

Studies on the Activity of the Hypoxia-Inducible Factor Hydroxylases Using An Oxygen Consumption Assay

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Summary

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

Abbreviations: HIF, hypoxia-inducible factor; FIH, factor inhibiting HIF; PHD, prolyl hydroxylase domain containing protein; NODD, N-terminal oxygen-dependent degradation domain; CODD, C-terminal oxygen-dependent degradation domain; CAD, C-terminal transcriptional activation domain; TauD, taurine dioxygenase; mPAHX; mature phytanoyl-CoA hydroxylase; K_{mapp} , apparent Michaelis-Menten constant.

Introduction

The α/β -heterodimeric protein hypoxia-inducible factor (HIF) is a central regulator in the response of metazoans to hypoxia [1, 2]. Human HIF regulates the transcription of genes encoding for 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 ischemic disease (promoting HIF activity). In contrast to 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 dimerisation 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 prolyl residues within LXXLAP motifs (Pro402, Pro564 in human HIF-1 α) [7, 8]. This modification enables binding of the HIF- α subunits to the von Hippel-Lindau tumour suppressor protein (pVHL) which targets HIF- α for ubiquitination and proteasomal degradation. Human HIF-1 α and HIF-2 α also possess a conserved site of asparaginyl hydroxylation (Asn803 of HIF-1 α) in their C-terminal transcriptional activation domains (CAD) [9]. Hydroxylation at the *pro-S* β -position of Asn803 [10] ablates the interaction between HIF and the transcriptional co-activator p300 thus blocking HIF mediated transcription [11, 12].

In humans HIF- α prolyl-hydroxylation is catalysed by three Fe(II) dependent dioxygenases PHD1-3 (prolyl hydroxylase domain containing enzymes, or EGLN2/1/3 or HPH3/2/1), with closely related catalytic domains [13, 14]. Asparaginyl hydroxylation of the HIF- α CAD is also catalysed by a dioxygenase, factor inhibiting HIF (FIH) [15, 16]. The four identified human HIF hydroxylases belong to the non-haem Fe(II) and 2-oxoglutarate (2OG) 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 coproduct [15, 19, 20].

Using baculovirus produced PHDs Hirsilä *et al.* reported $K_{m, \text{oxygen}}$ values for the PHDs (230-250 μM) that were higher than that of human procollagen prolyl-4-hydroxylase (40 μM), an observation interpreted as being significant in terms of the role of the PHDs as oxygen sensors [21]. Koivunen *et al.* reported a lower $K_{m, \text{oxygen}}$ value for FIH (90 μM) and argued that a larger decrease in oxygen concentration was required for a significant decrease in FIH activity compared to the PHDs [22]. 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 ca. 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 cosubstrate dependence of FIH and PHD2 using a range of substrates. Comparison of the data with other 2OG dependent dioxygenases (Fig. 1) indicates that the $K_{\text{mapp.oxygen}}$ values of the HIF hydroxylases are likely within the typical range for the 2OG dioxygenases. Together with biophysical data the results imply that PHD2 is selective in binding CODD over the NODD fragment of HIF-1 α , and that catalytic efficiency is dependent upon the substrate length. The work 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, U.K., synthetic peptides were from Peptide Protein Research Ltd., Fareham, UK.

Expression and purification of recombinant 2OG dependent dioxygenases and substrates – FIH and PHD2₁₈₁₋₄₂₆ were produced essentially as reported [15, 24]. In brief, the full length *fih* gene and an N-terminally truncated *phd2* gene encoding for PHD2₁₈₁₋₄₂₆ were subcloned into the pET-28a(+) vector (Novagen) as *NdeI/BamHI* or *NheI/BamHI* fragments. Proteins were expressed in *E. coli* BL21(DE3) in 2TY growth medium. Cells were induced with 0.5 mM IPTG when OD₆₀₀ = 0.8 - 1.0 and growth was continued at 37°C for 4 hrs before cell harvest. Purification of the N-terminally His₆-tagged (N-terminus: MGSSHHHHHSSGLVPRGSH) proteins were carried out using nickel-affinity chromatography on HisBind-IDA resin (Novagen) according to the manufacturer's instructions. Cleavage of the affinity tag was achieved by incubation of the fusion protein for 16 hrs at 4°C with thrombin (Novagen). Desalting on a 300 ml Superdex-75 column (GE Healthcare) yielded ca. 95% pure protein as judged by SDS-PAGE analysis. Proteins were characterised by ESI-MS: FIH calculated (c)40566 : observed (o)40569 Da; PHD2₁₈₁₋₄₂₆ c28056 : o28056; His₆-PHD2₁₈₁₋

⁴²⁶ c29807 : o29805 Da. For surface plasmon resonance analyses, the sequence encoding PHD2₁₈₁₋₄₂₆ 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 : o27646). The N-terminally truncated form of PHD2 was chosen as it can be easily produced in *E.coli*.

His₆-HIF-1 α ₃₄₄₋₅₀₃ NODD and His₆-HIF-1 α ₅₃₀₋₆₉₈ CODD were prepared as described [14, 15] (ESI-MS data: His₆-HIF-1 α ₃₄₄₋₅₀₃ NODD c19762 : o19763 Da; His₆-HIF-1 α ₅₃₀₋₆₉₈ CODD c20808 : o20808 Da). His₆-HIF-1 α ₅₃₀₋₆₅₂ CODD, His₆-HIF-1 α ₆₅₃₋₈₂₆ CAD and His₆-HIF-2 α 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 α ₅₅₆₋₅₇₄ CODD, HIF-1 α ₇₈₈₋₈₀₆ CAD, HIF-1 α ₇₈₈₋₈₂₂ CAD, HIF-2 α ₈₃₂₋₈₆₆ CAD) were prepared by synthesis.

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

Oxygen consumption assay – Assays were carried out under conditions of reduced light. Glucose oxidase (GOX) 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 NaOAc, pH 5.5. Rubber septum sealed reaction vials containing glucose at various concentrations in NaOAc buffer were exchanged with the appropriate gas mixture to give the required final oxygen concentration; catalase was then added. The reaction vial was placed in a stirred water bath and a FOXY AL-300 probe (OceanOptics, Inc.) 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, Inc.), 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 was analysed with SigmaPlot 2000.

Assays with the 2OG dependent dioxygenases were carried out essentially as described above at 37°C, in Tris·HCl buffer (50 mM, 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). Reaction was initiated by substrate addition. Assays of His₆-PHD2₁₈₁₋₄₂₆ followed the same

procedure using 50 μM PHD2₁₈₁₋₄₂₆ reconstituted with 50 μM FeSO₄ injected into a solution of 0.75 mM 2OG (unless otherwise stated). Assays using TauD were carried out in imidazole buffer (10 mM) pH 6.9 using 10 μM TauD, Fe(II) 100 μM , 0.5 mM 2OG and 0.5 mM taurine as a standard. Assays using mPAHX were carried out using 50 μM mPAHX, Fe(II) 50 μM , 1.25 mM 2OG and 0.25 mM isovaleryl-CoA.

$K_{\text{mapp,sub}}$ and $K_{\text{mapp,2OG}}$ values were determined at ambient oxygen concentrations (220 μM) by varying the concentration of either the substrate or the cosubstrate. For $K_{\text{mapp,oxygen}}$ 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 3 independent measurements. V_{max} values were derived from Michaelis-Menten curves for substrate dependence, and k_{cat} and $k_{\text{cat}}/K_{\text{mapp}}$ determined using this data.

Surface Plasmon Resonance – Binding studies were carried out on a BIAcore 2000™ biosensor (Biacore AB). His₆-HIF-1 α ₃₄₄₋₅₀₃ NODD or His₆-HIF-1 α ₅₃₀₋₆₉₈ CODD protein substrates were covalently coupled to a CM5 sensor chip using the BIAcore Amine Coupling Kit (BIAcore AB) at pH 4.5. After controls were performed to eliminate mass transport effects, ligands were bound such that response units increased by ~800. 15 μl analyte protein was injected over the experimental and control flow cells at 50 $\mu\text{l}/\text{min}$, 25°C in 10 mM HEPES pH 7.4, 150 mM NaCl, 0.005% surfactant-P20. PHD2₁₈₁₋₄₂₆ (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 analyzing 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, OceanOptics Inc.) for further evaluation as it is suited for assays in small volumes (<200 μl), 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 ($K_{\text{m,glc}}$ 23 \pm 2; $K_{\text{mapp,oxygen}}$ 140 \pm 10, Fig. 2) were in agreement with previous results obtained with a Clark electrode ($K_{\text{m,glc}}$ 26; $K_{\text{mapp,oxygen}}$ 200) [30]. Under reduced light conditions reproducible data

across a range of dissolved oxygen levels in small sample volumes (200 μ l) was 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 (ca. 1:5). The addition of potential stabilising and/or reducing compounds including dithiothreitol, bovine serum albumin and catalase did not make a significant difference to the initial rate measurements and were omitted (data not shown). Ascorbate is reported to be necessary for optimal stimulation of activity of several 2OG dependent oxygenases, including procollagen prolyl 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 (data 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 oxygen consumption 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_m values for 2OG and oxygen obtained with the HIF-1 α ₅₅₆₋₅₇₄ CODD peptide (Table I, entry 1) were consistent with previous reports employing the [¹⁴C]-2OG capture assay on *PHD2*₁₋₄₂₆ isolated from crude insect cell extracts (Table I, entry 2) [21]. The specific activity of 1.6 mol oxygen per min per mol of *PHD2*₁₈₁₋₄₂₆ when assayed with HIF-1 α ₅₅₆₋₅₇₄ CODD (Table I, entry 1) was the same as that reported previously for highly purified *PHD2*₁₈₁₋₄₂₆ [34] but was ca. 20 fold lower than specific activities of full length *PHD2*₁₋₄₂₆ in crude cell extracts in the presence of ascorbate (>40-55 mol oxygen per min per mol of full length *PHD2*₁₋₄₂₆) (Table I, entry 2) [21].

In comparison with the 19 residue HIF-1 α ₅₅₆₋₅₇₄ CODD fragment (Table I, entries 1+2), when longer HIF-1 α CODD fragments were used (His₆-HIF-1 α ₅₃₀₋₆₅₂ CODD and His₆-HIF-1 α ₅₃₀₋₆₉₈ CODD) lower $K_{mapp,sub}$ values of 2.3 μ M and 1.8 μ M respectively, were observed (Table I, entries 3+4; Fig. 3) demonstrating that the kinetic parameters can be affected by length of substrate. The low $K_{mapp,sub}$ value for His₆-HIF-1 α ₅₃₀₋₆₉₈ CODD obtained with this assay was confirmed by the [¹⁴C]-2OG capture assay (data not shown).

The $K_{mapp,sub}$ value for the CODD fragment (1.8 μM) was significantly lower than for the NODD fragment ($K_{mapp,sub}$ 24 μM) suggesting that the difference in specific activity for the two fragments was due to differential binding. To investigate this possibility surface plasmon resonance (SPR) analyses were carried out. Although conducted under different conditions to 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 ($1.56 \pm 0.49 \text{ M}^{-1}\text{s}^{-1}$ and $1.37 \pm 0.42 \text{ M}^{-1}\text{s}^{-1}$, respectively), but the observed dissociation rate constant for His₆-HIF-1 α ₅₃₀₋₆₉₈ CODD was lower than that of His₆-HIF-1 α ₃₄₄₋₅₀₃ NODD ($0.186 \pm 0.04 \text{ s}^{-1}$ compared to $0.697 \pm 0.24 \text{ s}^{-1}$, $n=6$, $p<0.001$, respectively) (Fig. 4). These data indicate that the differences in $K_{mapp,sub}$ values are due 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 $0.121 \pm 0.02 \text{ s}^{-1}$ and $0.68 \pm 0.28 \text{ s}^{-1}$ ($n=6$) respectively (data not shown).

The K_{mapp} values for 2OG and oxygen were then determined using the different length substrates. For the HIF-1 α substrates tested the $K_{mapp,2OG}$ values for His₆-PHD2₁₈₁₋₄₂₆ were in the range of 55-75 μM (Table I) 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 copurification with PHD2₁₈₁₋₄₂₆ cannot be excluded [34]; data from other assays implies the binding constant for 2OG is much lower than the K_{mapp} values reported here (ca. 1 μM). The apparent $K_{mapp,oxygen}$ for His₆-PHD2₁₈₁₋₄₂₆ was ca. 81 μM for His₆-HIF-1 α ₅₃₀₋₆₉₈ CODD (Fig. 5,A), which was about the same as for His₆-HIF-1 α ₃₄₄₋₅₀₃ NODD (85 μM) and lower than that for the 19 residue peptide HIF-1 α ₅₅₆₋₅₇₄ CODD of 229 μM , implying that, under these assay conditions, the length of the substrate influences the apparent $K_{mapp,oxygen}$ (Table I). Oxygen concentrations above 220 μM (ambient oxygen levels) caused substrate inhibition (data not shown), possibly due to oxidation of iron at the enzyme active site, though inactivating or inhibiting oxidation at other sites including exposed cysteines [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 to FIIH) consistent with the possibly important ability of this enzyme to form a relatively stable enzyme.Fe(II).2OG intermediate.

Kinetic parameters for PHD2₁₈₁₋₄₂₆ 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 α ₃₅₀₋₄₇₆ NODD. A soluble His₆-HIF-2 α ₅₀₂₋₆₉₇ CODD fragment was obtained, but it was found necessary to carry out assays at pH 8.0 and at high buffer concentrations (250 mM Tris-HCl) to maintain substrate solubility. Both the $K_{mapp,sub}$ and $K_{mapp,2OG}$ values for His₆-HIF-2 α ₅₀₂₋₆₉₇ CODD were significantly higher compared to the equivalent parameters for the His₆-

HIF-1 α ₅₃₀₋₆₉₈ CODD, but the different assay conditions make direct comparisons of limited value (Table I, entries 4 + 6). In contrast, the $K_{mapp,oxygen}$ value for the recombinant His₆-HIF-2 α ₅₀₂₋₆₉₇ CODD fragment of 67 μ M (Table I, entry 6) is below the value of the HIF-1 α equivalents of 81 μ M (His₆-HIF-1 α ₅₃₀₋₆₉₈ CODD) and 85 μ M (His₆-HIF-1 α 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 - $K_{mapp,sub}$ values (Table II) for each of the HIF-1 α CAD fragment substrates (19 residue and 35 residues, HIF-1 α ₇₈₈₋₈₀₆ CAD and HIF-1 α ₇₈₈₋₈₂₂ CAD) were at 395 μ M and 222 μ M, respectively, in reasonable agreement with the reported values, obtained with different techniques (Table II) [22, 24]. As with PHD2₁₈₁₋₄₂₆ there was a drop in the apparent $K_{mapp,sub}$ value (to 154 μ M) when using a longer substrate, His₆-HIF-1 α ₆₅₃₋₈₂₆ CAD (Table II, entry 6), but the effect was less pronounced than for PHD2 (Table I, entries 1+3+4). The $K_{mapp,2OG}$ value of FIH (64 μ M) observed with His₆-HIF-1 α ₆₅₃₋₈₂₆ CAD (Table II, entry 6) was a similar value to that obtained for PHD2₁₈₁₋₄₂₆ with a similar length substrate (compare Table I entries 4 and 5). The $K_{mapp,oxygen}$ for FIH was 237 μ M with His₆-HIF-1 α ₆₅₃₋₈₂₆ CAD (Fig. 5,B) and 145 μ M and 150 μ M with the 19 and 35 residue peptides (HIF-1 α ₇₈₈₋₈₀₆ CAD and HIF-1 α ₇₈₈₋₈₂₂ CAD), respectively (Table II, entries 1, 3 and 6), i.e. above the $K_{mapp,oxygen}$ values of PHD2₁₈₁₋₄₂₆ assayed with His₆-HIF-1 α ₅₃₀₋₆₉₈ CODD (110 μ M) and His₆-HIF-1 α ₃₄₄₋₅₀₃ NODD (85 μ M) (Table I, entries 4 + 5). FIH assayed with a HIF-2 α ₈₃₂₋₈₆₆ CAD 35 residue peptide revealed $K_{mapp,sub}$ (229 μ M), $K_{m,2OG}$ (119 μ M), $K_{mapp,oxygen}$ (110 μ M) values that were similar to the K_m values for the HIF-1 α equivalent HIF-1 α ₇₈₈₋₈₂₂ CAD peptide. (Table II, entries 3+7).

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_m values for the substrates and 2OG of these enzymes have been studied previously but not their direct dependence on oxygen [36, 37]. Due 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 solubilisation agents [25]. The isovaleryl-CoA substrate displayed Michaelis-Menten behaviour using the oxygen sensor assay but has a higher apparent K_{mapp} than phytanoyl-CoA (Table III entries 1+2). The apparent $K_{mapp,2OG}$ value was also higher for isovaleryl-CoA than phytanoyl-CoA and was high in comparison with reported values for other 2OG oxygenases (50-90 μ M). The results for TauD ($K_{mapp,sub}$ 54 μ M and $K_{mapp,2OG}$ 90 μ M) were also within the same order of magnitude of previously reported data measured with a different assay ($K_{mapp,sub}$ 55 μ M and $K_{m,2OG}$ 11 μ M) (Table III, entries 3+4) [37]. Under the assay conditions, the

apparent $K_{mapp,oxygen}$ values for PAHX (with isovaleryl-CoA as a substrate) and TauD were 93 μ M and 76 μ M respectively (Table III, entries 1+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 current 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 current work, the lack of ascorbate (required for optimal in vitro activity) in truncated PHD2₁₈₁₋₄₂₆ assays, the use of high enzyme to 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 copurification of 2OG with recombinant PHD2₁₈₁₋₄₂₆ [34] also indicates that the 2OG binding constant is significantly lower than the reported $K_{mapp,2OG}$ 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 work, 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.* reported that the activity of FIH with the 19 residue HIF-1 α ₇₈₈₋₈₀₆ CAD fragment was ca. 10% of that with a 35 residue HIF-1 α ₇₈₈₋₈₂₂ CAD fragment. Consistent with this report we observed a drop in apparent $K_{mapp,sub}$ as the length of the HIF-1 α substrate fragment was increased from 19 to 35 and to 173 residues (HIF-1 α ₇₈₈₋₈₀₆ CAD, HIF-1 α ₇₈₈₋₈₂₂ CAD, His₆-HIF-1 α ₆₅₃₋₈₂₆ CAD) with the $K_{mapp,sub}$ for the 173 residue fragment being less than half of the 19 residue fragment (Table II, entries 1+3+6). However, the k_{cat} value of the longest fragment tested, His₆-HIF-1 α ₆₅₃₋₈₂₆ CAD, was also reduced meaning there was actually a decrease in k_{cat}/K_{mapp} . The reasons for the apparent decrease in k_{cat} for His₆-HIF-1 α ₆₅₃₋₈₂₆ CAD are unclear and may reflect non-optimal assay conditions.

The variation in catalytic activity of PHD2₁₈₁₋₄₂₆ with respect to substrate length was more striking than for FIH. Whilst for PHD2₁₈₁₋₄₂₆ the $K_{mapp,2OG}$ values were reasonably consistent for the CODD fragments (55-75 μ M), there was a significant reduction in the $K_{mapp,sub}$ on going from the 19

residue to the 169 residue fragment, and a similar effect for the 123 residue fragment (Table I). This decrease in $K_{\text{mapp,sub}}$ 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 non-optimal, 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 $K_{\text{mapp,sub}}$ 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 $K_{\text{mapp,sub}}$ values for both FIH and the PHDs are significantly higher than the probable concentration range of HIF- α in cells (pM to nM) thus it is likely that the effective cellular binding constants for the HIF hydroxylases are significantly lower than the $K_{\text{mapp,sub}}$ 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 $\alpha_{530-698}$ CODD over the His₆-HIF-1 $\alpha_{344-503}$ NODD fragment (Table I, entries 4+5, Figure 4). This observation is consistent with the cellular work of Chan *et al.* [40] which 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 hydroxylations 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 $K_{\text{mapp,sub}}$ $K_{\text{mapp,2OG}}$ $K_{\text{mapp,oxygen}}$ were obtained for the HIF-1 α and HIF-2 α 35 residue fragments (HIF-1 $\alpha_{788-822}$ CAD, HIF-2 $\alpha_{832-866}$ CAD) (Table II, entries 3+7). 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 this data was acquired under different conditions due to

solubility problems. Hence, accurate comparisons cannot be made and, in contrast to the NODD/CODD selectivity, it is premature to state that PHD2₁₈₁₋₄₂₆ has a preference for the His₆-HIF-1 α ₅₃₀₋₆₉₈ CODD over the His₆-HIF-2 α ₅₀₂₋₆₉₇ CODD (Table I, entries 4+6).

Using data from crude baculovirus produced PHDs, Hirsilä *et al.* [21] concluded that the $K_{mapp,oxygen}$ values for the PHDs (230-250 μ M) are significantly higher than for the human 2OG dependent 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 $K_{mapp,oxygen}$ 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 $K_{mapp,oxygen}$ 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 $K_{mapp,oxygen}$ values for PHD2₁₈₁₋₄₂₆ and FIH are in the range of 65-240 μ M when using a variety of substrates (Tables I+II). 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 with 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 $K_{mapp,oxygen}$ values for TauD and PAHX were 76 μ M and 93 μ M (Table III, entries 1+3), respectively, in the same range as those obtained for PHD2₁₈₁₋₄₂₆ 85-110 μ M and FIH 90-240 μ M (Tables I+II). In the case of PHD2₁₈₁₋₄₂₆ 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 $K_{mapp,oxygen}$ for PHD2₁₈₁₋₄₂₆ 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 pO₂ 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 over-estimates as measurements made close to respiring tissue culture monolayers have indicated surprisingly low values [42]. The available data (albeit imperfect) thus implies the $K_{mapp,oxygen}$ 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 utilisation 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 $K_{mapp,oxygen}$ 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 to 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/cosubstrate/iron concentrations and the presence of reactive oxidising 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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Figure Legends

Fig. 1. Reactions catalysed by the non-haem iron (II) and 2-oxoglutarate dependent dioxygenases used in this study: (A) Prolyl hydroxylase domain containing enzymes (PHDs) (B) Factor inhibiting HIF (FIH) (C) Taurine dioxygenase (TauD) and (D) Mature phytanoyl-CoA hydroxylase (mPAHX).

Fig. 2. Kinetic studies of the glucose oxidase using the oxygen consumption assay at 25°C in 50 mM NaOAc, pH 5.5. Glucose oxidase activity with different concentrations of (A) glucose and (B) oxygen.

Fig. 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 with different concentrations of (A) His₆-HIF-1 α ₅₃₀₋₆₅₂ CODD and (B) HIF-1 α ₅₅₆₋₅₇₄ CODD.

Fig. 4. Surface plasmon resonance 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 α ₅₃₀₋₆₉₈ CODD: dissociation rate constant $0.186 \pm 0.04 \text{ s}^{-1}$. (B) PHD2 (1 μ M, 1.5 μ M, 2 μ M and 2.5 μ M) binding to His₆-HIF-1 α ₃₄₄₋₅₀₃ NODD: dissociation rate constant $0.697 \pm 0.24 \text{ s}^{-1}$.

Fig. 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 dependence 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.

Figure 1

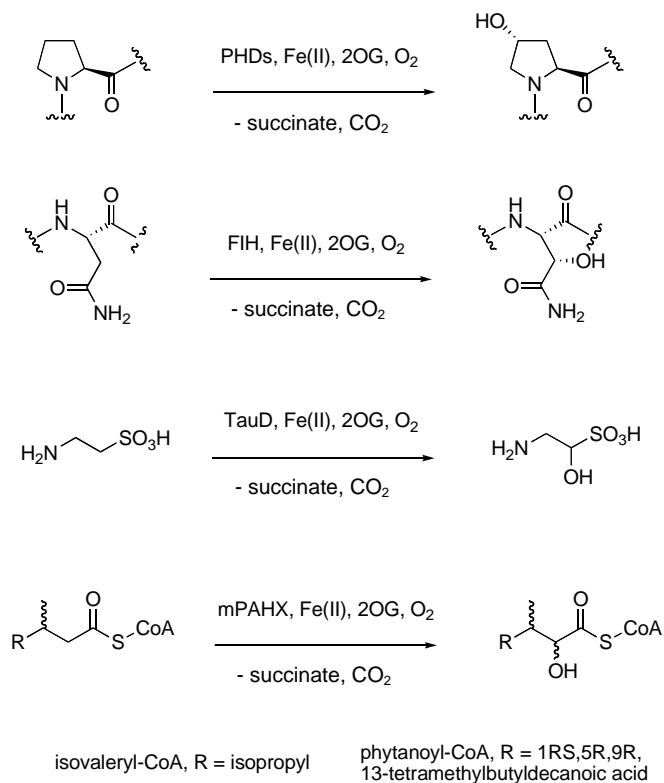


Figure 2

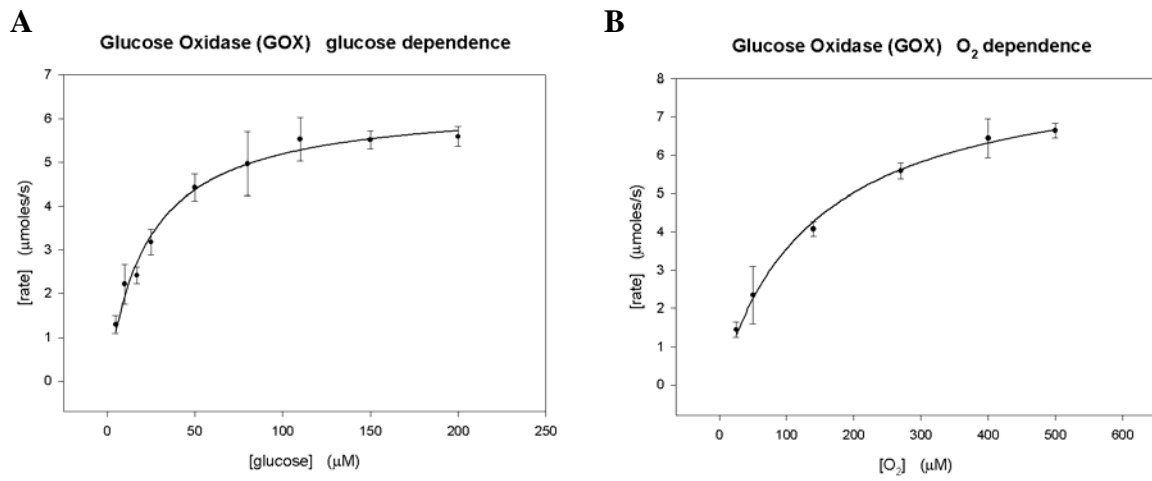


Figure 3

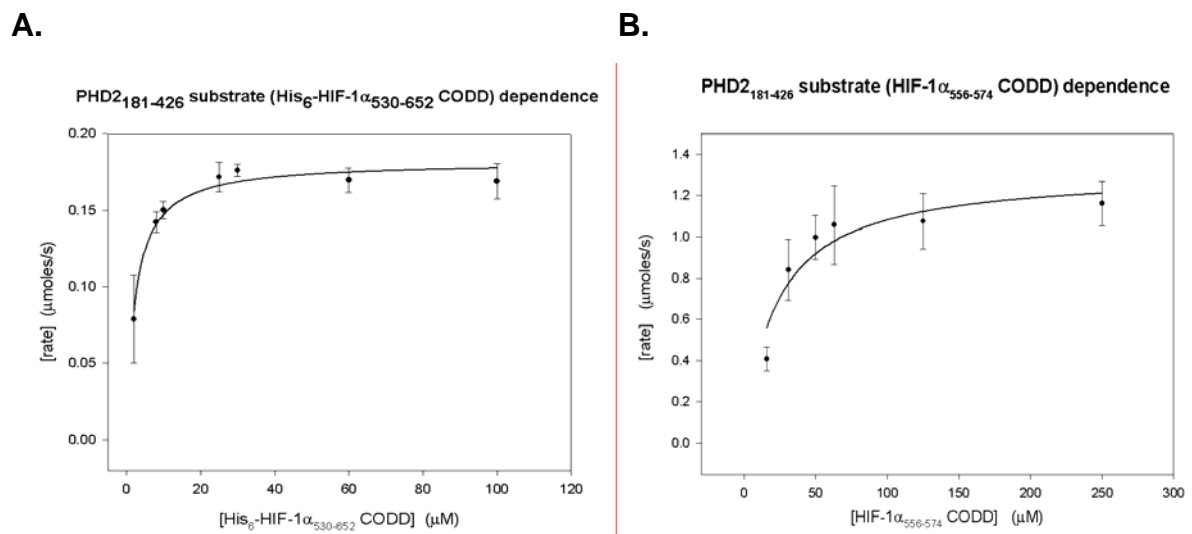


Figure 4

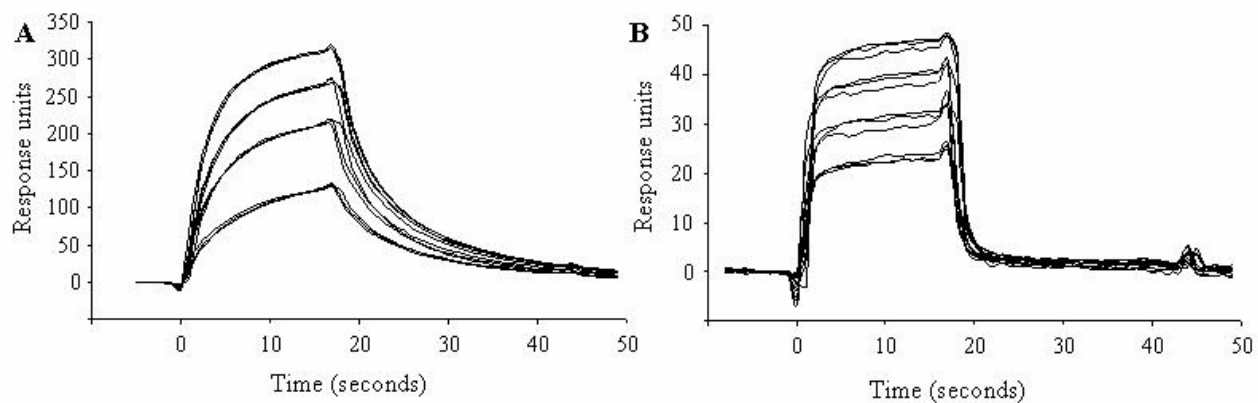


Figure 5

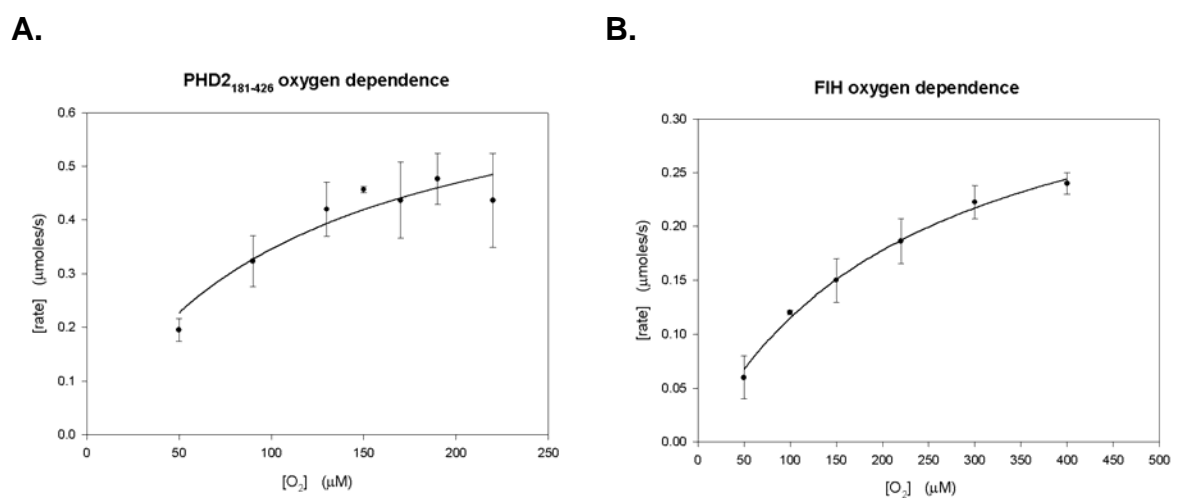


TABLE I

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

Assays were performed using the oxygen consumption assay in 50 mM Tris-HCl, 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-HCl, pH 8.0 (entry 6). Entry 2 refers to reported data obtained under different conditions.

entry	enzyme	substrate	length	V_{max} (μmols^{-1})	$K_{mapp,sub}$ (μM)	$K_{mapp,2OG}$ (μM)	$K_{mapp,oxygen}$ (μM)	k_{cat} (min^{-1})	k_{cat}/K_{mapp} ($\text{M}^{-1}\text{s}^{-1}$)
1	PHD2 ₍₁₈₁₋₄₂₆₎	HIF-1 α ₍₅₅₆₋₅₇₄₎ CODD	19	1.30±.30	21.6±7	55±11	229±60	1.6	1,204
2 [*]	PHD2 ₍₁₋₄₂₆₎	HIF-1 α ₍₅₅₆₋₅₇₄₎ CODD	19	ND	7	60	250	>40-55	>95-131·10 ³
3	His ₆ -PHD2 ₁₈₁₋₄₂₆	His ₆ -HIF-1 α ₅₃₀₋₆₅₂ CODD	123	0.180±.004	2.3±.3	ND	76±11	0.54	3863
4	His ₆ -PHD2 ₁₈₁₋₄₂₆	His ₆ -HIF-1 α ₅₃₀₋₆₉₈ CODD	169	0.074±.003	1.8±.5	75±20 ^a	81±28	0.22	1968
5	His ₆ -PHD2 ₁₈₁₋₄₂₆	His ₆ -HIF-1 α ₃₄₄₋₅₀₃ NODD	160	0.97±.07	24±7	58±20	85±17	1.16	805
6	His ₆ -PHD2 ₁₈₁₋₄₂₆	His ₆ -HIF-2 α ₅₀₂₋₆₉₇ CODD	195	0.3±.001	15±2	267±34	67±10	0.42	467

reported values for ^{*} ref. [22]

TABLE II

Kinetic parameters for FIH wt for the synthetic peptides HIF-1 α ₇₈₈₋₈₀₆ CAD and HIF-1 α 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-HCl, pH 7.5 at 37°C (entries 1+3+6) except His₆-HIF-1 α ₆₅₃₋₈₂₆ CAD which was assayed in 50 mM CHES, pH 9.9 (entry 6). Entries 2, 4 and 5 refer to reported data obtained under different conditions.

entry	enzyme	substrate	length	V_{max} (μmols^{-1})	$K_{mapp,sub}$ (μM)	$K_{mapp,2OG}$ (μM)	$K_{mapp,oxygen}$ (μM)	k_{cat} (min^{-1})	k_{cat}/K_{mapp} ($\text{M}^{-1}\text{s}^{-1}$)
1	FIH	HIF-1 α ₇₈₈₋₈₀₆ CAD	19	21±3	395±180	143±49	145±33	31.5	1,329
2 [*]	FIH	HIF-1 α ₇₈₈₋₈₀₆ CAD	19	ND	420±50	ND	ND	ND	ND
3	FIH	HIF-1 α ₇₈₈₋₈₂₂ CAD	35	9.7±0.5	222±30	147±46	150±30	14.55	1,092
4 [*]	FIH	HIF-1 α ₇₈₈₋₈₂₂ CAD	35	ND	205±30	114±25	ND	ND	ND
5 [†]	FIH	HIF-1 α ₇₈₈₋₈₂₂ CAD	35	ND	100±5	25±3	90±20	70-135	11-23·10 ³
6	FIH	His ₆ -HIF-1 α ₆₅₃₋₈₂₆ CAD	173	0.32±.04	154±37	64±25	237±28	1.92	208
7	FIH	HIF-2 α ₈₃₂₋₈₆₆ CAD	35	1.12±0.1	229±70	119±47	110±73	6.7	489

reported values for ^{*} ref. [24]; [†] ref. [22]

TABLE III

Kinetic parameters for other Fe(II) and 2OG dependent oxygenases.

Assays were performed using the oxygen consumption assay in 50 mM Tris-HCl, pH 7.5 at 37°C (mPAHX), or 10 mM imidazole, pH 6.9 at 30°C (TauD).

<i>entry</i>	<i>enzyme</i>	<i>substrate</i>	V_{\max} (μmols^{-1})	$K_{m,\text{sub}}$ (μM)	$K_{m,2\text{OG}}$ (μM)	$K_{m,\text{oxygen}}$ (μM)	k_{cat} (min^{-1})	k_{cat}/K_m ($\text{M}^{-1}\text{s}^{-1}$)
1	mPAHX	isovaleryl-CoA	0.77±.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

reported values for * Ref. [36]; [†] Ref. [37];