Kinetic Rationale for Selectivity toward N- and C-terminal Oxygen-dependent Degradation Domain Substrates Mediated by a Loop Region of Hypoxia-Inducible Factor Prolyl Hydroxylases

Received for publication, September 4, 2007, and in revised form, November 28, 2007. Published, JBC Papers in Press, December 5, 2007, DOI 10.1074/jbc.M707411200

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

From the Chemistry Research Laboratory, Department of Chemistry and the Oxford Centre for Integrative Systems Biology, University of Oxford, Mansfield Road, Oxford OX1 3TA, United Kingdom

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 (in competition experiments), as observed for wild-type PHD3. CODD was observed to bind much more tightly to this chimeric protein than the wild type PHD2 catalytic domain.

The heterodimeric transcription factor Hypoxia-Inducible Factor 1 (HIF1) 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 in Refs. 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, Pro–402 (N-terminal oxygen-dependent degradation domain, NODD) and Pro–564 (C-terminal oxygen-dependent degradation domain, CODD). HIF-1α is also hydroxylated in its C-terminal transactivation domain (C-TAD) at Asn–803, in a reaction catalyzed by another iron and 2OG-dependent oxygenase, Factor Inhibiting HIF (FIH) (11–13). Hydroxylation of Asn–803 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 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 (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 (8, 21, 22), and that PHD1 displays less activity toward 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 potentially 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 (Fig. 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, UK. The NODD peptide sequence used was DALTLLAPAGDTIISLDF (one-letter amino acid abbreviations), and the CODD peptide sequence LDLLEMLAPYIMPMDFFQQL. These peptides are referred to as NODD and CODD hereafter. A hydroxylated CODD peptide (CODD-Pro564-OH), LDLLEMLAHypYIMPMDFFQQL (Hyp represents the heating of 4-hydroxynorleucine, was prepared on a CS Bio 2 loop) was prepared by synthesizing using a standard 9-fluorenemethoxycarbonyl/N,N′-disopropylcarbodiimide/1-hydrobenzotriazole strategy, then purified by reversed-phase HPLC. DNA primers were from SigmaGenosys Ltd.

**Cloning of PHD2**<sub>181–402</sub> **PHD2<sub>loop</sub><**<br>**PHD2/1, and PHD2/3—**

The DNA sequence encoding PHD2<sub>181–402</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 Supplemental Table S1). A deletion variant (PHD2<sub>loop</sub>) was produced whereby residues 238–250 (the β2β3 loop) were removed by site-directed mutagenesis (Stratagene) (Supplemental Table S1). Variant chimeric proteins were generated whereby the β2β3 loop of PHD2<sub>181–402</sub> was replaced with that of either PHD1 or PHD3 (proteins termed PHD2/1 or PHD2/3, respectively). PHD2/1A forward and reverse primers (Supplemental Table S1) were designed and used to amplify the sequence encoding for PHD2<sub>181–243</sub> PHD2/1B forward and reverse primers (Supplemental Table S1) were used to amplify the sequence encoding for PHD2<sub>251–426</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 (Supplemental Table S1) was designed to mutate the DNA sequence encoding PHD2<sub>241–251</sub> to the sequence encoding PHD3<sub>62–73</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—**

PHD2<sub>181–426</sub> and variants were produced 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 100 μl (final volume) contained typically enzyme (4 μM), substrate (100 μM), 2OG (288 μM), [¹⁴C]-2OG (3.6 μM), Fe(II) (500 μM), ascorbate (4 mM), dithiothreitol (1 mM), and catalase (0.3 mg/ml). Unless stated, reactions took place for 15 min at 37 °C, then were stopped with 200 μl of methanol and incubated on ice for 20 min to allow complete absorption of all residual [¹⁴C]CO₂. For kinetic measurements, the optimum assay time for measuring initial velocity was determined by time course experiments, and assays were then carried out over a range of substrate concentrations. Data were fit by the Michaelis-Menten equation using SigmaPlot 2000, allowing *Kₘ* (or apparent *Kₘ*) and *v_max* 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. Assay solution (1 μl) was mixed with α-cyano-4-hydroxycinnamic acid MALDI matrix (1 μl) (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<sup>TM</sup> or BIAcore T100<sup>TM</sup> (GE Healthcare). His-NODD<sub>344–503</sub> or His-CODD<sub>530–698</sub> protein substrates (purified as described in Ref. 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 (15 μl) was injected over the experimental and control flow cells at 50 μl/min, 25 °C in 10 mM HEPES pH 7.4, 150 mM 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**

PHD2<sub>181–402</sub> Catalyzes 2OG Turnover and NODD/CODD Hydroxylation Similarly to PHD2<sub>181–426</sub>

Studies on some 2OG oxygenases from the subfamily 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 PHD2\textsubscript{181–426} (24) (Fig. 1) show high conservation and homology within their catalytic domains. Aside from their very different N termini, there are two regions where this conservation is not maintained: the C-terminus and the flexible \( \beta_{2} \beta_{3} \) loop region located near the active site, suggesting a potential role for these regions in determining selectivity (24). A C-terminal truncation variant, PHD2\textsubscript{181–402} was therefore targeted because significant sequence variation occurs to the C-terminal side of residue 402. Further truncations were also produced (PHD2\textsubscript{181–399} and PHD2\textsubscript{181–397}), but these showed a reduction in catalytic activity (Supplemental Fig. S1). We therefore focused on PHD2\textsubscript{181–402} for substrate selectivity studies. Circular dichroism (CD) spectroscopy indicated no significant differences between the secondary structures of PHD2\textsubscript{181–402} and PHD2\textsubscript{181–426} (Supplemental Fig. S2).

Activity assays, measuring total 2OG turnover during a standard 15-min 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 PHD2\textsubscript{181–426} to decarboxylate 2OG to succinate and CO\(_2\) when using either NODD or CODD peptide substrates (Fig. 2A). Similarly, no difference in 2OG turnover was seen between PHD2\textsubscript{181–426} and PHD2\textsubscript{181–402} when longer His\(_6\)-CODD\(_{530–698}\) and His\(_6\)-NODD\(_{344–503}\) protein substrates were used (Supplemental Fig. 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 (Fig. 2B). The C-terminally truncated PHD2\textsubscript{181–402} enzyme was found to hydroxylate both NODD and CODD substrates as efficiently as PHD2\textsubscript{181–426} under these assay conditions.

Further 2OG turnover assays, measuring initial velocity at substrate concentrations varying from 0–200 \( \mu \)M, gave \( K_m \) values for PHD2\textsubscript{181–426} of 44 \( \mu \)M and 37 \( \mu \)M for the NODD and CODD peptides, respectively (Table 1). Previously, PHD2\textsubscript{181–426} \( K_m \) values for NODD and CODD peptides were reported as 24 \( \mu \)M and 2 \( \mu \)M, respectively, as determined by an oxygen consumption assay (25), and 130 \( \mu \)M and 7 \( \mu \)M, respectively, as determined by 2OG turnover assays using crude cell lysates.
determine the preference of PHD2181–426 and PHD2181–402 for terminus also results in an increase in doubles on removal of the C-terminus. Truncation of the C-terminus results in little overall change in catalytic efficiency with a larger increase for CODD than NODD. When the NODD Hydroxylation to PHD2 181–426

To investigate whether the C-terminus has a role in substrate recognition to Both His6-CODD530–698 and His6-NODD344–503 binding, surface plasmon resonance (SPR) studies were carried out using purified His6-CODD530–698 and His6-NODD344–503 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 PHD2181–426 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). PHD2181–402 was found to have $K_m$ values for NODD and CODD of 50 $\mu$M and 77 $\mu$M 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 $k_{cat}$ 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 toward NODD.

PHD2181–402 Shows a Similar Preference for CODD over NODD Hydroxylation to PHD2181–426—To unequivocally determine the preference of PHD2181–426 and PHD2181–402 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 150 $\mu$M (Fig. 3A), PHD2181–426 preferentially hydroxylated CODD peptide, while PHD2181–402 showed less selectivity between the two substrates. A greater selectivity for CODD hydroxylation was apparent for both PHD2181–426 and PHD2181–402 when both peptides were present at 500 $\mu$M (Supplemental Fig. S4). When competition experiments were carried out over time periods ranging from 0–60 min (Fig. 3B, lower graphs), it appears that, when compared with single substrate experiments (Fig. 3B, upper graphs), CODD hydroxylation occurred at a faster rate than NODD hydroxylation for PHD2181–426 indicating selectivity for this substrate. The rate of NODD and CODD hydroxylation by PHD2181–402 is quite similar (compared with PHD2181–426) and this likely reflects the increased $k_{cat}/K_m$ for hydroxylation of NODD over CODD seen for this variant compared with PHD2181–426. 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.

PHD2181–402 and PHD2181–426 Show Similar Binding Characteristics to Both His6-NODD344–503 and His6-CODD530–698—To investigate whether the C-terminus has a role in substrate binding, surface plasmon resonance (SPR) studies were carried out using purified His6-CODD530–698 and His6-NODD344–503

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Substrate</th>
<th>Reaction time</th>
<th>$K_m$</th>
<th>$v_{max}$</th>
<th>$k_{cat}$</th>
<th>$k_{cat}/K_m$</th>
</tr>
</thead>
<tbody>
<tr>
<td>PHD2181–426</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) × 10²</td>
</tr>
<tr>
<td>PHD2181–402</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) × 10²</td>
</tr>
<tr>
<td>PHD2/1</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>13.8 (±3.1) × 10²</td>
</tr>
<tr>
<td>PHD2/3</td>
<td>19mer CODD</td>
<td>5</td>
<td>77.2 (±15.2)</td>
<td>25.7 (±2.2)</td>
<td>0.064 (±0.005)</td>
<td>8.29 (±2.4) × 10²</td>
</tr>
</tbody>
</table>

FIGURE 3. Substrate selectivity of PHD2181–426 and PHD2181–402. A, MALDI-TOF mass spectra showing selective hydroxylation of NODD and CODD when in competition with each other (peptide concentration 150 $\mu$M): (1) in the presence of PHD2181–426, (2) in the presence of PHD2181–402, (3) in the absence of enzyme. B, graphs showing % hydroxylation over time of NODD (black circles) and CODD (white circles), each at 150 $\mu$M, when only one substrate was available (upper graphs), and when in competition with each other (lower graph); (i) and (ii) in the presence of PHD2181–426 (iii) and (iv) in the presence of PHD2181–402.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Substrate</th>
<th>$k_a$</th>
<th>$k_d$</th>
<th>$n/s$</th>
</tr>
</thead>
<tbody>
<tr>
<td>PHD2181–426</td>
<td>His$_6$-NODD</td>
<td>1.46 (±0.41)</td>
<td>1.06 (±0.39)</td>
<td></td>
</tr>
<tr>
<td>PHD2181–402</td>
<td>His$_6$-CODD</td>
<td>1.34 (±0.63)</td>
<td>0.14 (±0.04)</td>
<td></td>
</tr>
<tr>
<td>PHD2/1</td>
<td>His$_6$-NODD</td>
<td>1.50 (±0.37)</td>
<td>0.64 (±0.13)</td>
<td></td>
</tr>
<tr>
<td>PHD2/3</td>
<td>His$_6$-CODD</td>
<td>2.09 (±0.87)</td>
<td>0.16 (±0.03)</td>
<td></td>
</tr>
</tbody>
</table>

TABLE 1
A comparison of the kinetics of 2OG turnover

TABLE 2
A comparison of the kinetics of the enzyme-substrate interaction

FEBRUARY 15, 2008 • VOLUME 283 • NUMBER 7

JOURNAL OF BIOLOGICAL CHEMISTRY

3811
HIF Prolyl Hydroxylase Substrate Selectivity

HIF-1α substrates (Table 2). As reported (25), PHD2181–426 binds to Hisα-CODD530–698 more strongly than it binds to Hisα-NODD344–503, as indicated by a slower dissociation rate constant (0.14 (±0.04) s⁻¹ compared with 1.06 (±0.39) s⁻¹, n = 7, p < 0.001). The association rate constants for the two substrates are very similar. PHD2181–402 also bound to Hisα-CODD530–698, more strongly than it did to Hisα-NODD344–503 again indicated by a slower dissociation rate constant (CODD: 0.16 (±0.03) s⁻¹ compared with NODD: 0.64 (±0.13) s⁻¹, n ≥ 5, p < 0.001). The rate of association for PHD2181–403 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 PHD2181–426 and PHD2181–402 with respect to each substrate, with the only statistically significant difference being a decrease in the dissociation constant for Hisα-NODD344–503 when the C-terminus is truncated (p < 0.05). These results reflect the similarities seen between PHD2181–426 and PHD2181–403 in catalysis.

**PHD2/1 and PHD2/3 Catalyze 2OG Turnover and NODD/CODD Hydroxylation**—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 PHD2181–426 were deleted and replaced by Ala-Ala, i.e. 237DGQLVSQKSDSSKDI251 was replaced with 237DAAI251. The Ala-Ala sequence was aimed at providing a short linker between β2 and β3 while 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 (Supplemental Fig. S5A), hydroxylation assays demonstrated that this variant catalyzed less than 5% substrate hydroxylation under the standard conditions of 15 min of incubation at 37 °C (Supplemental Fig. S5B). Non-denaturing protein MS assays (31) indicated that the loop deletion variant formed a less stable complex with Fe(II) and 2OG than PHD2181–426 but that it was capable of binding CODD and NODD even though hydroxylation was low (<5%) (Supplemental Fig. S6, a and b). Together, these observations indicate that the β2β3 loop is involved in coupling 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 PHD2181–426 (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 with PHD2181–426 (Supplemental Fig. S2).

The activities of PHD2/1 and PHD2/3 were compared with that of PHD2181–426 for CODD and NODD substrates. Both PHD2/1 and PHD2/3 variants behaved similarly to PHD2181–426 in terms of decarboxylation of 2OG to succinate and CO₂ with both NODD and CODD peptide substrates under standard conditions (Fig. 4A). The PHD2 loop chimeras also appeared to hydroxylate both NODD and CODD substrates as efficiently as PHD2181–426 under standard hydroxylation assay conditions (Fig. 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 0–200 μM for NODD and 0–100 μM for CODD were then used to determine Kₘ and Vₘₐₓ values for PHD2/1 and PHD2/3 (Table 1). The Kₘ (~50 μM) and kₐₜ (~0.05 s⁻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 kₐₜ value approximately half of that for PHD2181–426 and PHD2/1. However, there was also a drop in the Kₘ value for PHD2/3 with CODD of ~10-fold compared with PHD2/1 and ~5-fold compared with PHD2181–426. Importantly, such a drop in Kₘ was not observed for NODD. This decrease in Kₘ for CODD means that the kₐₜ/Kₘ value for PHD2/3 was at least 2-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 further investigate whether 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 150 μM (Fig. 5A). Under these conditions both PHD2/1 and
PHD2/3 preferentially hydroxylated CODD as also seen for PHD2181–426. A greater selectivity for CODD hydroxylation was again seen when both peptides were present at 500 μM (Supplemental Fig. S4). When competition experiments in which NODD and CODD were simultaneously present were carried out for PHD2/1 and PHD2/3 (peptides at 150 μM) over time periods ranging from 0–60 min (Fig. 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 min. In single substrate experiments (Fig. 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$_{530–698}$ and His$_6$-NODD$_{344–503}$ substrates (Table 2). When compared with PHD2181–426, 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 (± 9.5) × 10$^5$ M$^{-1}$ s$^{-1}$ compared with 1.46 (±0.41) × 10$^5$ M$^{-1}$ s$^{-1}$, $n$ ≥ 3, $p < 0.05$) and a decreased dissociation rate constant for both PHD2/1 and PHD2/3 (0.22 (± 0.17) s$^{-1}$ and 0.54 (± 0.16) s$^{-1}$ compared with 1.06 (± 0.39) s$^{-1}$, $n$ ≥ 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 $K_m$ values, implying the loop is predominantly involved in the binding rather than the hydroxylation step. Additionally, the results reveal binding of both PHD2/1 and PHD2/3 to His$_6$-CODD$_{530–698}$ is stronger than PHD2$_{181–426}$ with particularly higher association rate constants (29.5 (± 11.7) × 10$^5$ M$^{-1}$ s$^{-1}$ and 61.3 (± 28.0) × 10$^5$ M$^{-1}$ s$^{-1}$ respectively, compared with just 1.34 (± 0.63) × 10$^5$ M$^{-1}$ s$^{-1}$, $n$ ≥ 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 electro spray ionization mass spectrometry binding experiments with peptide substrates (Supplemental Fig. 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 CODD and CODD$_{Pro564-OH}$ were present at equal concentrations, equivalent to the $K_m$ for NODD (Fig. 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 PHD2β3 loop region for CODD. Experiments were also carried out with an excess of CODD$_{Pro564-OH}$ (5 times the $K_m$ for NODD) with preincubation of the inhibiting peptide with the enzyme for 15 min at room temperature. Under these conditions, reduced NODD hydroxylation was seen for PHD2$_{181–426}$, PHD2$_{181–402}$, PHD2/1, and PHD2/3 (Fig. 6C), showing that for each of the enzymes, CODD$_{Pro564-OH}$ is capable of inhibiting activity toward 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 a relatively minor role in determining substrate selectivity. In the case of crystallographic studies on FIH, its overall fold changes very little on reduction of the catalytic domain (29–31). This is consistent with the observation that the C-terminus plays a minor role in determining substrate selectivity. In the case of crystallographic studies on FIH, its overall fold changes very little on reduction of the catalytic domain (29–31). This is consistent with the observation that the C-terminus plays a minor role in determining substrate selectivity.
HIF Prolyl Hydroxylase Substrate Selectivity

Figure 6. Inhibition of NODD hydroxylation by CODD_{Pro564-OH} for PHD2_{181-426} (1), PHD2_{181-402} (2), PHD2/1 (3), and PHD2/3 (4). A, NODD hydroxylation with no CODD_{Pro564-OH} present; B, NODD hydroxylation with CODD_{Pro564-OH} present at a concentration equal to NODD {K_m}; C, NODD hydroxylation after 15 min of preincubation with CODD_{Pro564-OH} present at a concentration of five times the NODD {K_m}.

Additionally 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 with 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 PHD2_{181-426} in complex with iron and an inhibitor reveal that the β2β3 loop is located near the active site, but directed away from the metal center (24). However, in these structures PHD2_{181-426} 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 PHD2_{181-426}. In contrast, with the PHD2/3 chimera only binding of the CODD was increased, apparently due to a ~45-fold increase in {k_cat} 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 6 μM 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 toward the overall {K_m} of each chimera toward 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 PHD2_{181-426}. 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 PHD2_{181-426} 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 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 effects 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 (Arg-71) in this region compared with PHD2 and PHD1 (Fig. 1) may correlate with the unusual preference of the latter for negatively charged CODD (His_{Pro564-CODD_{530-698}} 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 rather than CODD hydroxy-
loration 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) or to selectively target inhibition of NODD and CODD hydroxylation. Given the broad physiological role of hypoxic regulation, alternative approaches may be appropriate for different therapeutic objectives. An understanding of the exact nature of the interaction between each enzyme and substrate is desirable to target future therapeutics most effectively.

REFERENCES