

Determination and comparison of specific activity of the HIF-prolyl hydroxylases

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Abstract Hypoxia-inducible factor (HIF) is a transcriptional complex that is regulated by oxygen sensitive hydroxylation of its α subunits by the prolyl hydroxylases PHD1, 2 and 3. To better understand the role of these enzymes in directing cellular responses to hypoxia, we derived an assay to determine their specific activity in both native cell extracts and recombinant sources of enzyme. We show that all three are capable of high rates of catalysis, in the order PHD2 = PHD3 > PHD1, using substrate peptides derived from the C-terminal degradation domain of HIF- α subunits, and that each demonstrates similar and remarkable sensitivity to oxygen, commensurate with a common role in signaling hypoxia.

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1. Introduction

Hypoxia-inducible factor (HIF), comprising a heterodimer of α and β subunits [1], is a central mediator of many transcriptional adaptations to hypoxia. The α subunit is rapidly degraded in the presence of oxygen, leading to inactivation of the hypoxic response [2]. In normoxia HIF- α binds the von Hippel–Lindau tumor suppressor protein (pVHL), which forms the recognition component of an E3 ubiquitin ligase complex, leading to ubiquitination of HIF- α and consequent degradation by the ubiquitin-proteasome pathway [3,4]. The targeting event is an oxygen dependent enzymatic hydroxylation of specific prolyl residues [5–7], which uses 2-oxoglutarate as a co-substrate. Analysis of HIF-prolyl hydroxylase activity is therefore an important focus for understanding how cells respond to hypoxia.

Following identification of cDNAs encoding three human HIF-prolyl hydroxylases, termed PHD (prolyl hydroxylase domain) 1–3 [8], recombinant enzyme preparations produced in bacteria were shown to hydroxylate HIF peptides in vitro, but observed rates of catalysis were low (approximately 7×10^{-3} mol/mol/min) [9]. More recently, recombinant PHD from insect cells has been used in kinetic analyses using the rate of [^{14}C]CO₂ released on decarboxylation of [1- ^{14}C] α -ketoglutarate, to measure enzyme activity [9,10]. These studies provided data on a number of important kinetic parameters, and indicated that the catalytic rate is most probably much higher than that estimated above, but did not measure enzyme concentration, and therefore could not provide measurements of specificity activity.

To understand HIF-prolyl hydroxylation better, we derived an assay that could measure the specific activity of HIF-prolyl hydroxylases and could be applied to small quantities of unpurified enzyme in biological samples. Since [1- ^{14}C] α -ketoglutarate decarboxylation assays use large amounts of enzyme and require purified or overexpressed enzyme to obviate background release of [^{14}C]CO₂ by other pathways, they are not suitable for assaying activity in samples such as crude cell extracts. In another type of assay, pVHL capture has been used to detect the production of hydroxylated HIF- α through the specific interaction of pVHL with hydroxylated but not unmodified peptide. However, though sensitive and specific, such assays have not, to date, been used in a quantitative format.

Here we show, using a synthetic hydroxylated HIF-1 α peptide for calibration and methods for enzyme quantification, that the pVHL capture assay provides a method for measurement of specific activity with sufficient sensitivity to measure PHD activity in crude cell extracts. We report the use of this assay to measure the specific activity of endogenous HIF-prolyl hydroxylase in mammalian cell extracts, to compare the specific activities of different PHD isoforms and their response to hypoxia, and to compare the activity of different recombinant enzyme preparations.

2. Materials and methods

2.1. Cell Culture and preparation of endogenous PHD2

HEK293T and MDA-MB-435 cells were cultured at 37 °C in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum. Sf9 cells, were maintained in Sf-900 II serum-free medium (Life Technologies) at 27 °C. Endogenous PHD2 was prepared by

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Abbreviations: HIF, hypoxia-inducible factor; PHD, prolyl hydroxylase domain containing protein; pVHL, von Hippel–Lindau tumor suppressor protein; IVTT, in vitro transcription/translation; BT19, N-terminal biotinylated 19'mer peptide (HIF-1 α 556–574); BT19OH, BT19 with proline 564 substituted by hydroxyproline; siRNA, small interfering RNA; RNAi, RNA interference.

incubating MDA-MB-435 cells in 1% hypoxia for 36 h prior to harvest by sonication into 150 mM sodium chloride, 20 mM Tris (pH 7.5) and 1 mM dithiothreitol.

2.2. RNA interference

MDA-MB-435 cells were transfected at 0 and 24 h with PHD2 specific small interfering RNA (siRNA) (corresponding to nucleotides 4077–4097) [11], using Oligofectamine (Ambion), and extracts prepared 24 h later, as above. Efficacy and specificity of RNA interference (RNAi) were confirmed by immunoblotting for PHD1, PHD2 and PHD3 (see below).

2.3. In vitro transcription/translation

[³⁵S]Methionine labeled proteins were synthesized by in vitro transcription/translation (IVTT) reactions using TNT T7 Quick Coupled Rabbit Reticulocyte Lysate (Promega). Plasmids encoding each PHD in pcDNA3 (Invitrogen) have been described previously [8].

2.4. Expression and purification of recombinant PHD enzymes from Sf9 cells

Recombinant baculoviruses containing genes encoding the N-terminal FLAG-tagged and untagged PHD1, 2, and 3 were prepared using the Bac-to-Bac (Life Technologies) or Sapphire (Orbigen) baculovirus expression systems. FLAG-tagged PHD2 was prepared by sonicating Sf9 cells, 70 h post-infection, into 50 mM Tris, pH 7.4, 150 mM NaCl, 0.1% Triton X-100, and Complete Protease Inhibitors (Roche). The soluble fraction was applied to an anti-FLAG M2 affinity column (Sigma) and eluted using FLAG octapeptide (Sigma). Crude extracts of Sf9 cells expressing untagged PHD2 were harvested as for MDA-MB-435 cells.

2.5. Expression of recombinant PHD2 enzyme in HEK293T cells

HEK293T cells were transfected with pcDNA3 PHD2 expression vector [8], using polyethylenimine. Cells were harvested 48 h post-transfection and extracts prepared as for MDA-MB-435 cells.

2.6. Antibodies and immunoblotting

Mouse monoclonal antibody to PHD2 [11] was used in immunoblots to quantify PHD2 in extracts, by comparison with purified FLAG-tagged PHD2. Relative concentrations of each PHD in MDA-MB-435 extract were determined, by comparison with equal molar amounts of PHD (as judged by [³⁵S]methionine incorporation) produced in IVTT reactions, using rabbit polyclonal antibodies to PHD1 and PHD2, and mouse monoclonal antibody to PHD3 [11].

2.7. Biotinylated peptides

N-terminal biotinylated peptides corresponding to HIF-1 α residues 556–574 (biotin-DLDLEMLAPYIPMDDDFQL)(BT19), HIF-2 α (biotin-ELDLETLAPYIPMDGEDFQL) and HIF-3 α (biotin-ALDLEMLAPYISMDDDFQL) were used (Peptide Protein Research Ltd). The equivalent HIF-1 α peptide with Pro564 substituted by hydroxyproline (BT19OH) was used to calibrate the assay.

2.8. Enzyme assays

Biotinylated synthetic HIF- α peptides (1 μ M) were incubated, at 37 $^{\circ}$ C, in a reaction mix containing the enzyme, 50 mM Tris (pH 7.5), 2 mg/ml BSA, 1 mM DTT, 1 mM α -ketoglutarate, 1 mM ascorbate, 50 μ M FeCl₂, and 0.3 mg/ml catalase. Reactions were commenced by addition of the enzyme. Aliquots were removed at various time points and further reaction stopped by dilution in ice-cold ‘stop buffer’ (150 mM NaCl, 20 mM Tris (pH 7.5) and 0.5% Igepal, 300 μ M desferrioxamine mesylate (DFO)). The biotinylated peptide was captured using streptavidin-conjugated magnetic beads in ‘capture buffer’ (150 mM NaCl, 20 mM Tris (pH 7.5), 0.5% Igepal and 100 μ M DFO) followed by washing. The peptide-coated beads were then incubated at 4 $^{\circ}$ C with excess [³⁵S]pVHL produced by IVTT reaction, diluted in ‘capture buffer’. Following two further washes, captured [³⁵S]pVHL was quantified by SDS-PAGE and autoradiography using a phosphorimager.

3. Results and discussion

3.1. Design of quantitative pVHL capture assay

The pVHL capture method reports hydroxyproline content based on the highly specific and well characterized interaction

between pVHL and HIF-1 α hydroxylated at position Pro564 [5,8,12,13]. A minimal HIF-1 α peptide (residues 556–574) was used, since previous studies have established that, for this hydroxylation site, binding affinities to pVHL [13] and PHD enzyme kinetics [10] are unaffected by the use of longer peptides.

Calibration of peptide hydroxylation was performed by mixing known amounts of biotinylated synthetic peptide, containing hydroxyproline, with non-hydroxylated peptide, in capture buffer. This allowed the signal obtained from captured [³⁵S]pVHL to be calibrated against the amount of hydroxylated peptide present (Fig. 1A and B). Background binding to non-hydroxylated peptide was undetectable. The calibration curves demonstrated good linearity and, in all reactions, the proportion of peptide hydroxylated was low, such that observed rates approximated initial reaction rates.

PHD2 concentration in lysates was determined using known quantities of FLAG-PHD2, purified to homogeneity from Sf9 cells, to calibrate immunoblot signals obtained using monoclonal antibodies against PHD2 (Fig. 1C). The N-terminal FLAG tag did not affect antigenicity (data not shown). Purity was verified by SDS-PAGE (Fig. 1D) and protein concentration

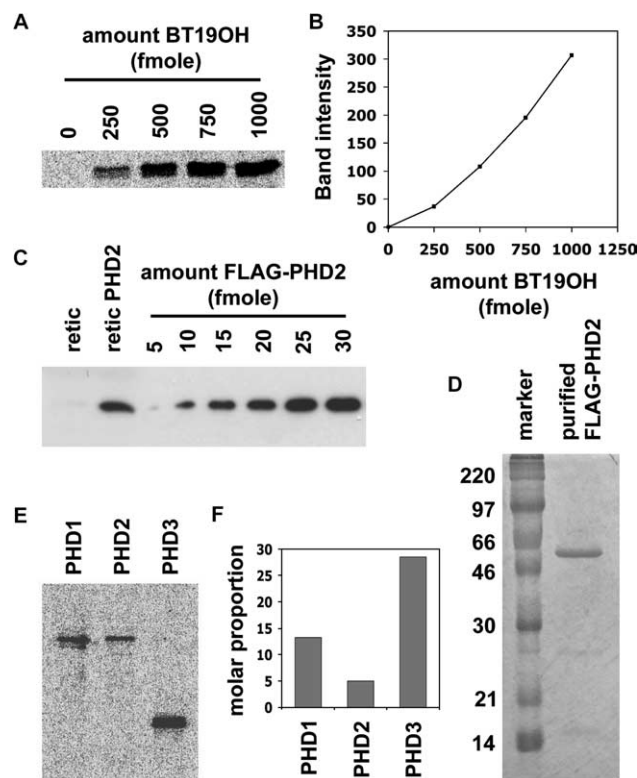


Fig. 1. (A) and (B) Calibration of captured pVHL against amount of hydroxylated peptide. Known amounts of BT19OH were added to a fixed quantity of BT19, before binding to streptavidin coated beads, and [³⁵S]pVHL capture. Following SDS-PAGE, [³⁵S]pVHL was quantified by autoradiography using a phosphorimager and calibrated against the amount of hydroxylated peptide. (C) Quantification of PHD enzymes. The amount of reticulocyte IVTT PHD2 was quantified against known amounts of purified FLAG-PHD2, by comparative immunoblot. (D) Purity of the FLAG-PHD2 is demonstrated. (E) and (F) The concentrations of IVTT PHD1 and 3 were quantified by comparison with IVTT PHD2 using autoradiography and phosphorimaging of [³⁵S]PHD IVTTs, making allowance for relative methionine content.

determined using Bio-Rad Dc protein assay. From this, the absolute concentration of PHD2 was calculated.

Initial work to produce purified recombinant PHD enzymes using a baculoviral expression system had revealed that PHD3 was almost entirely insoluble when expressed in the Sf9 cells and that PHD1 was only marginally soluble. Therefore, standard preparations for calibrating anti-PHD1 and anti-PHD3 signals on immunoblots could not be obtained. However, by incorporating a [³⁵S]methionine radiolabel in IVTT preparations, the relative amounts of each PHD isoform could be measured, and hence, by comparison with PHD2, the absolute concentration of each determined (Fig. 1E and F).

The method of detection is extremely sensitive allowing the activity of very small quantities of enzyme to be determined (currently 4–10 fmol of enzyme in a volume of 10–25 μ l). Furthermore, assaying peptide hydroxylation permits determination of PHD activity in crude cell lysates, without the confounding issue of non-PHD related α -ketoglutarate turnover. This obviates the need for purification of soluble enzyme, where effects on activity may be substantial [10] (and personal observation).

3.2. Measurement of the specific activity of endogenous and recombinant PHD enzymes

Endogenous PHD2 activity. Circumstances under which either PHD1 or PHD3 are expressed in isolation have not been determined [11]. We therefore sought to determine endogenous PHD2 activity, in order to provide a comparison for preparations of recombinant enzyme. Analysis was performed on extracts prepared from hypoxic MDA-MB-435 cells. Immunoblotting experiments, comparing this extract with equal molar quantities of each [³⁵S]methionine labeled PHD, produced in reticulocyte lysate IVTT reactions, indicate that under these circumstances PHD2 accounts for >95% total PHD immunoreactivity (Fig. 2A), comparable with published results [11]. Cell extracts prepared from hypoxic MDA-MB-435 cells were estimated to contain approximately 4 nM PHD2 by comparative immunoblotting (Fig. 2B).

Enzyme assays were performed in triplicate using these extracts (Fig. 2C). The initial reaction was linear over a 6-min period and yielded a value for specific activity of approximately 20 mol/mol/min. This result is much greater than that reported for purified PHD1 (7×10^{-3} mol/mol/min) [9], although lower than for FIH (85–135 mol/mol/min) [14] or collagen prolyl 4-hydroxylase (400 mol/mol/min) [15]. The value represents a lower limit for V_{\max} as the concentration of peptide used in the assays (1 μ M) was below the reported K_m (7 μ M) [10]. However, when the peptide concentration was increased above 1 μ M a paradoxical fall in specific activity was recorded. Reduction in peptide concentration to 0.05 μ M resulted in a proportionate fall in turnover consistent with these concentrations being below the K_m .

Finally, to further confirm that the measured activity was due to PHD2, its level was suppressed by RNAi (Fig. 2D). This abrogated pVHL capture in the assay (Fig. 2E) to a level commensurate with the reduction in PHD2 immunoreactivity, indicating that it is the predominant PHD activity in this extract.

Activity of recombinant PHD enzymes. Next, PHD2 activity from different eukaryotic expression systems, that expressed largely soluble enzyme, was compared. Since preliminary ex-

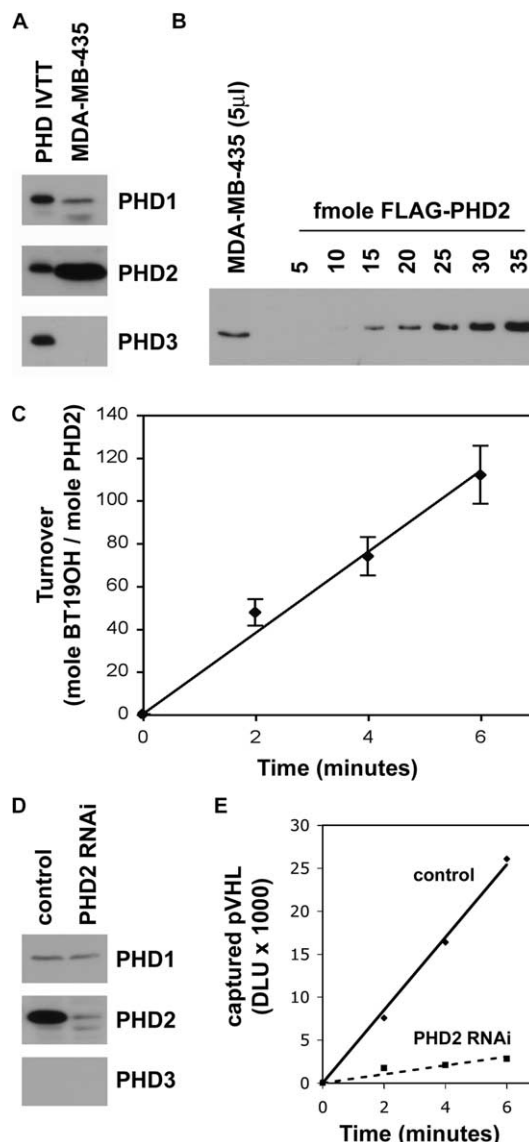


Fig. 2. Determination of endogenous PHD2 activity in hypoxically incubated MDA-MB-435 cell extracts. (A) Immunoblots comparing equivalent amounts of PHD IVTTs with hypoxic MDA435 extract demonstrate that PHD2 is the dominant isoform expressed. (B) PHD2 from MDA435 extract was quantified by comparative immunoblot with purified FLAG-PHD2. (C) Enzyme assays were performed on MDA-MB-435 extract. Means and standard deviations are for three independent experiments. (D) PHD1, PHD2 and PHD3 immunoblots demonstrate (6–8-fold) suppression of PHD2 in MDA-MB-435 cells by RNAi, but no effect on PHD1. (E) Suppression of PHD2 by RNAi results in loss of activity in pVHL capture assay.

periments indicated that both addition of an affinity tag and purification procedures altered enzyme activity, measurements were performed on unpurified preparations of untagged enzyme. All extracts were diluted to comparable PHD2 concentrations, in extract buffer containing 2 mg/ml BSA and known co-factors, prior to assay, to avoid saturating the assay. Activity of recombinant PHD2 prepared in a reticulocyte IVTT system, HEK293T mammalian cells, and Sf9 insect cells was compared with that of native PHD2 from hypoxic MDA-MB-435 cells. All preparations were catalytically active (Fig. 3).

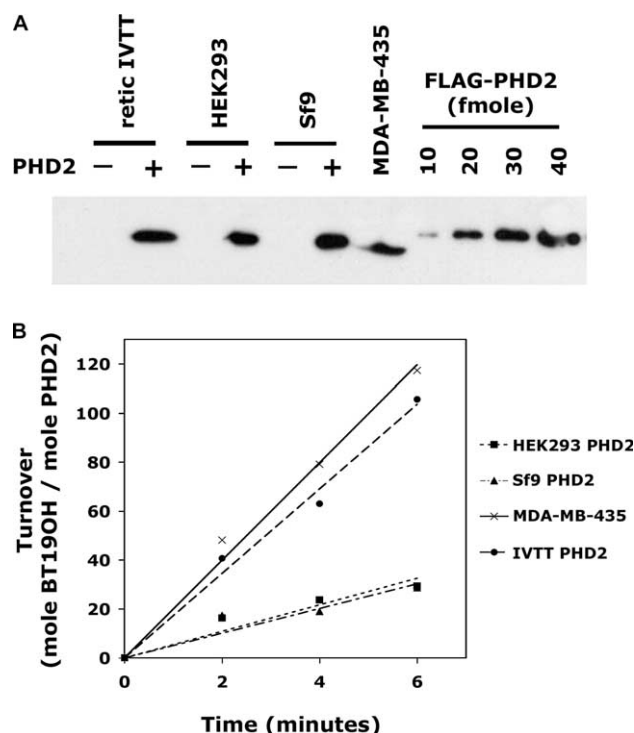


Fig. 3. Comparison of PHD2 from different sources. Enzyme assays were performed using reticulocyte IVTT PHD2, and identically prepared crude cell lysates of hypoxic MDA-MB-435 cells, transiently transfected HEK293T cells, and baculovirus infected Sf9 cells (+). Unprogrammed reticulocyte lysate IVTT, untransfected HEK293T cells, and uninfected Sf9 cells (-) were used to determine baseline activity for each source. All lysates were diluted to comparable PHD2 concentrations prior to assay. (A) Comparative immunoblot. (B) Initial reaction rates.

However, whilst specific activity of PHD2 from reticulocyte IVTT preparation was comparable to that of native PHD2 in MDA-MB-435 extracts (approximately 20 mol/mol/min), somewhat lower specific activity was observed in preparations from HEK293T cells and Sf9 cells. Both these systems expressed PHD2 at very high levels and it is possible that reduction in activity is due to a non-specific effect of dilution or dilution of a specific component required for activity. Similar reductions in activity with mammalian and insect cell preparation mitigate against a failure of a species specific post-translational modification causing the effect. In keeping with this, further experiments showed that PHD2, expressed in wheat germ IVTT, had comparable activity to that produced in the reticulocyte system (not shown).

Comparison of activity of PHD1, 2, and 3. Since PHD2 produced in reticulocyte IVTT had comparable activity to native protein, and testing of other expression systems had revealed solubility problems with PHD1 and PHD3, we focused on the reticulocyte system for comparative studies. Each PHD isoform was produced identically in the IVTT system. [35 S]methionine labeling was used to normalize molar equivalent enzyme quantities. Full solubility of each isoform, in the reaction mix, was confirmed by autoradiography before and after centrifugation. Results using the 19-residue (556–574) HIF-1 α peptide as substrate are shown (Fig. 4A) and reveal very similar specific activities for PHD2 and PHD3, and substantially lower activity for PHD1.

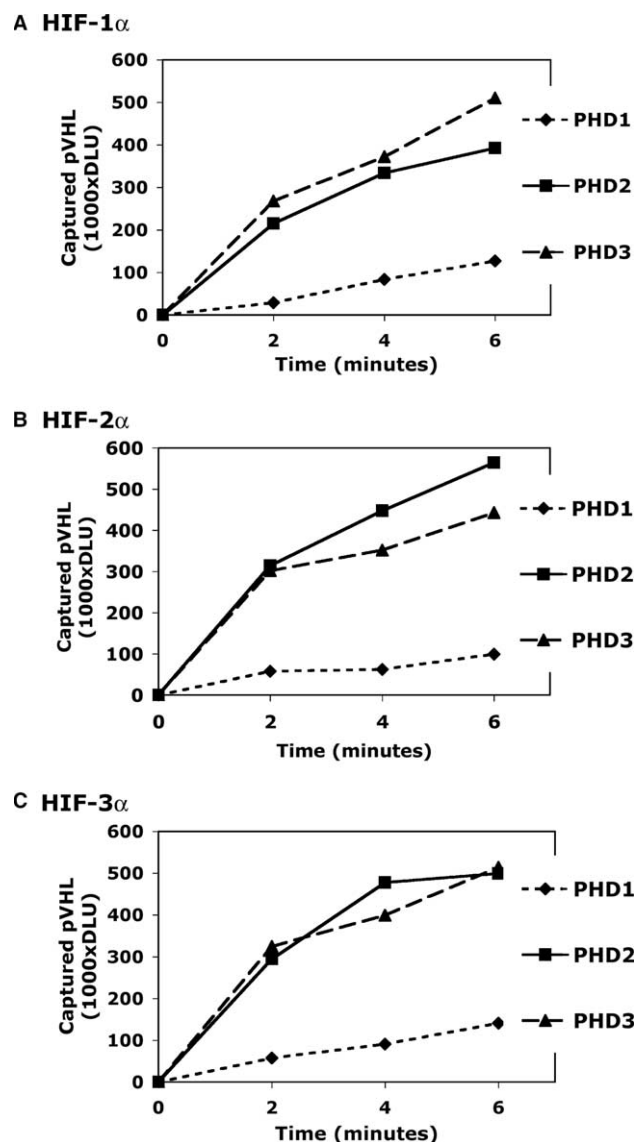


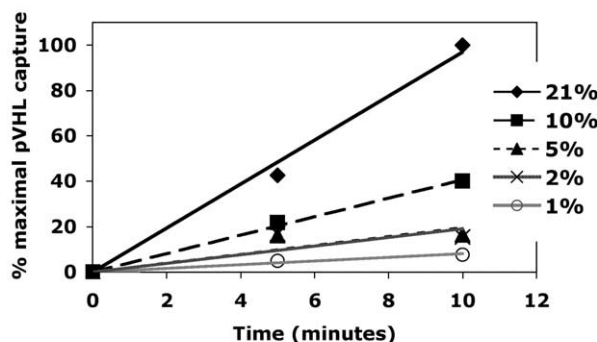
Fig. 4. Comparison of PHD isoforms. (A) Enzyme assays were performed, using HIF-1 α BT19 substrate and [35 S]PHD1, 2, and 3 produced in rabbit reticulocyte lysate IVTTs, with unprogrammed lysate determining baseline. [35 S]pVHL capture was normalized for the relative amount of each PHD. (B) and (C) The experiment was repeated using HIF-2 α and HIF-3 α peptides.

Further experiments were performed using the equivalent 19 residue peptides from HIF-2 α and HIF-3 α (Fig. 4B and C). Very similar relative activities in the order PHD2 = PHD3 > PHD1 were observed for all peptides, though since calibrating hydroxylated peptides were not used for the HIF-2 α and HIF-3 α based assays, the figures are relative, rather than absolute, measures of activity.

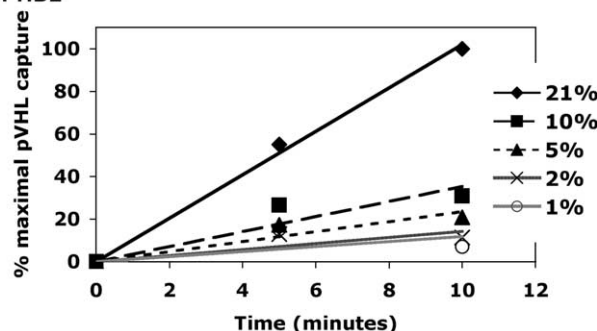
3.3. Oxygen dependence of PHD activity

Reticulocyte IVTT preparations of the PHD enzymes were next used to compare the dependence of activity on oxygen concentration for each isoform. In these experiments, both the reaction mix and enzyme preparation were pre-equilibrated at the designated ambient oxygen concentration in an Invivo2 Hypoxia Workstation (Ruskin/Biotrace International) prior to commencing the reaction. Results are shown (Fig. 5) and

A PHD1



B PHD2



C PHD3

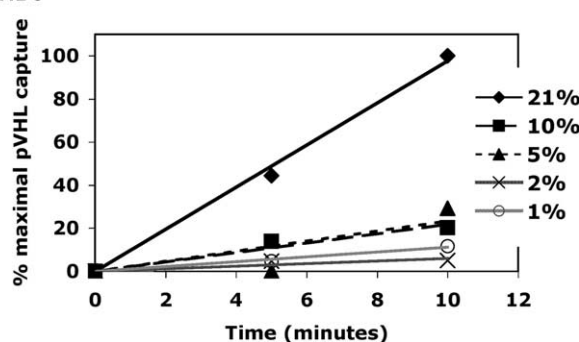


Fig. 5. Oxygen dependence of PHD isoforms. (A), (B) and (C) Enzyme assays were performed, using HIF-1 α , for each PHD isoform, after pre-equilibration across a range of ambient oxygen concentrations. [35 S]pVHL capture is expressed as percent maximal capture.

reveal similar oxygen dependence for the activity of each PHD isoform. In each case, the reaction rate was oxygen dependent over the full range of concentrations used, from 1% to 21% oxygen, with no indication of saturation.

Our data complement recent comparative studies of the PHD enzymes conducted both *in vitro* and *in vivo*. Hirsila et al. [10] studied the kinetics of recombinant PHD enzymes in unpurified insect cell lysates, deriving K_m values for substrates and co-factors. Of particular interest, in these [14 C] α -ketoglutarate decarboxylation assays, each PHD isoform manifests unusual sensitivity to hypoxia, with very high K_m values (230–250 μ M). The current work, using quantitative VHL capture assays, shows no evidence of saturation under ambient oxygen concentrations up to 21%. Assuming oxygen solubility at 37 °C of 1.30 μ mol/l/mmHg [16], this is equivalent to a concentration of roughly 200 μ M. These values are significantly in excess of tissue pO_2 . Thus, two different types of assays reveal that PHD

enzyme activity is oxygen concentration dependent over the entire physiological range, consistent with a role in regulating homeostatic responses to oxygen.

Our data indicate that all PHD enzymes are capable of acting catalytically to generate many molecules of hydroxylated HIF- α for each PHD molecule, and give a specific activity of approximately 20 mol/mol/min for PHD2 and PHD3 and somewhat lower for PHD1. These values provide only a lower limit for V_{max} , since the concentrations of HIF peptide and oxygen used were less than the reported K_m for this substrate. Superficially, this might suggest that cells possess an excess capacity for HIF hydroxylation that could not be rate limiting in HIF regulation. However, the HIF peptide concentrations used in these assays (1 μ M) remain greatly in excess of the concentration of HIF- α in cells (estimated to be in the sub-nanomolar range). Assuming an approximately linear reduction in catalytic rate with substrate concentration, capacity for hydroxylation of native HIF- α subunits would be much lower, potentially compatible with a regulatory function.

The importance of PHD2 function in the regulation of normoxic HIF level has recently been demonstrated *in vivo* by suppression of PHD expression by siRNA [17]. However, another study has demonstrated that when expressed at comparable levels, PHD1 and PHD3 could also influence the level of HIF-1 α consistent with our data. It is likely that, *in vivo*, many other factors (particularly the relative abundance of the enzymes in cells under analysis) will contribute to the observed effect of suppression by siRNA. Our results are however somewhat different from those reported by Huang et al. [18], which gave relative activities in the order PHD2 > PHD3 > PHD1, using an uncalibrated VHL capture assay and nickel affinity chromatography purified, tagged recombinant PHDs expressed in insect cells. Lower activity for PHD3 in the latter system might result from tagging and purification. Alternatively, recent work has identified the chaperone complex TRiC as a PHD3 binding complex [19], and this association may be required for production of fully active enzyme in some circumstances.

Overall, our data showing high levels of HIF-hydroxylase activity and striking oxygen sensitivity for the three PHD isoforms support a role for each in the oxygen dependent regulation of HIF. The calibrated VHL capture assay should provide a means for analyzing the biological control of this pathway in greater detail.

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References

- [1] Wang, G.L. and Semenza, G.L. (1995) *J. Biol. Chem.* 270, 1230–1237.
- [2] Huang, L.E., Arany, Z., Livingston, D.M. and Bunn, H.F. (1996) *J. Biol. Chem.* 271, 32253–32259.
- [3] Maxwell, P.H., Wiesener, M.S., Chang, G.W., Clifford, S.C., Vaux, E.C., Cockman, M.E., Wykoff, C.C., Pugh, C.W., Maher, E.R. and Ratcliffe, P.J. (1999) *Nature* 399, 271–275.
- [4] Cockman, M.E., Masson, N., Mole, D.R., Jaakkola, P., Chang, G.W., Clifford, S.C., Maher, E.R., Pugh, C.W., Ratcliffe, P.J. and Maxwell, P.H. (2000) *J. Biol. Chem.* 275, 25733–25741.
- [5] Jaakkola, P., Mole, D.R., Tian, Y.M., Wilson, M.I., Gielbert, J., Gaskell, S.J., Kriegsheim, A., Hebestreit, H.F., Mukherji, M.,

- Schofield, C.J., Maxwell, P.H., Pugh, C.W. and Ratcliffe, P.J. (2001) *Science* 292, 468–472.
- [6] Ivan, M., Kondo, K., Yang, H., Kim, W., Valiando, J., Ohh, M., Salic, A., Asara, J.M., Lane, W.S. and Kaelin Jr, W.G. (2001) *Science* 292, 464–468.
- [7] Masson, N., Willam, C., Maxwell, P.H., Pugh, C.W. and Ratcliffe, P.J. (2001) *EMBO J.* 20, 5197–5206.
- [8] Epstein, A.C., Gleadle, J.M., McNeill, L.A., Hewitson, K.S., O'Rourke, J., Mole, D.R., Mukherji, M., Metzen, E., Wilson, M.I., Dhanda, A., Tian, Y.M., Masson, N., Hamilton, D.L., Jaakkola, P., Barstead, R., Hodgkin, J., Maxwell, P.H., Pugh, C.W., Schofield, C.J. and Ratcliffe, P.J. (2001) *Cell* 107, 43–54.
- [9] McNeill, L.A., Hewitson, K.S., Gleadle, J.M., Horsfall, L.E., Oldham, N.J., Maxwell, P.H., Pugh, C.W., Ratcliffe, P.J. and Schofield, C.J. (2002) *Bioorg. Med. Chem. Lett.* 12, 1547–1550.
- [10] Hirsila, M., Koivunen, P., Gunzler, V., Kivirikko, K.I. and Myllyharju, J. (2003) *J. Biol. Chem.* 278, 30772–30780.
- [11] Appelhoff, R.J., Tian, Y.-M., Raval, R.R., Turley, H., Harris, A.L., Pugh, C.W., Ratcliffe, P.J. and Gleadle, J.M. (2004) *J. Biol. Chem.* 279, 38458–38465.
- [12] Ivan, M., Haberberger, T., Gervasi, D.C., Michelson, K.S., Gunzler, V., Kondo, K., Yang, H., Sorokina, I., Conaway, R.C., Conaway, J.W. and Kaelin Jr, W.G. (2002) *Proc. Natl. Acad. Sci. USA* 99, 13459–13464.
- [13] Hon, W.C., Wilson, M.I., Harlos, K., Claridge, T.D., Schofield, C.J., Pugh, C.W., Maxwell, P.H., Ratcliffe, P.J., Stuart, D.I. and Jones, E.Y. (2002) *Nature* 417, 975–978.
- [14] Koivunen, P., Hirsila, M., Gunzler, V., Kivirikko, K.I. and Myllyharju, J. (2004) *J. Biol. Chem.* 279, 9899–9904.
- [15] Vuori, K., Pihlajaniemi, T., Marttila, M. and Kivirikko, K.I. (1992) *Proc. Natl. Acad. Sci. USA* 89, 7467–7470.
- [16] Kaye, G.W.C. and Laby, T.H. (1973) Longman, London and New York.
- [17] Berra, E., Benizri, E., Ginouves, A., Volmat, V., Roux, D. and Pouyssegur, J. (2003) *EMBO J.* 22, 4082–4090.
- [18] Huang, J., Zhao, Q., Mooney, S.M. and Lee, F.S. (2002) *J. Biol. Chem.* 277, 39792–39800.
- [19] Masson, N., Appelhoff, R.J., Tuckerman, J.R., Tian, Y.M., Demol, H., Puype, M., Vanderkerchove, J., Ratcliffe, P.J. and Pugh, C.W. (2004) *FEBS Lett.* 570, 166–170.