Hydroxylation of two conserved prolyl residues in the N- and C-terminal oxygen dependent degradation domains (NODD and CODD) of the α-subunit of hypoxia-inducible factor (HIF) signals for its degradation via the ubiquitin-proteasome pathway. In human cells, three prolyl hydroxylases (PHDs 1-3) belonging to the Fe(II) and 2-oxoglutarate (2OG) dependent oxygenase family catalyze prolyl hydroxylation with differing selectivity for CODD and NODD. Sequence analysis of the catalytic domains of the PHDs in the light of crystal structures for PHD2, and results for other 2OG oxygenases, suggested that either the C-terminal region or a loop linking two β-strands (β2 and β3 in human PHD2) are important in determining substrate selectivity. Mutation analyses on PHD2 revealed that the β2β3 loop is a major determinant in conferring selectivity for CODD over NODD peptides. A chimeric PHD in which the β2β3 loop of PHD2 was replaced with that of PHD3 displayed an almost complete selectivity for CODD, as observed for wild type PHD3. Kinetic studies suggest this is effected by both changes in strength of binding and catalytic efficiency.

The heterodimeric transcription factor Hypoxia Inducible Factor (HIF) plays a central role in the response of metazoans to hypoxia (1,2). Levels of the HIF-β subunit are independent of oxygen (3), but under conditions of normoxia, the HIF-α subunit is hydroxylated in its oxygen dependent degradation domain (ODDD), enabling recognition by the von Hippel-Lindau (pVHL) ubiquitin ligase complex and subsequent degradation by the proteasome (4,5). Under hypoxic conditions, hydroxylation of the HIF-α subunit is reduced and it dimerizes with HIF-β, resulting in the increased expression of hypoxically regulated target genes such as erythropoietin and vascular endothelial growth factor, which enable adaptation to low oxygen concentrations (see reviews (6,7)). Hydroxylation of the three HIF-α isoforms is catalyzed by prolyl hydroxylases (in humans, PHDs 1-3) (8-10), belonging to the family of iron(II) and 2-oxoglutarate (2OG) dependent oxygenases, and occurs at two specific proline residues in the HIF-1α ODDD, Pro402 (N-terminal oxygen dependent degradation domain, NODD) and Pro564 (C-terminal oxygen dependent degradation domain, CODD). HIF-1α is also hydroxylated in its C-terminal transactivation domain (C-TAD) at Asn803, in a reaction catalyzed by another iron and 2OG dependent oxygenase, Factor Inhibiting HIF (FIH) (11-13). Hydroxylation of Asn803 prevents binding of HIF to the transcriptional co-activator p300/CBP in an oxygen dependent fashion.

PHD1 is localized to the nucleus, PHD2 and FIH are commonly found in the cytoplasm, and PHD3 is distributed homogeneously in both the nucleus and cytoplasm (14). One study on relative activities of recombinant human PHDs, produced by rabbit reticulocyte in vitro transcription and translation, on HIF-1α, 2α and 3α has given an order of activity of PHD2=3>1 (15) although a different study proposes that PHD2 has the greatest activity with HIF-1α (16). The role of PHD3 may be more important than that of PHD2 under hypoxia as observed by its striking induction under these conditions (17). It has been suggested, however, that PHD2 is the dominant human PHD in normoxia (18), with the greatest expression observed across a range of cell types. Importantly,
significant effects on the HIF system are observed when each of the PHDs is individually suppressed (17), so all three PHDs can make a non-redundant contribution to the hypoxic response.

Although hydroxylation at one of the two HIF-α prolines is sufficient for pVHL-mediated recognition and degradation (19,20), the presence of two proline hydroxylation sites in the ODDD of HIF-1α and HIF-2α suggests a differential role for each site. It is proposed that under normoxia hydroxylation of CODD precedes and is necessary for efficient hydroxylation of NODD, thereby resulting in two hydroxyprolines for pVHL-mediated degradation (20); NODD hydroxylation is proposed to become more sensitive to oxygen than CODD hydroxylation under conditions of hypoxia (20). Importantly, the different human PHD isozymes have differing specificities for NODD and CODD, with reports that PHD3 does not catalyze hydroxylation or interact with NODD NODD and CODD, with reports that PHD3 does not catalyze hydroxylation or interact with NODD (8,21,22), and that PHD1 displays less activity towards NODD than CODD relative to PHD2 (22).

Presently, it is unclear whether structural differences between the PHDs enable their selectivity for CODD and NODD. An understanding of how this regulation occurs is essential for developing a molecular understanding of the HIF system and important for developing specific therapeutic targets. All characterized 2OG oxygenases possess a catalytic domain containing a core double-stranded β-helix (DSBH) fold which supports a highly conserved HXD/E…H iron binding motif (23). In contrast, the substrate binding residues are poorly conserved and can involve residues from the DSBH and surrounding regions, sometimes including the C-terminus (23). Although the catalytic domains of the PHDs are highly conserved, sequence analyses (Figure 1) reveal unconserved regions in the flexible “finger-like” loop between β-strands 2 and 3, and in their C-terminal regions (24). Here we report mutation studies on the catalytic domain of human PHD2, an N-terminally truncated PHD that can be efficiently produced in an active recombinant form in *Escherichia coli*. The results reveal that the β2β3 “finger-like” loop has an important role in determining the differing selectivity of the PHDs for CODD relative to NODD.

**EXPERIMENTAL PROCEDURES**

*Materials -* HIF-1α peptide substrates were obtained from Peptide Protein Research Ltd., Fareham, U.K. The NODD peptide sequence used was DALTLLAPAGDTHLSLF (one-letter amino acid abbreviations), and the CODD peptide sequence DLDLEMLAPYIPMDDDFQL. These peptides are referred to as NODD and CODD hereafter. A hydroxylated CODD peptide (CODD<sub>Pro564-OH</sub>) DLDLEMLAHypYIPMDDDFQL (Hyp represents trans-4-hydroxyproline), was prepared on a CS Bio CS336 solid phase peptide synthesizer using a standard 9-Fluorenylmethoxycarbonyl/ N,N'-Diisopropylcarbodiimide / 1-<br>Hydroxybenzotriazole strategy, then purified by reversed-phase HPLC. DNA primers were from SigmaGenosys Ltd.

**Cloning of PHD2<sub>181-402</sub>, PHD2<sub>Δloop</sub> PHD2/1 and PHD2/3** - The DNA sequence encoding PHD2<sub>181-426</sub> has previously been ligated into the pET-24a vector (Novagen) (25). The C-terminally truncated PHD2<sub>181-402</sub> was produced using site-directed mutagenesis (Stratagene) (for primers see Table S1). A deletion variant (PHD2<sub>Δloop</sub>) was produced whereby residues 238 to 250 (the β2β3 loop) were removed by site-directed mutagenesis (Stratagene) (Table S1). Variant chimeric proteins were generated whereby the β2β3 loop of PHD2<sub>181-426</sub> was replaced with that of either PHD1 or PHD3 (proteins termed PHD2/1 or PHD2/3 respectively). PHD2/1A forward and reverse primers (Table S1) were designed and used to amplify the sequence encoding for PHD2<sub>181-243</sub>. PHD2/1B forward and reverse primers (Table S1) were used to amplify the sequence encoding for PHD2<sub>241-251</sub>. The overhangs on primers PHD2/1A reverse and PHD2/1B forward, which encoded for PHD1 residues 222-232, were then able to self-prime in a further amplification reaction. *NheI* and *BamHI* restriction enzymes were used to digest and ligate the full PHD2/1 sequence into the pET-24a(+) vector. A set of primers (Table S1) was designed to mutate the DNA sequence encoding PHD2<sub>241-251</sub> to the sequence encoding PHD3<sub>62-23</sub> by six rounds of site-directed mutagenesis (Stratagene). All constructs were verified by DNA sequencing (Sir William Dunn School of Pathology, University of Oxford).
Expression and Purification of PHD2 Proteins - PHD2181-426 and variants were expressed in E. coli BL21(DE3) and purified by cation exchange and size exclusion chromatography, as described (26). Protein purity was assessed by SDS-PAGE and electrospray ionization (ESI) or matrix-assisted laser desorption/ionization (MALDI)-TOF mass spectrometry.  

2OG Turnover Assays - Enzyme activity from 2OG turnover was measured by assaying [14C]-CO₂ production, as described (12): Assay mixes of 100µl (final volume) contained typically enzyme (4µM), substrate (100µM), 2OG (288µM), [14C]-2OG (3.6µM), Fe(II) (500µM), ascorbate (4mM), dithiothreitol (DTT, 1mM) and catalase (0.3mg/ml). Unless stated, reactions took place for 15 minutes at 37°C, then were stopped with 200µl methanol and incubated on ice for 20 minutes to allow complete absorption of all residual [14C]-CO₂. For kinetic measurements, the optimum assay length for measuring initial velocity was determined by time course experiments, and assays were then carried out over a range of substrate concentrations. Data were fitted by the Michaelis-Menten equation using SigmaPlot 2000, allowing Kₘ (or apparent Kₘ) and vₘₐₓ to be determined.  

Hydroxylation Assays - Enzyme activity in terms of peptide substrate hydroxylation was measured by MALDI-TOF mass spectrometry. Experimental conditions were as for the 2OG turnover assay. Product (1µl) was mixed with α-cyano-4-hydroxycinnamic acid MALDI matrix (1µl) (LaserBio Labs) and spotted onto the target plate. Samples were analyzed using a MALDI-TOF micro MX mass spectrometer (Waters Micromass).  

Surface Plasmon Resonance - Binding studies were carried out using a BIACore 2000™ or BIACore T100™ machine (GE Healthcare). His-NODD344-503 or His-CODD530-698 protein substrates (purified as described in (8)) were covalently coupled to a CM5 sensor chip using the BIACore Amine Coupling Kit (GE Healthcare) at pH 4.5. After controls were performed to eliminate mass transport effects, ligands were bound such that response units increased by 600-800. Analyte protein (15µl) was injected over the experimental and control flow cells at 50µl/min, 25°C in 10mM HEPES pH 7.4, 150mM NaCl, 0.005% surfactant-P20. Binding experiments were performed over a range of concentrations and kinetic data were calculated using the BIAevaluation software (GE Healthcare).  

RESULTS  

PHD2181-402 Catalyses 2OG Turnover and NODD/CODD Hydroxylation Similarly to PHD2181-426 - Studies on some 2OG oxygenases from the sub-family to which the PHDs belong have revealed a role for the C-terminus of the enzyme in substrate binding and selectivity (23,27-30). Alignment of the three human PHD isoform sequences combined with information from the crystal structure of PHD2181-426 (24) (Figure 1) show high conservation and homology within their catalytic domains. However, aside from their very different N-termini, there are two regions where this conservation is not maintained, the C-terminus and the flexible βββ “finger-like” loop region located near the active site, suggesting a potential role for these regions in determining selectivity (24). A C-terminal truncation variant, PHD2181-402 was therefore targeted because significant sequence variation occurs to the C-terminal side of residue 402. Further truncations were also produced (PHD2181-399- PHD2181-397 and PHD2181-395), but these showed a reduction in catalytic activity (Figure S1). We therefore focused on PHD2181-402 for substrate selectivity studies. Circular dichroism (CD) spectroscopy indicated no significant differences between the secondary structures of PHD2181-402 and PHD2181-426 (Figure S2).  

Activity assays, measuring total 2OG turnover during a standard 15 minute incubation, were carried out to determine whether the C-terminal truncation had any effect on activity with the CODD and NODD substrates. The results revealed that truncation of the C-terminus did not significantly alter the ability of PHD2181-426 to decarboxylate 2OG to succinate and CO₂ when using either NODD or CODD peptide substrates (Figure 2A). Similarly, no difference in 2OG turnover was seen between PHD2181-426 and PHD2181-402 when longer His₅₆-NODD530-698 and His₅₆-NODD344-503 protein substrates were used (Figure S3).  

It is possible that removal of the C-terminus of the enzyme actually promotes uncoupled turnover of 2OG (28), i.e. without peptide hydroxylation. To ensure that the activity seen in
this assay was coupled to peptide substrate hydroxylation, assays were repeated and the resultant peptides analyzed by MALDI mass spectrometry to detect hydroxylation, i.e. a +16 mass shift of the peptide substrate (Figure 2B). The C-terminally truncated PHD2_{181-402} enzyme was found to hydroxylate both NODD and CODD substrates as efficiently as PHD2_{181-426} under these assay conditions.

Further 2OG turnover assays, measuring initial velocity at substrate concentrations ranging from 0-200µM, gave $K_m$ values for PHD2_{181-426} of 44µM and 37µM for the NODD and CODD peptides, respectively (Table 1). Previously, PHD2_{181-426} $K_m$ values for NODD and CODD peptides were reported as 24µM and 2µM, respectively, as determined by an oxygen consumption assay (25), and 130µM and 7µM, respectively, as determined by 2OG turnover assays using crude cell lysates containing expressed PHD2 (22). Thus, although the difference in $K_m$ values seen with this assay is minimal, other reported assays support a preference of PHD2_{181-426} for CODD over NODD (at least in terms of $K_m$ values). The differences in absolute values may reflect differences in assay conditions/substrates (including the presence or absence of ascorbate). PHD2_{181-402} was found to have $K_m$ values for NODD and CODD of 50µM and 77µM respectively, revealing that the NODD $K_m$ does not significantly alter when the C-terminus is removed from PHD2; the CODD $K_m$ however, approximately doubles on removal of the C-terminus. Truncation of the C-terminus also results in an increase in $k_{cat}/K_m$ for both substrates, with a larger increase for CODD than NODD. When the $k_{cat}/K_m$ values are examined, it appears that removal of the C-terminus results in little overall change in catalytic efficiency and only a small bias in the CODD/NODD selectivity towards NODD.

**PHD2\_181-402 shows a similar preference for CODD over NODD hydroxylation to PHD2\_181-426.** To unequivocally determine the preference of PHD2_{181-426} and PHD2_{181-402} for CODD over NODD in hydroxylation assays, competition assays in which both peptides were simultaneously present were carried out. Under standard conditions, with both substrates present at 150µM (Figure 3A), PHD2_{181-426} preferentially hydroxylated CODD peptide, while PHD2_{181-402} showed less selectivity between the two substrates. A greater selectivity for CODD hydroxylation was seen for both PHD2_{181-426} and PHD2_{181-402} when both peptides were present at 500µM (Figure S4). When competition experiments were carried out over time periods ranging from 0-60 minutes (Figure 3B, lower graphs), it appears that, when compared to single substrate experiments (Figure 3B upper graphs), CODD hydroxylation occurred at a faster rate than NODD hydroxylation for PHD2_{181-426}, indicating selectivity for this substrate. The rate of NODD and CODD hydroxylation by PHD2_{181-402} is quite similar (compared with PHD2_{181-426}) and this likely reflects the increased $k_{cat}/K_m$ for hydroxylation of NODD over CODD seen for this variant compared to PHD2_{181-426}. The results thus indicate that the C-terminal region, although it may contribute, is not the only factor or a dominant factor in determining substrate selectivity.

**PHD2\_181-402 and PHD2\_181-426 Show Similar Binding Characteristics to both His\_6-NODD\_344-503 and His\_6-CODD\_530-698.** To investigate whether the C-terminus has a role in substrate binding, surface plasmon resonance (SPR) studies were carried out using purified His\_6-CODD\_530-698 and His\_6-NODD\_344-503 HIF-1α substrates (Table 2). As reported (25), PHD2\_181-426 binds to His\_6-CODD\_530-698 more strongly than it binds to His\_6-NODD\_344-503, as indicated by a slower dissociation rate constant (0.14 (±0.04) s\(^{-1}\) compared to 1.06 (±0.39) s\(^{-1}\), n=7, P<0.001). The association rate constants for the two substrates are very similar. PHD2\_181-402 also bound to His\_6-CODD\_530-698 more strongly than it did to His\_6-NODD\_344-503, again indicated by a slower dissociation rate constant (CODD: 0.16 (±0.03) s\(^{-1}\) compared to NODD: 0.64 (±0.13) s\(^{-1}\), n≥5, P<0.001). The rate of association for PHD2\_181-402 binding to CODD was slightly greater than that for NODD binding, though this difference was not statistically significant. The association and dissociation constants were also very similar for PHD2\_181-426 and PHD2\_181-402 with respect to each substrate, with the only statistically significant difference being a decrease in the dissociation constant for His\_6-NODD\_344-503 when the C-terminus is truncated (P<0.05). These results reflect the similarities seen between PHD2\_181-426 and PHD2\_181-402 kinetic parameters.

**PHD2\_1 and PHD2\_3 catalyze 2OG turnover and NODD/CODD hydroxylation similarly to**
PHD2$_{181-426}$. As the results on deletion of the C-terminus did not explain the observed CODD/NODD selectivity of the different PHDs, the β2β3 loop region was next targeted. Initially residues 238-250 of PHD2$_{181-426}$ were deleted and replaced by Ala-Ala, i.e. D(237)GQLVSQKSDDSKD(I251) was replaced with D(237)AAI(251). The Ala-Ala sequence was aimed at providing a short linker between β2 and β3 whilst still maintaining the overall fold. Although deleting the thirteen loop residues actually altered 2OG turnover in the presence of substrate peptides, with a marked increase in 2OG turnover in the presence of CODD (Figure S5A), hydroxylation assays demonstrated that this variant catalyzed less than 5% substrate hydroxylation under the standard conditions of 15 minutes incubation at 37°C (Figure S5B). Non-denaturing protein MS assays (31) indicated that the loop deletion variant formed a less stable complex with Fe(II) and 2OG than PHD2$_{181-426}$, but that it was capable of binding CODD and NODD even though hydroxylation was low (5%) (Figure S6, (a) and (b)). Together, these observations indicate that the β2β3 loop is involved in ensuring coupling of 2OG decarboxylation to substrate hydroxylation.

To investigate the proposal that the β2β3 “finger-like” loop sequences of the different PHDs are involved in selectivity, chimeras were produced where the β2β3 loop of PHD2$_{181-426}$ (residues 238-251) were substituted with that of either PHD1 or PHD3 (PHD2/1 and PHD2/3). After purification, CD spectroscopy indicated no significant change in the secondary structure of the proteins when compared to PHD2$_{181-426}$ (Figure S2).

The activities of PHD2/1 and PHD2/3 were compared to that of PHD2$_{181-426}$ for CODD and NODD substrates. Both PHD2/1 and PHD2/3 variants behaved similarly to PHD2$_{181-426}$ in terms of decarboxylation of 2OG to succinate and CO$_2$ with both NODD and CODD peptide substrates under standard conditions (Figure 4A). The PHD2 loop chimeras also appeared to hydroxylate both NODD and CODD substrates as efficiently as PHD2$_{181-426}$ under standard hydroxylation assay conditions (Figure 4B).

Replacement of the PHD2 β2β3 “finger-like” loop with that of PHD3 has a significant effect on the kinetics of CODD Hydroxylation. 2OG turnover assays, measuring initial velocity with substrate concentrations varying between 0-200µM for NODD and 0-100µM for CODD were then used to determine K$_m$ and v$_{max}$ values for PHD2/1 and PHD2/3 (Table 1). The K$_m$ (~50µM) and k$_{cat}$ (~ 0.05s$^{-1}$) values for the chimeric enzymes with NODD were similar, at least within the range of experimental error. In contrast, for the CODD substrates striking differences were apparent. With CODD as substrate, PHD2/3 had a k$_{cat}$ value approximately half of that for PHD2$_{181-426}$ and PHD2/1. However, there was also a drop in the K$_m$ value for PHD2/3 with CODD of ~ 10 fold compared to PHD2/1 and ~ 5 fold compared to PHD2$_{181-426}$. Importantly, such a drop in K$_m$ was not observed for NODD. This decrease in K$_m$ for CODD means that the k$_{cat}$/K$_m$ value for PHD2/3 was at least two fold greater than all other combinations, and indicated that differential binding of CODD and NODD with the β2β3 loop could play an important role in the reported near absolute preference of PHD3 for CODD as a substrate rather than NODD (8,21,22).

The β2β3 “Finger-Like” loop appears to influence selectivity in substrate hydroxylation. To unequivocally demonstrate that the difference in kinetic parameters seen for the PHD2/1 and PHD2/3 loop variants were reflected in their substrate specificity, competition experiments using the hydroxylation assay were carried out, at NODD and CODD concentrations of 150µM (Figure 5A). Under these conditions both PHD2/1 and PHD2/3 preferentially hydroxylated CODD as substrate rather than NODD (8,21,22).

When competition experiments in which NODD peptides were present at 500µM (Figures S4). When competition experiments in which NODD and CODD were simultaneously present were carried out for PHD2/1 and PHD2/3 (peptides at 150µM) over time periods ranging from 0-60 minutes (Figure 5B, lower graphs), CODD hydroxylation occurred at a notably faster rate than NODD hydroxylation. A significant decrease in overall NODD hydroxylation was seen for PHD2/3, even after 60 minutes. In single substrate experiments (Figure 5B, upper graphs), the substrate selectivity was less apparent. These results suggest that the effect of the β2β3 loop on substrate binding involves the selectivity of the enzyme for substrate hydroxylation with a preference of the PHD3 loop for CODD.
The β2β3 loop has an important role in substrate binding. To investigate whether the changes in $K_m$ were due, at least in part, to substrate binding, SPR analyses were then carried out using HIF-1α His$_6$-CODD$_{330-698}$ and His$_6$-NODD$_{344-503}$ substrates (Table 2). When compared to PHD2$_{181-426}$ the association and dissociation constants for His$_6$-NODD$_{344-503}$, PHD2/1 and PHD2/3 displayed an increase in the affinity of the enzyme-substrate interaction, as demonstrated by an increased association rate constant for PHD2/1 ($12.0 \pm 9.5 \times 10^5$ M$^{-1}$s$^{-1}$ compared to $1.46 \pm 0.41 \times 10^5$ M$^{-1}$s$^{-1}$, $n \geq 3$, $P<0.05$) and a decreased dissociation rate constant for both PHD2/1 and PHD2/3 ($0.22 \pm 0.17$ s$^{-1}$ and $0.54 \pm 0.16$ s$^{-1}$ compared to $1.06 \pm 0.39$ s$^{-1}$, $n \geq 3$, $P<0.005$ and $P<0.05$ respectively). This increased strength in binding with substituted β2β3 loop regions, however, is not reflected in differences in $k_{cat}$ values, implying the loop is predominantly involved in binding rather than hydroxylation. Additionally, the results reveal binding of both PHD2/1 and PHD2/3 to His$_6$-CODD$_{330-698}$ is stronger than PHD2$_{181-426}$, with particularly higher association rate constants ($29.5 \pm 11.7 \times 10^5$ M$^{-1}$s$^{-1}$ and $61.3 \pm 28 \times 10^5$ M$^{-1}$s$^{-1}$ respectively, compared to just $1.34 \pm 0.63 \times 10^5$ M$^{-1}$s$^{-1}$, $n \geq 3$, $P<0.001$), an effect that is even more marked than that seen with His$_6$-NODD$_{344-503}$ binding. The relative differences in strength of binding of protein substrates seen by SPR were also apparent in qualitative electrospray ionization mass spectrometry binding experiments with peptide substrates (Figure S6).

**Hydroxylated CODD is a weak inhibitor of NODD hydroxylation.** To investigate the possibility that the observed selectivity of PHD2$_{181-426}$ and its chimeras for CODD over NODD in competition experiments was caused by inhibition of NODD hydroxylation by hydroxylated CODD product binding to the active site, hydroxylation assays were carried out to assay NODD hydroxylation in the presence of a hydroxylated CODD peptide (CODD$_{Pro564-OH}$). Assay conditions were such that NODD and CODD$_{Pro564-OH}$ were present at equal concentrations, equivalent to the $K_m$ for NODD (Figure 6B). NODD hydroxylation was reduced in the presence of the CODD$_{Pro564-OH}$ peptide for PHD2/3, but not PHD2$_{181-426}$, PHD2$_{181-402}$ or PHD2/1, consistent with the strong preference of the PHD3 β2β3 loop region for CODD. Experiments were also carried out with an excess of CODD$_{Pro564-OH}$ (5 times the $K_m$ for NODD) with pre-incubation of the inhibiting peptide with the enzyme for 15 minutes at room temperature. Under these conditions, reduced NODD hydroxylation was seen for PHD2$_{181-426}$, PHD2$_{181-402}$, PHD2/1 and PHD2/3 (Figure 6C), showing that for each of the enzymes, CODD$_{Pro564-OH}$ is capable of inhibiting activity towards NODD hydroxylation.

**DISCUSSION**

There are precedents for a role of the C-terminus of 2OG oxygenases in substrate binding and selectivity (27-30). However, removal of the C-terminal residues 403-426 from PHD2$_{181-426}$ affects neither its ability to decarboxylate 2OG, nor its ability to hydroxylate CODD or NODD substrates when peptide substrates were assayed separately. Competition experiments under limiting substrate conditions indicated that in the presence of CODD, the truncation variant was slightly better than PHD2$_{181-426}$ at catalyzing NODD hydroxylation. We thus conclude that the C-terminal region has, at most, a minor role in the different NODD/CODD selectivities of the PHDs.

The results reveal that the β2β3 loop is an important region in determining substrate selectivity. In the case of crystallographic studies on FIH, its overall fold changes very little on CAD substrate (HIF-1α$_{786-826}$) binding, though localized changes are observed at the active site (32). In contrast the evidence presented here suggests that significant conformational changes occur upon HIF-1α binding to the PHDs. Precedent for such changes comes from crystallographic work on other 2OG oxygenases and related enzymes. In the case of the oxidase isopenicillin N synthase, which although not using 2OG as a cosubstrate belongs to the same superfamily, substrate binding occurs with movement of an arginine residue that interacts with the tripeptide substitute carboxylate (33).

With the 2OG oxygenases AlkB (34) and AsnO (35), there is also evidence for movement of flexible loops that fold to enclose the substrate. The loops in AlkB and AsnO are positioned differently compared to the β2β3 loop in the PHDs. It seems possible that the PHD β2β3 loops act in a similar way to isolate intermediates during catalysis.
The reported crystal structures of PHD26, in complex with iron and an inhibitor reveal that the β2β3 loop is located near the active site, but directed away from the metal centre (24). However, in these structures PHD26 crystallizes in a ‘head to tail’ trimeric form in which the C-terminus of one molecule interacts closely with the active site of another, and they may not fully reflect a conformation to which HIF–α binds. Similarly, the β2β3 loop interacts with a symmetry related molecule, possibly restricting its flexibility. The results presented here lead us to propose that, relative to the reported crystal structures, significant conformational changes are likely to occur upon substrate binding involving the β2β3 loop. With the PHD2/1 loop chimera, SPR data revealed that binding of both NODD and CODD was significantly increased relative to PHD26. In contrast, with the PHD2/3 chimera only binding of the CODD was increased, apparently due to a ~45 fold increase in $k_v$ and significantly more so than for the PHD2/1 chimera. Notably in the case of the PHD2/3 chimera this was reflected in a very much reduced $K_m$ (~6 fold, from ~37 to 6µM with CODD); such a reduction was not observed for any of the other variants studied. The combined SPR and kinetic results highlight the differing contributions of substrate binding and/or other catalytic processes towards the overall $K_m$ of each chimera towards each substrate.

Despite the significantly increased binding strength of NODD and CODD to PHD2/1 and of CODD to PHD2/3 there was little overall change in catalytic efficiency as judged by $k_{cat}/K_m$ values. For PHD2/1 the $k_{cat}/K_m$ values decreased, whereas for PHD2/3 with NODD it was the same as for PHD26. Only for the PHD2/3-CODD combination was a significant increase in $k_{cat}/K_m$ observed. Thus, the data imply that the β2β3 loop is involved in both binding of the substrate and subsequent catalytic events; evidence for the latter came from a loop deletion variant that oxidized 2OG but did not hydroxylate NODD or CODD under standard conditions.

Although our results are based on mutations within the context of an N-terminally truncated PHD26, and care must be taken in correlating analyses of purified proteins and peptide substrates with in vivo data, particularly when the proteins of interest are part of a complex system involving adjunct molecules, overall our study has provided biochemical evidence that the β2β3 loop is a significant factor in enabling the observed preference of PHD3 for CODD over NODD hydroxylation. Evidence that our kinetic data with isolated protein is relevant to in cell selectivity of the PHDs comes from the work of Villar et al (36), reported during the preparation of our manuscript. On the basis of yeast two hybrid and luciferase reporter assays with PHD and HIF variants they also propose that residues corresponding to the β2β3 loop are important in enabling the preference of PHD3 for CODD. Our kinetic analyses reveal that the β2β3 loop affects both the strength of the interaction between the enzyme and substrate, and catalytic efficiency.

If indeed a conformational change in the β2β3 loop is involved in substrate selectivity, an extra positively-charged residue in PHD3 (Arg71) in this region compared to PHD2 and PHD1 (Figure 1) may correlate with the unusual preference of the latter for negatively charged CODD (His530,CODD calculated pI = 5.5, CODD peptide calculated pI = 3.2). Alternatively, the effect of the extra residue in the PHD3 β2β3 loop may result in altered local structure, affecting changes in substrate binding/catalysis, e.g. presentation of the target proline to the active site in a catalytically productive manner.

Data are still emerging regarding the roles of different PHD isoforms, HIF–α isoforms and NODD/CODD hydroxylation within hypoxic responses in different cellular and physiological contexts. There is evidence that the role of PHD3 may become more important under hypoxia, corresponding with the greater decrease in NODD hydroxylation than CODD hydroxylation at limiting oxygen concentrations (17,20). With respect to the goal of therapeutically upregulating HIF–α via PHD inhibition, e.g. for the treatment of anemia or ischemic disease, it is presently unclear whether it is best to selectively inhibit specific PHDs (or PHD combinations), to selectively target inhibition of NODD and CODD hydroxylation, or to directly intervene at the level of HIF target genes; indeed given the broad physiological role of hypoxic regulation, all avenues may be individually pursued for specific therapeutic advantages. An understanding of the exact nature of the interaction between each enzyme and
substrate is desirable in order to target future therapeutics most effectively.

REFERENCES


FOOTNOTES

The work was supported by ReOx Ltd., the B.B.S.R.C., the Wellcome Trust and the Newton-Abraham Fund.

The abbreviations used are: Da, Dalton; HIF, hypoxia-inducible factor; PHD, prolyl hydroxylase domain containing; FIH, factor inhibiting HIF; NODD, N-terminal oxygen-dependent degradation domain; CODD, C-terminal oxygen-dependent degradation domain; 2OG, 2-oxoglutarate; CD, circular dichroism; SPR, surface plasmon resonance; MALDI-TOF, matrix-assisted laser desorption/ionization time of flight.

FIGURE LEGENDS

**FIGURE 1.** Sequence and Structural Features of PHD2. A, Sequence alignment of human PHD isoforms. Alignment generated with programs CLUSTAL W (37) and ESPript (ExPASy Molecular biology Server, www.expasy.org). The catalytic domain of each isozyme begins at PHD2181, as indicated. Residues with a black background are identical across isoforms and those outlined indicate homologous residues. The β2β3 loop and C-terminal tail regions are indicated, as is the MYND-type Zinc finger domain in the PHD2 N-terminus (38). The PHD1 N-terminus is predicted to be disordered. B, A ribbon representation of the PHD2181-426 structure (PDB I.D. 2G1M) (24). The flexible β2β3 loop residues
mutated are highlighted in black showing proximity to the active site, where iron is coordinated by the HXD…H motif.

**FIGURE 2.** *Comparison of the activity of PHD2<sub>181-426</sub> and PHD2<sub>181-402</sub>.* A, 2OG turnover (dark grey PHD2<sub>181-426</sub>, light grey PHD2<sub>181-402</sub>). B, Substrate hydroxylation as determined by MALDI-TOF mass spectrometry: NODD and CODD peptides were found to be best detected in negative ion mode. This gives m/z of ~1913 Da for NODD, and ~2249 Da for CODD, which increase to ~1929 Da and ~2265 Da respectively upon hydroxylation. Spectra 1-3 show NODD hydroxylation, and 4-6 show CODD hydroxylation, in the presence of PHD2<sub>181-426</sub> (spectra 1 and 4), PHD2<sub>181-402</sub> (spectra 2 and 5). Spectra 3 and 6 show the results of assays which took place in the absence of enzyme.

**FIGURE 3.** *Substrate Selectivity of PHD2<sub>181-426</sub> and PHD2<sub>181-402</sub>.* A, MALDI-TOF mass spectra showing selective hydroxylation of NODD and CODD when in competition with each other (peptide concentration 150µM): (1) in the presence of PHD2<sub>181-426</sub>, (2) in the presence of PHD2<sub>181-402</sub>, (3) in the absence of enzyme. B, Graphs showing % hydroxylation over time of NODD (black circles) and CODD (white circles), each at 150µM, when only one substrate was available (upper graphs), and when in competition with each other (lower graph): (i) and (ii) in the presence of PHD2<sub>181-426</sub>, (iii) and (iv) in the presence of PHD2<sub>181-402</sub>.

**FIGURE 4.** *Comparison of the activity of PHD2<sub>181-426</sub>, PHD2/1 and PHD2/3.*** A, 2OG turnover (dark grey PHD2<sub>181-426</sub>; light grey PHD2/1; stripes PHD2/3). B, substrate hydroxylation as determined by MALDI-TOF mass spectrometry: Spectra 1-4 show NODD hydroxylation, and 5-8 show CODD hydroxylation, in the presence of PHD2<sub>181-426</sub> (spectra 1 and 5), PHD2/1 (spectra 2 and 6), PHD2/3 (spectra 3 and 7). Spectra 4 and 8 show the results of assays which took place in the absence of enzyme.

**FIGURE 5.** *Substrate Selectivity of PHD2/1 and PHD2/3.* A, MALDI-TOF mass spectra showing selective hydroxylation of NODD and CODD when in competition with each other (peptide concentration 500µM): (1) in the presence of PHD2<sub>181-426</sub>, (2) in the presence of PHD2/1, (3) in the presence of PHD2/3, (4) in the absence of enzyme. B, Graphs showing % hydroxylation over time of NODD (black circles) and CODD (white circles), each at 150µM, when only one substrate was available (top), and when in competition with each other (bottom): (i) and (ii) in the presence of PHD2/1, (iii) and (iv) in the presence of PHD2/3.

**FIGURE 6.** Inhibition of NODD hydroxylation by CODD<sub>Pro564-OH</sub> for PHD2<sub>181-426</sub> (1), PHD2<sub>181-402</sub> (2), PHD2/1 (3) and PHD2/3 (4). A, NODD hydroxylation with no CODD<sub>Pro564-OH</sub> present; B, NODD hydroxylation with CODD<sub>Pro564-OH</sub> present at a concentration equal to NODD K<sub>m</sub>; C, NODD hydroxylation after 15 minutes pre-incubation with CODD<sub>Pro564-OH</sub> present at concentration five times NODD K<sub>m</sub>.

**TABLE LEGENDS**

**TABLE 1.** A comparison of the kinetics of 2OG turnover. Kinetic parameters were obtained measuring initial velocity for PHD2<sub>181-426</sub>, PHD2<sub>181-402</sub>, PHD2/1 and PHD2/3 with NODD and CODD substrates, n=2-4.

**TABLE 2.** A comparison of the kinetics of the enzyme-substrate interaction. The kinetics of each interaction were determined by SPR for PHD2<sub>181-426</sub>, PHD2<sub>181-402</sub>, PHD2/1 and PHD2/3 with His<sub>6</sub>-CODD<sub>530-698</sub> and His<sub>6</sub>-NODD<sub>344-503</sub> protein substrates, n=3-8.
FIGURES

Figure 1A

Figure 1B
Figure 3

A  NODD

1912.30  1928.27

1912.36  1928.33

m/z

(1)

CODD

2248.55  2264.44

2248.61  2264.51

(2)

(3)

B  PHD2₁₈₁-₄₂₆

% Hydroxylation

0  10  20  30  40  50  60

Time (mins)

(i)

(iii)

PHD2₁₈₁-₄₀₂

% Hydroxylation

0  10  20  30  40  50  60

Time (mins)

(ii)

(iv)
Figure 5

A. NODD

1912.24
1912.27
1912.36
1928.18
1928.21
1928.27

(1) CODD

2247.56
2247.63
2248.48
2264.35
2264.38
2264.51

B. PHD2/1

(i)

% Hydroxylation

Time (mins)
0 10 20 30 40 50 60

(ii)

% Hydroxylation

Time (mins)
0 10 20 30 40 50 60

B. PHD2/3

(iii)

% Hydroxylation

Time (mins)
0 10 20 30 40 50 60

(iv)

% Hydroxylation

Time (mins)
0 10 20 30 40 50 60
### Figure 6

![Figure 6](image)

### TABLES

#### Table 1

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Substrate</th>
<th>Reaction time (mins)</th>
<th>$K_M$ (µM)</th>
<th>$v_{max}$ (pmol s$^{-1}$)</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
<th>$k_{cat}/K_M$ (M$^{-1}$ s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PHD2$_{181-426}$</td>
<td>19mer NODD</td>
<td>5</td>
<td>44.4 (±8.4)</td>
<td>17.7 (±1.2)</td>
<td>0.044 (±0.003)</td>
<td>9.91 (±3.0)x10$^2$</td>
</tr>
<tr>
<td></td>
<td>19mer CODD</td>
<td>5</td>
<td>36.7 (±9.0)</td>
<td>13.2 (±1.3)</td>
<td>0.033 (±0.003)</td>
<td>8.99 (±3.2) x10$^3$</td>
</tr>
<tr>
<td>PHD2$_{181-402}$</td>
<td>19mer NODD</td>
<td>5</td>
<td>50.1 (±8.1)</td>
<td>27.6 (±1.6)</td>
<td>0.069 (±0.004)</td>
<td>13.77 (±3.1) x10$^2$</td>
</tr>
<tr>
<td></td>
<td>19mer CODD</td>
<td>5</td>
<td>77.2 (±15.2)</td>
<td>25.74 (±2.2)</td>
<td>0.064 (±0.005)</td>
<td>8.29 (±2.4) x10$^2$</td>
</tr>
<tr>
<td>PHD2/1</td>
<td>19mer NODD</td>
<td>7</td>
<td>56.9 (±11.6)</td>
<td>16.1 (±1.2)</td>
<td>0.040 (±0.003)</td>
<td>7.03 (±2.0) x10$^2$</td>
</tr>
<tr>
<td></td>
<td>19mer CODD</td>
<td>7</td>
<td>63.0 (±15.7)</td>
<td>12.6 (±1.6)</td>
<td>0.031 (±0.004)</td>
<td>4.92 (±2.0) x10$^2$</td>
</tr>
<tr>
<td>PHD2/3</td>
<td>19mer NODD</td>
<td>7</td>
<td>56.0 (±9.2)</td>
<td>23.0 (±1.4)</td>
<td>0.057 (±0.003)</td>
<td>10.2 (±2.3) x10$^2$</td>
</tr>
<tr>
<td></td>
<td>19mer CODD</td>
<td>7</td>
<td>6.4 (±0.2)</td>
<td>6.5 (±1.4)</td>
<td>0.016 (±0.003)</td>
<td>24.9 (±5.5) x10$^2$</td>
</tr>
<tr>
<td>Enzyme</td>
<td>Substrate</td>
<td>$k_a (M^{-1}s^{-1}) \times 10^7$</td>
<td>$k_d (s^{-1})$</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>------------</td>
<td>------------</td>
<td>---------------------------------</td>
<td>-----------------</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PHD2_{181-425}</td>
<td>His$_6$-NODD</td>
<td>1.46 (±0.41)</td>
<td>1.06 (±0.39)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>His$_6$-CODD</td>
<td>1.34 (±0.63)</td>
<td>0.14 (±0.04)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PHD2_{181-402}</td>
<td>His$_6$-NODD</td>
<td>1.50 (±0.37)</td>
<td>0.64 (±0.13)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>His$_6$-CODD</td>
<td>2.09 (±0.87)</td>
<td>0.16 (±0.03)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PHD2/1</td>
<td>His$_6$-NODD</td>
<td>12.0 (±9.5)</td>
<td>0.22 (±0.17)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>His$_6$-CODD</td>
<td>29.5 (±11.7)</td>
<td>0.20 (±0.11)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PHD2/3</td>
<td>His$_6$-NODD</td>
<td>1.54 (±0.33)</td>
<td>0.54 (±0.16)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>His$_6$-CODD</td>
<td>61.3 (±28)</td>
<td>0.17 (±0.03)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Kinetic rationale for selectivity towards N- and C-terminal oxygen dependent degradation domain substrates mediated by a loop region of HIF prolyl hydroxylases

Emily Flashman, Eleanor A. L. Bagg, Rasheduzzaman Chowdhury, Jasmin Mecinovic, Christoph Loenarz, Michael A. McDonough, Kirsty S. Hewitson and Christopher J. Schofield

J. Biol. Chem. published online December 5, 2007

Access the most updated version of this article at doi: 10.1074/jbc.M707411200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

Supplemental material:
http://www.jbc.org/content/suppl/2007/12/06/M707411200.DC1

This article cites 0 references, 0 of which can be accessed free at
http://www.jbc.org/content/early/2007/12/05/jbc.M707411200.citation.full.html#ref-list-1