity compared with the PHDs. These studies on the PHDs and FIH were conducted using crude baculovirus extracts and peptide fragments of HIF- α . However, there is evidence that short fragments of HIF- α may not be representative substrates for the intact protein, e.g. a 35-residue fragment of the HIF- α CAD is reported to be a better substrate for FIH than a 19-residue fragment, and the minimum effective substrate reported for PHD2 is approx. 17 residues long [21–23].

To address the question of whether, particularly in terms of their oxygen-binding characteristics, the HIF hydroxylases have unusual features within the 2OG dioxygenase superfamily, we developed an assay that continuously measures oxygen consumption to investigate the oxygen and co-substrate dependence of FIH and PHD2 using a range of substrates. Comparison of the data with other 2OG-dependent dioxygenases (Figure 1) indicates that the K_m (app; oxygen) values of the HIF hydroxylases are likely within

Abbreviations used: c, calculated; CAD, C-terminal transcriptional activation domain; CODD, C-terminal oxygen-dependent degradation domain; ESI-MS, electrospray ionization MS; FIH, factor inhibiting HIF; GOX, glucose oxidase; HIF, hypoxia-inducible factor; NODD, N-terminal oxygen-dependent degradation domain; o, observed; 2OG, 2-oxoglutarate; PAHX, phytanoyl-CoA hydroxylase; mPAHX, mature PAHX; PHD, prolyl hydroxylase domaincontaining protein; SPR, surface plasmon resonance; TauD, taurine dioxygenase.

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Figure 1 Reactions catalysed by the non-haem Fe(II)- and 2-OG-dependent dioxygenases used in the present study

(A) PHDs, (B) FIH, (C) TauD and (D) mPAHX.

the typical range for the 2OG dioxygenases. Together with biophysical data, the results imply that PHD2 is selective in binding CODD (C-terminal oxygen-dependent degradation domain) over the NODD (N-terminal oxygen-dependent degradation domain) fragment of HIF-1 α , and that catalytic efficiency is dependent on the substrate length. The present study has implications both for modelling the role of the HIF hydroxylases in the hypoxic response and for the design of *in vitro* assays to identify HIF hydroxylase inhibitors aimed at inducing the hypoxic response.

EXPERIMENTAL

Materials

Chemicals were obtained from Sigma–Aldrich, oxygen/nitrogen gas mixtures were from BOC Gases, and synthetic peptides were from Peptide Protein Research Ltd, Fareham, Hants. U.K.

Expression and purification of recombinant 20G-dependent dioxygenases and substrates

FIH and PHD2^{181–426} were produced essentially as reported in [15,24]. In brief, the full-length *fih* gene and an N-terminally truncated *phd2* gene encoding PHD2^{181–426} were subcloned into the pET-28a(+) vector (Novagen) as NdeI/BamHI or NheI/BamHI fragments. Proteins were expressed in *Escherichia coli* BL21(DE3) in 2YT [1.6% (w/v) tryptone, 1% (w/v) yeast extract and 0.5% (w/v) NaCI] growth medium. Cells were induced with 0.5 mM isopropyl β -D-thiogalactoside when $D_{600} = 0.8-1.0$ and growth was continued at 37°C for 4 h before cell harvest. Purification of the N-terminally His₆-tagged (N-MGSSHHHHH-HSSGLVPRGSH) proteins were carried out using nickel-affinity chromatography on HisBind-IDA resin (Novagen) according to the manufacturer's instructions. Cleavage of the affinity tag

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was achieved by incubation of the fusion protein for 16 h at 4 °C with thrombin (Novagen). Desalting on a 300 ml Superdex-75 column (GE Healthcare) yielded approx. 95% pure protein as judged by SDS/PAGE analysis. Proteins were characterized by ESI–MS (electrospray ionization MS): FIH c (calculated) 40566, o (observed) 40569 Da; PHD2^{181–426} c28056, o28056 Da; and His₆–PHD2^{181–426} c29807, o29805 Da. For SPR (surface plasmon resonance) analyses, the sequence encoding PHD2^{181–426} was subcloned into the pET-24a(+) vector (Novagen) to produce protein lacking an N-terminal His₆ tag, expressed as described above, then purified by cation-exchange and size-exclusion chromatography to >95% purity as judged by SDS/PAGE analysis (ESI-MS data: c27643 and o27646 Da). The N-terminally truncated form of PHD2 was chosen as it can be easily produced in *E. coli*.

His₆–HIF-1 $\alpha^{344-503}$ NODD and His₆–HIF-1 $\alpha^{530-698}$ CODD were prepared as described in [14,15] (ESI-MS data: His₆–HIF-1 $\alpha^{344-503}$ NODD c19762, o19763 Da; His₆–HIF-1 $\alpha^{530-698}$ CODD c20808, o20808 Da). His₆–HIF-1 $\alpha^{530-652}$ CODD, His₆–HIF-1 $\alpha^{653-826}$ CAD and His₆–HIF-2 $\alpha^{502-697}$ CODD were expressed from pET-28a(+) constructs in *E. coli* BL21(DE3) and purified using nickel-affinity chromatography as reported for the other HIF fragments [14,15]. Short HIF fragments (35 residues or less: HIF-1 $\alpha^{556-574}$ CODD, HIF-1 $\alpha^{788-806}$ CAD, HIF-1 $\alpha^{788-822}$ CAD and HIF-2 $\alpha^{832-866}$ CAD) were prepared by synthesis.

The mature form (i.e. lacking the N-terminal peroxisomal targeting sequence) of mPAHX (mature phytanoyl-CoA hydroxylase) [25] and TauD (taurine dioxygenase) [26] were produced as reported (ESI–MS data: mPAHX, c35435, o35436 Da; and TauD c32278, o32282 Da).

Oxygen consumption assay

Assays were carried out under conditions of reduced light. GOX (glucose oxidase) assays were carried out in a final volume of 200 μ l at 25 °C. The final reaction mixture consisted of 32 pM GOX (Sigma-Aldrich), 10 nM catalase and 5-200 mM glucose in 50 mM sodium acetate (pH 5.5). Rubber-septum-sealed reaction vials containing glucose at various concentrations in sodium acetate buffer were exchanged with the appropriate gas mixture to give the required final oxygen concentration, and catalase was then added. The reaction vial was placed in a stirred water bath and a FOXY AL-300 probe (Ocean Optics Ltd.) inserted through the septum into the vial. The mixture was equilibrated and the reaction then initiated by the injection of GOX using a Hamilton syringe (50 μ l). Oxygen levels were monitored in the absence of ambient light using a fibre-optic oxygen-sensor system (FOXY, OceanOptics), calibrated using oxygen-saturated water [27], and an oxygen-depleted aqueous solution (prepared by adding crystalline Na₂SO₃). Only the rate of consumption within the first 5-20% of conversion (linear) was considered. Kinetic data were analysed with SigmaPlot 2000.

Assays with the 2OG-dependent dioxygenases were carried out essentially as described above at 37 °C, in 50 mM Tris/HCl (pH 7.5) unless otherwise stated. FIH (40 μ M) was reconstituted with 40 μ M FeSO₄ and injected into a solution of 1.5 mM 2OG (unless otherwise stated). The reaction was initiated by substrate addition. Assays of His₆–PHD2^{181–426} followed the same procedure using 50 μ M PHD2^{181–426} reconstituted with 50 μ M FeSO₄ injected into a solution of 0.75 mM 2OG (unless otherwise stated). Assays using TauD were carried out in 10 mM imidazole buffer (pH 6.9) using 10 μ M TauD, 100 μ M Fe(II), 0.5 mM 2OG and 0.5 mM taurine as a standard. Assays using mPAHX were carried out using 50 μ M mPAHX, 50 μ M Fe(II), 1.25 mM 2OG and 0.25 mM isovaleryl-CoA.



Figure 2 Kinetic studies of the GOX using the oxygen consumption assay at 25 °C in 50 mM NaOAc sodium acetate (pH 5.5)

GOX activity with different concentrations of (A) glucose and (B) oxygen.

 $K_{\rm m}$ (app; sub) and $K_{\rm m}$ (app; 2OG) values were determined at ambient oxygen concentrations (220 μ M) by varying the concentration of either the substrate or the co-substrate. For $K_{\rm m}$ (app; oxygen) value determination, different oxygen concentrations were obtained by exchanging the 2OG and Tris/HCl buffer solution with oxygen/nitrogen gas mixtures. The Michaelis– Menten equation was fitted directly to the data using SigmaPlot 2000, and values are quoted as a mean of at least three independent measurements. $V_{\rm max}$ values were derived from Michaelis–Menten curves for substrate dependence, and $k_{\rm cat}$ and $k_{\rm cat}/K_{\rm m}$ (app) determined using these data.

SPR

Binding studies were carried out on a BIAcore 2000TM biosensor (Biacore AB). His₆–HIF-1 $\alpha^{344-503}$ NODD or His₆–HIF-1 $\alpha^{530-698}$ CODD protein substrates were covalently coupled with a CM5 sensor chip using the BIAcore Amine Coupling kit (BIAcore AB) at pH 4.5. After controls were performed to eliminate masstransport effects, ligands were bound such that response units increased by approx. 800. Analyte protein (15 µl) was injected over the experimental and control flow cells at 50 µl/min and 25 °C in 10 mM Hepes (pH 7.4), 150 mM NaCl and 0.005 % surfactant P20. PHD2^{181–426} [pET-24a(+)] binding experiments were performed over a range of concentrations and kinetic data were calculated using the BIAevaluation software (BIAcore AB).

RESULTS

Development of an oxygen consumption assay

None of the reported HIF hydroxylase assays *in vitro* have measured oxygen consumption; instead they analyse 2OG/succinate production or HIF- α hydroxylation. To investigate their oxygen dependency it was desirable to develop an assay that directly measured oxygen consumption. Following initial work with Clark electrodes, we selected a fibre-optic oxygen-sensing system (FOXY, Ocean Optics Ltd.) for further evaluation as it is suited for assays in small volumes (< 200 μ l), can continuously monitor oxygen levels and allows the use of sealed vials, so enabling assays under different oxygen concentrations [28]. As it displays well-defined Michaelis–Menten kinetics, GOX was used to validate the technique [29]. The results [K_m (glucose) 23 ± 2 mM; K_m (app; oxygen) $140 \pm 10 \ \mu$ M, Figure 2] were in agreement with previous results obtained with a Clark electrode [K_m (glucose) = 26 mM; K_m (app; oxygen) $= 200 \ \mu$ M] [30]. Under reduced light conditions

reproducible data across a range of dissolved oxygen levels in small sample volumes ($200 \ \mu$ l) were obtained. Thus, although the interference by ambient light is a limitation, the fibre-optic sensor is a viable alternative to Clarke electrode assays.

Oxygen consumption assay conditions for HIF hydroxylases

Using the conditions described above, when applied to the HIF hydroxylases, sufficient rates of oxygen consumption for reliable assays were only achieved using micromolar concentrations of enzymes and a relatively high enzyme/substrate ratio (~ 1.5). The addition of potential stabilizing and/or reducing compounds including DTT (dithiothreitol), BSA and catalase did not make a significant difference to the initial rate measurements and were omitted (results not shown). Ascorbate is reported to be necessary for optimal stimulation of activity of several 20G-dependent oxygenases, including procollagen proline hydroxylase [31] and PHD (and to a lesser extent FIH) [21,22], and stimulation of PHD activity in cells [32]. The addition of ascorbate to a $PHD2^{181-426}\ assay \ containing \ Fe(II)\ resulted\ in\ significant\ oxygen$ consumption in the absence of enzyme (results not shown); ascorbate is a reducing agent and can reduce molecular oxygen to water, a reaction stimulated by transition-metal ions, including Fe(III) [33]. Ascorbate was therefore excluded from the oxygenconsumption assay mixtures with a consequent possible decrease in catalytic efficiency.

Kinetic parameters for PHD2¹⁸¹⁻⁴²⁶ and HIF-1 α CODD and NODD substrates

The oxygen-sensor method was then used to assay PHD2^{181–426} with HIF1- α and HIF2- α fragments of different length. The apparent $K_{\rm m}$ values for 2OG and oxygen obtained with the HIF- $1\alpha^{556-574}$ CODD peptide (Table 1) were consistent with those of previous experiments employing the [¹⁴C]2OG capture assay on PHD2^{1–426} isolated from crude insect cell extracts (Table 1) [21]. The specific activity of 1.6 mol of oxygen per min per mol of PHD2^{181–426} when assayed with HIF- $1\alpha^{556-574}$ CODD (Table 1) was the same as that reported previously for highly purified PHD2^{181–426} [34] but was approx. 20-fold lower than specific activities of full-length PHD2^{1–426} in crude cell extracts in the presence of ascorbate (> 40–55 mol of oxygen/min per mol of full-length PHD2^{1–426}) (Table 1) [21].

In comparison with the 19-residue HIF- $1\alpha^{556-574}$ CODD fragment (Table 1), when longer HIF- 1α CODD fragments were used (His₆-HIF- $1\alpha^{530-652}$ CODD and His₆-HIF- $1\alpha^{530-698}$ CODD) lower $K_{\rm m}$ (app; sub) (substrate) values of 2.3 and 1.8 μ M respectively

Table 1 Kinetic parameters for PHD2¹⁸¹⁻⁴²⁶ for the synthetic peptide HIF-1 α ⁵⁵⁶⁻⁵⁷⁴ CODD (19 residues) and recombinant substrates (> 120 residues; sub) from the HIF- α ODDD

The oxygen consumption assays were performed in 50 mM Tris/HCI (pH 7.5) at 37 °C (entries 1 + 3 + 4 + 5), except for His₆–HIF-2 α ^{502–697} CODD, which was assayed in 250 mM Tris/HCI (pH 8.0) (entry 6). Entry 2 refers to data reported in [21], obtained under different conditions. aa, amino acid.

Entry	Enzyme	Substrate	Length (aa)	V _{max} (µM/s)	K _m (app; sub) (μM)	K _m (app; 20G) (μΜ)	K _m (app, oxygen) (µM)	k _{cat} (min ⁻¹)	k_{cat}/K_m (app) (M ⁻¹ · s ⁻¹)
1	PHD2 ¹⁸¹⁻⁴²⁶	HIF-1a ⁵⁵⁶⁻⁵⁷⁴ CODD	19	1.30 + 0.30	21.6 + 7	55 + 11	229+60	1.6	1,204
2	PHD21-426	HIF-1 $\alpha^{556-574}$ CODD	19	ND -	7	60	250	> 40-55	$>(95-131)\times10^{3}$
3	His ₆ -PHD2 ¹⁸¹⁻⁴²⁶	His ⁶ –HIF-1a ^{530–652} CODD	123	0.180 ± 0.004	2.3 ± 0.3	ND	76 + 11	0.54	3863
4	His ₆ -PHD2 ¹⁸¹⁻⁴²⁶	His ₆ –HIF-1α ^{530–698} CODD	169	0.074 + 00.003	1.8 ± 0.5	75 + 20	81 + 28	0.22	1968
5	His6-PHD2181-426	His6-HIF-1a ³⁴⁴⁻⁵⁰³ NODD	160	0.97 + 0.07	24 + 7	58 + 20	85 + 17	1.16	805
6	His ₆ -PHD2 ¹⁸¹⁻⁴²⁶	His_6 -HIF-2 $\alpha^{502-697}$ CODD	195	0.3 ± 0.001	15 ± 2	267 ± 34	67 ± 10	0.42	467



Figure 3 Kinetic studies of the HIF hydroxylases using the oxygen consumption assay at 37 °C in 50 mM Tris/HCl (pH 7.5)

His₆-PHD2¹⁸¹⁻⁴²⁶ activity was assayed with different concentrations of (A) His₆-HIF-1a⁵³⁰⁻⁶⁵² CODD and (B) HIF-1a⁵⁵⁶⁻⁵⁷⁴ CODD.



Figure 4 SPR data for PHD2¹⁸¹⁻⁴²⁶ binding to His₆-HIF-1 α ⁵³⁰⁻⁶⁹⁸ CODD and His₆-HIF-1 α ³⁴⁴⁻⁵⁰³ NODD

(A) PHD2 (250 nM, 500 nM, 750 nM and 1 μ M) binding to His₆-HIF-1 $\alpha^{530-698}$ CODD: dissociation rate constant 0.186 \pm 0.04 s⁻¹. (B) PHD2 (1, 1.5, 2 and 2.5 μ M) binding to His₆-HIF-1 $\alpha^{344-503}$ NODD: dissociation rate constant 0.697 \pm 0.24 s⁻¹.

were observed (Table 1; Figure 3), demonstrating that the kinetic parameters can be affected by length of substrate. The low $K_{\rm m}$ (app; sub) value for His₆–HIF-1 α ^{530–698} CODD obtained with this assay was confirmed by the [¹⁴C]2OG capture assay (results not shown).

The K_m (app; sub) value for the CODD fragment (1.8 μ M) was significantly lower than for the NODD fragment [K_m (app; sub) = 24 μ M], suggesting that the difference in specific activity for the two fragments was due to differential binding. To investigate this possibility, SPR analyses were carried out. Although conducted under conditions different from the oxygen sensing assay [notably in the absence of added Fe(II)], the SPR data indicated that PHD2¹⁸¹⁻⁴²⁶ preferentially binds His₆-HIF-1 α ⁵³⁰⁻⁶⁹⁸ CODD over His₆-HIF-1 α ³⁴⁴⁻⁵⁰³ NODD. The association rate constants for the two fragments were very similar $(1.56 \pm 0.49 \text{ and } 1.37 \pm 0.42 \text{ M}^{-1} \cdot \text{s}^{-1}$ respectively), but the observed dissociation rate constant for His₆–HIF-1 $\alpha^{530-698}$ CODD was lower than that of His₆–HIF-1 $\alpha^{344-503}$ NODD ($0.186 \pm 0.04 \text{ s}^{-1}$ compared with $0.697 \pm 0.24 \text{ s}^{-1}$, n = 6, P < 0.001 respectively) (Figure 4). These data indicate that the differences in K_{m} (app; sub) values are due, at least in part, to differences in binding. The variation between the rate constants for PHD2 dissociating from CODD and NODD were more pronounced in the presence of equimolar (with respect to enzyme) 2OG, at 0.121 ± 0.02 and $0.68 \pm 0.28 \text{ s}^{-1}$ (n = 6) respectively (results not shown).

The $K_{\rm m}$ (app) values for 2OG and oxygen were then determined using the different length substrates. For the HIF-1 α substrates tested the $K_{\rm m}$ (app; 2OG) values for His₆-PHD2¹⁸¹⁻⁴²⁶ were in



Figure 5 Kinetic studies of the HIF hydroxylases using the oxygen depletion assay at 37 °C in 50 mM Tris/HCl (pH 7.5)

The oxygen dependencies of (A) His₆-PHD2¹⁸¹⁻⁴²⁶ with His₆-HIF-1 α ⁵³⁰⁻⁶⁹⁸ CODD and (B) His₆-FIH with His₆-HIF-1 α ⁶⁵³⁻⁸²⁶ CAD

Table 2 Kinetic parameters for FIH wild-type, for the synthetic peptides HIF-1 $\alpha^{788-806}$ CAD and HIF-1 $\alpha^{788-822}$ CAD (19 and 35 residues) and a recombinant substrate (> 120 residues) representing the HIF- α CAD region

Assays were performed using the oxygen consumption assay in 50 mM Tris/HCI (pH 7.5) at 37 °C (entries 1 + 3 + 6) except His₆-HIF-1a⁶⁵³⁻⁸²⁶ CAD, which was assayed in 50 mM CHES [2-(*N*-cyclohexylamino)ethanesulfonic acid] (pH 9.9) (entry 6). Entries 2, 4 and 5 refer to reported data obtained under different conditions. aa, amino acid.

Entry	Enzyme	Substrate	Length (aa)	V _{max} (µM/s)	$K_{ m m}(m app; m sub)$ (μ M)	$K_{ m m}(m app; 20G)$ (μ M)	$K_{ m m}(m app; m oxygen)$ (μ M)	k _{cat} (min ^{−1})	$k_{cat}/K_m(app)$ (M ⁻¹ · s ⁻¹)
1	FIH	HIF-1α ⁷⁸⁸⁻⁸⁰⁶ CAD	19	21 <u>+</u> 3	395 <u>+</u> 180	143 <u>+</u> 49	145 <u>+</u> 33	31.5	1329
2*	FIH	HIF-1α ⁷⁸⁸⁻⁸⁰⁶ CAD	19	ND	420 ± 50	ND	ND	ND	ND
3	FIH	HIF-1α ⁷⁸⁸⁻⁸²² CAD	35	9.7 ± 0.5	222 ± 30	147 ± 46	150 ± 30	14.55	1092
4*	FIH	HIF-1a ⁷⁸⁸⁻⁸²² CAD	35	ND	205 + 30	114 + 25	ND	ND	ND
5†	FIH	HIF-1a788-822 CAD	35	ND	100 + 5	25 + 3	90 + 20	70–135	$(11-23) \times 10^{3}$
6	FIH	His ₆ -HIF-1a ⁶⁵³⁻⁸²⁶ CAD	173	0.32 ± 0.04	154 + 37	64 + 25	237 + 28	1.92	208
7	FIH	HIF-220832-866 CAD	35	1.12 ± 0.1	229 ± 70	119 ± 47	110 ± 73	6.7	489
*Reporte †Report	ed values in [24 ed values in [22	4]. 2].							

the range of 55–75 μ M (Table 1) and apparently unaffected by either the length or type (NODD or CODD) of substrate. A caveat on the 2OG data is that the possibility of some 2OG co-purification with PHD2¹⁸¹⁻⁴²⁶ cannot be excluded [34]; data from other assays imply the binding constant for 2OG is much lower than the $K_{\rm m}$ (app) values reported here (~1 μ M). The apparent $K_{\rm m}$ (app; oxygen) for His₆–PHD2^{181–426} was approx. 81 μ M for His₆–HIF- $1\alpha^{530-698}$ CODD (Figure 5A), which was about the same as for His₆–HIF-1 $\alpha^{344-503}$ NODD (85 μ M) and lower than that for the 19-residue peptide HIF-1 $\alpha^{556-574}$ CODD of 229 μ M, implying that, under these assay conditions, the length of the substrate influences the apparent K_m (app; oxygen) (Table 1). Oxygen concentrations above 220 μ M (ambient oxygen levels) caused substrate inhibition (results not shown), possibly due to oxidation of iron at the enzyme active site, although inactivating or inhibiting oxidation at other sites including exposed cysteine residues [35] on the surface of PHD2 is also possible. Notably for His₆-PHD2¹⁸¹⁻⁴²⁶ there was little oxygen consumption prior to the addition of substrate (in contrast with FIH) consistent with the possibly important ability of this enzyme to form a relatively stable enzyme \cdot Fe(II) \cdot 2OG intermediate.

Kinetic parameters for PHD2^{181–426} and HIF-2 α substrates

It has not yet been possible to produce a sufficiently soluble (without additives for the current assay conditions) recombinant substrate fragment of His₆–HIF- $2\alpha^{350-476}$ NODD. A soluble His₆–HIF- $2\alpha^{502-697}$ CODD fragment was obtained, but it was found necessary to carry out assays at pH 8.0 and at high buffer con-

centrations (250 mM Tris/HCl) to maintain substrate solubility. Both the K_m (app; sub) and K_m (app; 2OG) values for His₆– HIF- $2\alpha^{502-697}$ CODD were significantly higher compared with the equivalent parameters for the His₆–HIF- $1\alpha^{530-698}$ CODD, but the different assay conditions make direct comparisons of limited value (Table 1, entries 4 + 6). In contrast, the K_m (app; oxygen) value for the recombinant His₆–HIF- $2\alpha^{502-697}$ CODD fragment of 67 μ M (Table 1) is below the value of the HIF- 1α equivalents of 81 μ M (His₆–HIF- $1\alpha^{530-698}$ CODD) and 85 μ M (His₆–HIF- $1\alpha^{344-503}$ NODD). However, given the variations in conditions between the two assays, this difference should probably not be regarded as being significant.

Kinetic parameters for FIH

 $K_{\rm m}$ (app; sub) values (Table 2) for each of the HIF-1 α CAD fragment substrates (19 and 35 residues, HIF-1 $\alpha^{788-806}$ CAD and HIF-1 $\alpha^{788-802}$ CAD) were at 395 and 222 μ M respectively, in reasonable agreement with the reported values, obtained with different techniques (Table 2) [22,24]. As with PHD2¹⁸¹⁻⁴²⁶ there was a decrease in the apparent $K_{\rm m}$ (app; sub) value (to 154 μ M) when using a longer substrate, His₆–HIF-1 $\alpha^{653-826}$ CAD (Table 2), but the effect was less pronounced than for PHD2 (Table 1). The $K_{\rm m}$ (app; 2OG) value of FIH (64 μ M) observed with His₆–HIF-1 $\alpha^{653-826}$ CAD (Table 2) was a similar value to that obtained for PHD2¹⁸¹⁻⁴²⁶ with a similar length substrate (Table 1). The $K_{\rm m}$ (app; oxygen) for FIH was 237 μ M with His₆–HIF-1 $\alpha^{-653-826}$ CAD (Figure 5B) and 145 and 150 μ M with the 19- and 35-residue peptides (HIF-1 $\alpha^{788-806}$ CAD and HIF-1 $\alpha^{788-822}$ CAD) respectively

Table 3 Kinetic parameters for other Fe(II)- and 20G-dependent oxygenases

Assays were performed using the oxygen consumption assay in 50 mM Tris/HCI (pH 7.5) at 37 °C (mPAHX), or 10 mM imidazole (pH 6.9) at 30 °C (TauD). ND, not determined.

Entry	Enzyme	Substrate	$V_{ m max}$ (μ M/s)	$K_{ m m}(m sub)~(\mu M)$	$K_{ m m}(m 20G)~(\mu M)$	$K_{ m m}({ m oxygen})(\mu{ m M})$	$k_{\rm cat}$ (min ⁻¹)	$k_{cat}/K_{m} (M^{-1} \cdot s^{-1})$
1	mPAHX	IsovalervI-CoA	0.77 + 0.10	356 + 118	186 + 46	93+43	0.98	46
2*	mPAHX	Phytanoyl-CoA	ND	~ 30	50 + 15	ND	ND	ND
3	TauD	Taurine	4.8 ± 0.3	54 + 13	90 + 20	76 + 17	28.8	8888.9
4†	TauD	Taurine	ND	55	11	ND	ND	ND
*Repor †Repo	ted values for [36]. rted values for [37].							

(Table 2), i.e. above the $K_{\rm m}$ (app; oxygen) values of PHD2^{181–426} assayed with His₆–HIF-1 $\alpha^{530-698}$ CODD (110 μ M) and His₆–HIF-1 $\alpha^{344-503}$ NODD (85 μ M) (Table 1). FIH assayed with a HIF-2 $\alpha^{832-866}$ CAD 35-residue peptide revealed $K_{\rm m}$ (app; sub) (229 μ M), $K_{\rm m}$ (app; 2OG) (119 μ M), $K_{\rm m}$ (app; oxygen) (110 μ M) values that were similar to the $K_{\rm m}$ values for the HIF-1 α equivalent HIF-1 $\alpha^{788-822}$ CAD peptide. (Table 2).

Results for mPAHX and TauD

We assayed two other 2OG-dependent oxygenases; E. coli TauD and human PAHX, for comparison with the HIF hydroxylases. The $K_{\rm m}$ values for the substrates and 2OG of these enzymes have been studied previously, but not their direct dependence on oxygen [36,37]. Owing to the scarcity of the natural phytanoyl-CoA substrate and complications with kinetic studies due to its lack of solubility in aqueous solvents [36], isovaleryl-CoA was used as a substrate for the PAHX work as it does not require solubilization agents [25]. The isovaleryl-CoA substrate displayed Michaelis-Menten behaviour using the oxygen sensor assay, but has a higher apparent $K_{\rm m}$ (app) than phytanoyl-CoA (Table 3). The apparent $K_{\rm m}$ (app; 2OG) value was also higher for isovaleryl-CoA than phytanoyl-CoA and was high in comparison with reported values for other 2OG oxygenases (50–90 $\mu\mathrm{M}).$ The results for TauD [$K_{\rm m}$ (app; sub) = 54 μ M and $K_{\rm m}$ (app; 2OG) = 90 μ M] were also within the same order of magnitude of previously reported data measured with a different assay $[K_m \text{ (app; sub)} = 55 \ \mu\text{M}$ and $K_{\rm m}$ (app; 2OG) = 11 μ M; Table 3] [37]. Under the assay conditions, the apparent $K_{\rm m}$ (app; oxygen) values for PAHX (with isovaleryl-CoA as a substrate) and TauD were 93 and 76 μ M respectively (Table 3).

DISCUSSION

There are important limitations imposed by the use of non-physiological conditions and the constraints imposed by the assays on the absolute kinetic values reported in the present and, likely, previous studies [21,22] on the oxygen-dependency of isolated HIF hydroxylases. These include use of unnatural truncated forms of HIF- α with a single hydroxylation site, use of a truncated form of PHD2 and likely non-optimal assay conditions including, in the present study, the lack of ascorbate (required for optimal in vitro activity) in truncated PHD2¹⁸¹⁻⁴²⁶ assays, the use of high enzymeto-substrate ratios and the fact that other proteins may contribute to the interactions being examined, e.g. OS-9 [38]. Most of the activity values reported here are lower than those for the enzymes in crude baculovirus extracts, possibly due to the presence of unassigned stimulatory factors in the extracts. The co-purification of 2OG with recombinant PHD2181-426 [34] also indicates that the 20G binding constant is significantly lower than the reported $K_{\rm m}$ (app; 2OG) values presented here and elsewhere [21,22]; thus the

absolute values should be treated with caution, especially with respect to estimation of binding constants. However, since the aims of the present study, in addition to the development of an oxygen consumption assay for the hydroxylases, were to investigate whether their catalytic efficiency varies with substrate length and whether they have unusual properties within the family of 2OG oxygenases, comparisons between substrates and enzymes are of some value.

An important observation that arises from the results on the oxygen-dependency of the HIF hydroxylases is the variation in catalytic efficiency with different length substrates. Koivunen et al. [22] reported that the activity of FIH with the 19-residue HIF- $1\alpha^{788-806}$ CAD fragment was approx. 10% of that with a 35residue HIF-1 $\alpha^{788-822}$ CAD fragment. Consistent with this report we observed a decrease in apparent K_m (app; sub) as the length of the HIF-1 α substrate fragment was increased from 19 to 35 and to 173 residues (HIF-1 $\alpha^{788-806}$ CAD, HIF-1 $\alpha^{788-822}$ CAD and His₆-HIF-1 $\alpha^{653-826}$ CAD) with the $K_{\rm m}$ (app; sub) for the 173residue fragment being less than half of the 19-residue fragment (Table 2). However, the k_{cat} value of the longest fragment tested, His₆-HIF-1 $\alpha^{653-826}$ CAD, was also reduced, meaning there was actually a decrease in k_{cat}/K_m (app). The reasons for the apparent decrease in k_{cat} for His₆–HIF-1 $\alpha^{653-826}$ CAD are unclear and may reflect non-optimal assay conditions and/or product inhibition.

The variation in catalytic activity of PHD2¹⁸¹⁻⁴²⁶ with respect to substrate length was more striking than for FIH. Whereas for PHD2^{181–426} the $K_{\rm m}$ (app; 2OG) values were reasonably consistent for the HIF-1 α CODD fragments (55–75 μ M), there was a significant reduction in the K_m (app; sub) on going from the HIF-1 α 19-residue to the 169-residue fragment, and a similar effect for the 123-residue fragment (Table 1). This decrease in $K_{\rm m}$ (app; sub) resulted in a level of activity for His₆-HIF-1 $\alpha^{530-698}$ CODD similar to that observed for a 19-residue substrate when using crude baculovirus extracts or human cell extracts [21,39]. Further, the k_{cat} values reported here are likely underestimates, as the catalytic assay conditions used for the oxygen sensor are nonoptimal, i.e. ascorbate was absent and a high enzyme to substrate ratio was used. These results indicate that there are important enzyme-substrate recognition processes outside of the 19-residue HIF-1 $\alpha^{556-574}$ CODD sequence that has been previously used as a substrate. It may be that mechanistic studies or efforts to identify inhibitors or activators of the PHDs should utilize substrates reflecting significant fragments of HIF- α or complexes of HIF- α with partner proteins.

There appears to be agreement that all the reported K_m (app; sub) values for CAD fragments for FIH are high both in absolute terms and in comparison with the values for PHD2 [21,22]. The cellular significance, if any, of this observation is unclear; however, the K_m (app; sub) values for both FIH and the PHDs are significantly higher than the probable concentration range of HIF- α in cells (picomolar to nanomolar); thus it is likely that the

effective cellular binding constants for the HIF hydroxylases are significantly lower than the K_m (app; sub) values observed with isolated enzymes.

There is evidence that PHD3 is selective for the CODD over the NODD of HIF-1 α [14,21]. Both the kinetic and SPR results for PHD2¹⁸¹⁻⁴²⁶ imply a preferential binding of the His₆-HIF-1 α ⁵³⁰⁻⁶⁹⁸ CODD over the His₆-HIF-1 $\alpha^{344-503}$ NODD fragment (Table 1; Figure 4). This observation is consistent with the cellular work of Chan et al. [40] that demonstrates that under normoxic conditions the CODD is more efficiently hydroxylated by the PHDs than the NODD in full-length HIF-1 α . Since the catalytic domains of all three PHDs are highly conserved, it thus seems possible that they are all selective for the CODD over the NODD sequences, but vary in the degree of selectivity. However, Chan et al. [40] have also provided evidence that the hydroxylation at the two sites is linked (at least modification at the CODD sites affects hydroxylation at the NODD) and it may be that it will be difficult or impossible to obtain full molecular understanding of the relative importance of the roles of the two hydroxylation sites from experiments with isolated CODD and NODD fragments.

For FIH it is noteworthy that with the exception of a reduction in k_{cat} very similar kinetic parameters, K_m (app; sub), K_m (app; 2OG) and K_m (app; oxygen), were obtained for the HIF-1 α and HIF-2 α 35-residue fragments (HIF-1 $\alpha^{788-822}$ CAD and HIF-2 $\alpha^{832-866}$ CAD) (Table 2). In contrast, the HIF-2 α 195-residue fragment His₆–HIF-2 $\alpha^{502-697}$ CODD was a significantly poorer substrate for PHD2¹⁸¹⁻⁴²⁶ than the HIF-1 α CODD fragment; however, these data were acquired under different conditions, owing to solubility problems. Hence, accurate comparisons cannot be made and, in contrast with the NODD/CODD selectivity, it is premature to state that PHD2¹⁸¹⁻⁴²⁶ has a preference for the His₆–HIF-1 $\alpha^{530-698}$ CODD over the His₆–HIF-2 $\alpha^{502-697}$ CODD (Table 1).

Using data from crude baculovirus-produced PHDs, Hirsilä et al. [21] concluded that the K_m (app; oxygen) values for the PHDs (230–250 μ M) are significantly higher than for the human 2OGdependent oxygenase procollagen prolyl-4-hydroxylase (40 μ M), a difference in terms of the oxygen-sensing role of the PHDs that was interpreted as being significant. It was argued that the reported K_m (app; oxygen) values were slightly above the concentration of dissolved oxygen (atmospheric) and thereby enabled the functions of the PHDs as effective oxygen sensors since "even small decreases in oxygen are likely to influence their [PHD] activities" [21]. Koivunen et al. [22] reported a lower K_m (app; oxygen) value for FIH (90 μ M) and argued that a larger decrease in oxygen concentration was required for a significant decrease in FIH activity, but noted that the conclusions arising from cell culture experiments may not be valid in tissues.

The present results indicate that the $K_{\rm m}$ (app; oxygen) values for PHD2¹⁸¹⁻⁴²⁶ and FIH are in the range of 65–240 μ M when using a variety of substrates (Tables 1 and 2). For comparison, we carried out the same analyses on two other family members, bacterial TauD and human mPAHX. Although differences were apparent, overall the data, including those obtained using different substrates for PHD2 and FIH, suggest that the HIF hydroxylases are likely to be reasonably typical members of the 2OG oxygenase family, at least with respect to the relationship between catalytic activity and oxygen concentration; the apparent $K_{\rm m}$ (app; oxygen) values for TauD and PAHX were 76 and 93 μ M (Table 3) respectively, in the same range as those obtained for PHD2¹⁸¹⁻⁴²⁶ $85-110 \,\mu\text{M}$ and FIH 90-240 μM (Tables 1 and 2). In the case of PHD2^{181–426} it was observed that oxygen levels above 220 μ M had an inhibitory effect on efficiency, suggesting that PHD2 is limited towards oxygen concentrations that are higher than ambient levels.

Assuming that the values for K_m (app; oxygen) for PHD2^{181–426} and FIH reflect their oxygen-dependency, the question arises as to whether they are suited to their proposed role as oxygen sensors. It has been argued that, in order for them to act as oxygen-sensing components of the HIF system, the capacity for HIF hydroxylation must be rate-limited by oxygen availability [41]. For this to be the case, the threshold physiological pO_2 for a hypoxic response must be below saturating levels for enzymatic activity. Further, the enzymes must not bind oxygen so tightly that they cannot respond appropriately. Reports of oxygen concentrations within cells are in the 30–60 μ M range, but may be overestimates as measurements made close to respiring tissue-culture monolayers have indicated surprisingly low values [42]. The available data (albeit imperfect) thus imply the $K_{\rm m}$ (app; oxygen) values for FIH and PHD2¹⁸¹⁻⁴²⁶ values might be above most physiological concentrations of oxygen they will encounter, indicating that, even under conditions where other factors are not limiting, the oxygen utilization properties of the enzymes are suited to their proposed role as oxygen sensors. If intracellular oxygen concentrations in intact organisms are sufficiently low and the K_m (app; oxygen) values of the HIF hydroxylases are sufficiently high for the hydroxylation rate to be oxygen-dependent in any physiological condition, it is still possible that rate is limiting for the overall HIF signalling system over a limited range of oxygen concentrations compared with the full range of oxygen-dependency for the enzymes [41]. Inputs affecting the total hydroxylation capacity of the HIF pathway could effectively match the system to a hypoxic window appropriate for the physiology of a particular cell type or tissue. Such inputs could include relative enzyme/co-substrate/iron concentrations and the presence of reactive oxidizing species present in different redox conditions [43,44]. Indeed it may be that any 2OG oxygenase with a reasonably typical oxygen-sensitivity, or other enzyme similarly endowed, could act as an oxygen sensor if suitably positioned within a signalling pathway.

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