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Studies on the Substrate Selectivity of the Hypoxia-Inducible Factor Prolyl Hydroxylase 2 Catalytic Domain

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Abstract: In animals, the response to chronic hypoxia is mediated by upregulation of the α,β-heterodimeric hypoxia inducible factors (HIFs). Levels of HIFα isoforms, but not HIFβ, are regulated by their post-translational modification as catalysed by prolyl hydroxylase domain enzymes (PHDs). Different roles for human HIF-1/2α isoforms and their two oxygen dependent degradation domains (ODDs) are proposed. We report kinetic and NMR analyses on the ODD selectivity of the catalytic domain of wildtype PHD2 (which is conserved in nearly all animals) and clinically observed variants. Studies using Ala-scanning and 'hybrid' ODD peptides imply the relatively rigid conformation of the (hydroxylated) proline plays an important role in ODD binding. They also reveal differential roles in binding for the residues on the N- and C-terminal sides of the substrate proline. The overall results inform on how the PHDs achieve selectivity for HIFα ODDs and may be of use in identifying substrate selective PHD inhibitors.

In animals, the response to chronic hypoxia is mediated by upregulation of the α,β-heterodimeric hypoxia inducible factors (HIFs). Levels of HIFα isoforms, of which there are three in humans, but not HIFβ, are negatively regulated by post-translational modification of their oxygen dependent degradation domains (ODDs) as catalysed by the prolyl hydroxylase domain enzymes (PHD1-3 or EGLN1-3).[1] The three human PHDs (PHD1-3 or EGLN1-3) are Fe^{II} and 2-oxoglutarate (2OG) dependent oxygenases, whose activity is normally limited by oxygen availability. PHD-catalysed HIFα prolyl hydroxylation substantially promotes binding of HIFα isoforms to the von Hippel Lindau protein (pVHL), which is a targeting component of a ubiquitin ligase complex (Fig. 1A).[1]

Different, sometimes overlapping, roles of HIF-1α and HIF-2α have been identified and emerging results imply the PHDs may have different roles.[1a, 1c, 2] The two ODDs in the HIF-1α and 2α isoforms have prolyl hydroxylation sites.[1b, 1c] Hydroxylation of either the N- (NODD) or the C- (CODD) terminal ODD can signal for HIFα proteolysis,[2-3] with substitution of either $Pro402_{NODD}$ or $Pro564_{CODD}$ being sufficient to stabilise HIFα in normoxia.[2] There is evidence that CODD is preferentially hydroxylated over NODD, including in cells.[4] Bioinformatics imply a CODD, but not a NODD, type ODD is present in HIFα of early animals, which usually contains only one PHD, a PHD2 homologue.[5] The ODD selectivity of the PHDs varies; CODD is generally preferred over NODD, with PHD3 being particularly selective for CODD.[4a, 4b, 6] Crystallographic studies on complexes of the catalytic domain of $PHD2_{181-426}$ (tPHD2) with CODD and NODD have provided insight into how the PHDs bind to the ODDs (Fig. 1B), including how clinically observed PHD2 variants can manifest altered ODD selectivities.[1a, 9] They also reveal that induced fit during PHD:ODD binding involves a loop (β2β3 loop) and the C-terminal region of PHD2.[1a, 4a, 9a]

The precise reasons for the presence of two ODDs within the HIFα proteins in higher animals are unclear,[7] but they may help modulate the kinetics of PHD catalysis.[1a] PHD inhibitors are presently in clinical trials for anaemia treatment in chronic kidney disease because erythropoietin is a HIF target gene,[8] and hence, understanding how PHDs achieve ODD selectivity is of medical interest. We report kinetic and binding studies on the HIF-1α ODD selectivity of human tPHD2 and clinically observed tPHD2 variants, using alanine-scanning and HIF-1α CODD-NODD/NODD-CODD 'hybrid' peptides.

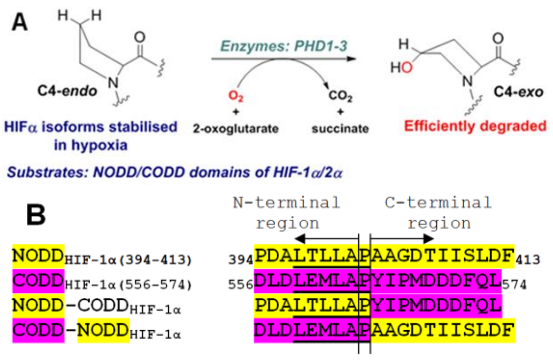

Figure 1. A. Prolyl hydroxylase domain enzymes (PHDs) catalyse oxygen availability limited hydroxylation of N- and C-terminal oxygen dependent degradation domains (NODD and CODD, respectively) of hypoxia inducible factor α (HIFα) isoforms. **B.** Sequences of CODD and NODD of HIF-1α and the hybrid peptides used in this study. The conserved ODD LxxLAP motif in the ODDs is underlined.

To investigate the selectivity of ODD HIF-1α binding to tPHD2, we carried out 18 'alanine-scanning' and other substitutions of clinical or mechanistic interest on an 19-mer HIF-1α $CODD_{556-574}$ peptide substrate (DLDLEMLAPYIPMDDDFQL-NH_2), which has been extensively used in prior work on the PHDs.[4a, 10] The alanine substituted HIF-1α $CODD_{556-574}$ peptides were tested as tPHD2 substrates using HIF-1α $CODD_{556-574}$ and HIF-1α $NODD_{394-413}$ (PDALTLLAPAAGDTIISLDF-NH_2) for comparison; an

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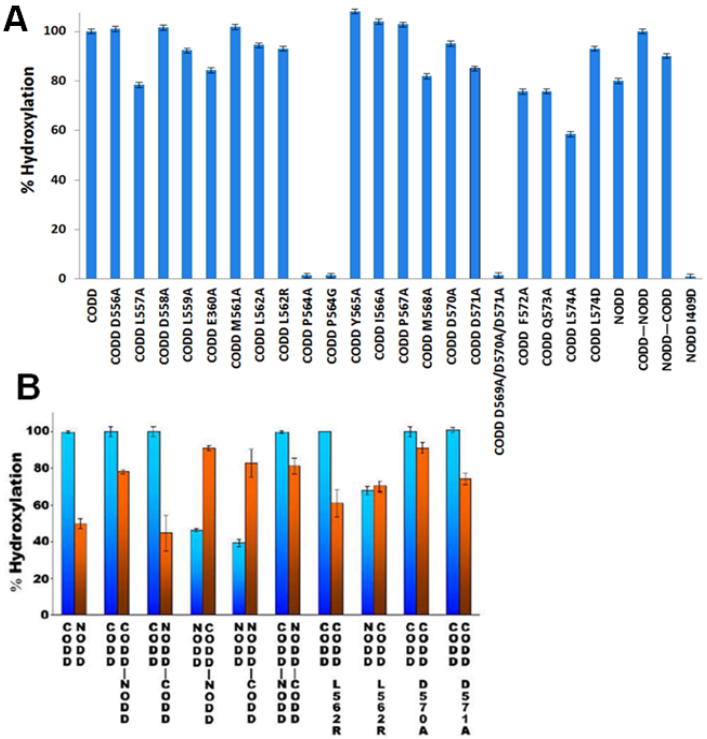

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$CODD_{HIF-1\alpha}$ variant	PHD2 residues with $\Delta\delta > 0.1$ ppm	Possible molecular reasons
D556A	T268	
L557A	None	
D558A	S248; T268; D320	
L559A	None	
E560A	T268; G321	
M561A	D246; S248; D250; T268; G321	D246, S248 and D250 are on the β2β3 loop which is important in ODD binding
L562A	S248; T268; R312	$R312_{tPHD2}$-$L562_{CODD}$ = ~3.3 Å
P564A/G	None (weak binding)	Importance of the proline conformation for binding
Y565A	T268; G321; K400; V401	$Y565_{CODD}$ interacts with C-terminal α4
I566A	F391	$F391_{tPHD2}$-$I566_{CODD}$ = ~3.5 Å
P567A	T268	
M568A	K400; V401	$K400_{tPHD2}$-$M568_{CODD}$ = ~3.4 Å; C-terminal α4
D569A/D570A/D571A	None (weak binding)	Importance of salt-bridge interactions with PHD C-terminal region
F572A	E260; G261; K262; R295; F391	$K262_{tPHD2}$-$F572_{CODD}$ = ~4.2 Å; $R295_{tPHD2}$-$F572_{CODD}$ = ~2.8 Å; $F391_{tPHD2}$-$F572_{CODD}$ = ~5.6 Å
Q573A	N293; G294	$N293_{tPHD2}$-$Q573_{CODD}$ = ~3.6 Å ($\Delta\delta > 0.05$)
L574A	S248; H374; A393; D394; A397; A398	H374: Fe^{II} ligand; D394, A397 and A398 located on C-terminal α4

Figure 2. Hydroxylation and binding of HIF-1α ODD variant peptides assayed by MS (turnover) and NMR (binding). **A**. 'Individual substrate assays' showing % hydroxylation of ODD variant peptides by tPHD2. **B**. 'Competition experiments' with substrate pairs. tPHD2 (2 μM) was incubated with $(NH_4)_2Fe(II)(SO_4)_2$ (50 μM), 2OG disodium salt (60 μM), sodium L-ascorbate (300 μM) and HIF-1α CODD peptides (50 μM), in Tris (50 mM), pH 7.5/25 °C. Reactions (15 min) were quenched with 1% ($^{v}/_{v}$) aqueous formic acid. Errors: standard deviations (n = 3). 100% corresponds to full substrate hydroxylation. **C.** Summary of differences in ^{1}H-^{15}N HSQC spectra for ^{15}N-$PHD2_{181-402}$-Zn(II)-2OG-$CODD_{HIF-1\alpha}$ and ^{15}N-$PHD2_{181-402}$-Zn^{II}-2OG with Ala-substituted CODD HIF-1α variants**.** Residues with $\Delta\delta > 0.1$ ppm difference between the 2 forms are listed, except for $Q573A_{CODD}$, where $\Delta\delta$ cut-off was 0.05 ppm due to small overall changes in the spectra. Samples contained ^{15}N-$PHD2_{181-402}$ (50 μM), $(NH_4)_2Fe(II)(SO_4)_2$ (50 μM), 2OG disodium salt (200 μM), HIF-1α CODD variant peptides (200 μM), in Tris-D_{11} (50 mM), pH 6.5, 310 K.

using an established MS based assay (Fig. 2A).[4a] Consistent with prior reports indicating the importance of the (subsequently hydroxylated) prolyl-residue (Pro564 in HIF-1α CODD) for tPHD2 catalysis,[4b, 6b, 11] the results revealed all the singly substituted HIF-1α peptides were hydroxylated under standard conditions, except for the P564A and P564G variants (Fig. 2A). In agreement with crystallographic analyses indicating that the 3 aspartyl-residues in $CODD_{HIF-1\alpha}$ (Fig. 3B) are important in binding to tPHD2,[11] the triple D569A/D570A/D571A $CODD_{HIF-1\alpha}$ variant was not hydroxylated by tPHD2 under our assay conditions.[4a, 10] Notably, $NODD_{HIF-1\alpha}$ I409D was not hydroxylated by tPHD2, consistent with the proposed importance of hydrophobic interactions between $I409_{NODD}$ and $F391_{tPHD2}$[1a][11] (Fig. 3C).

To further investigate these observations, competition assays between the variant peptides and wildtype HIF-1α NODD and CODD sequences were employed (Fig. 2B). The results provided further insight into important elements in substrate binding. Whilst D571A $CODD_{HIF-1\alpha}$ ($D571_{CODD}$ is part of the DDD motif) in isolation was almost completely hydroxylated under standard conditions, when in competition with $CODD_{HIF-1\alpha}$, D571A was <60% hydroxylated. This observation is consistent with the crystallographically observed salt-bridge between $D571_{CODD}$ and tPHD2 (Fig. S3B).[1a] An L562R $CODD_{HIF-1\alpha}$ variant (L562 is the second Leu of the LxxLAP motif) is proposed to manifest increased binding of $CODD_{HIF-1\alpha}$ to PHD2, and to a lesser extent, to PHD3, based on yeast-two hybrid assay results.[12] However, in our competition assays (Fig. 2B) the L562R $CODD_{HIF-1\alpha}$ variant was hydroxylated with lower efficiency than $CODD_{HIF-1\alpha}$, and with almost equal efficiency to $NODD_{HIF-1\alpha}$.

We then used NMR (Fig. 2C and S1-2) to investigate binding of the alanine-substituted CODD peptides to the ^{15}N-labelled $PHD2_{181-402}$-Zn^{II}-2OG complex using the minimal shift approximation method,[13] in which the assignments of the slow exchange perturbed peaks were made on a proximity basis by considering ^{1}H and ^{15}N shifts between the two spectra. A cut-off of 0.1 ppm was used for analyses and peaks not observed to have an apparent slow exchange partner were not considered. All the tested variants, except P564A $CODD_{HIF-1\alpha}$ and the triple substituted D569A/D570A/D571A $CODD_{HIF-1\alpha}$ variant, fully saturate $PHD2_{181-402}$-Zn(II)-2OG at the same concentration as $CODD_{HIF-1\alpha}$ (using a 2.7 fold molar excess of peptide) as judged by ^{1}H-^{15}N HSQC NMR, indicating binding is not disrupted by alanine substitution (under these assay conditions). The triply substituted D569A/D570A/D571A $CODD_{HIF-1\alpha}$ variant manifested weak binding, and is likely in fast exchange with the $PHD2_{181-402}$-Zn^{II}-2OG complex. These results are in agreement with the observations with catalytic assays that (most) single amino acid substitutions on $CODD_{HIF-1\alpha}$ did not cause substantial loss of tPHD2 activity (Fig 2A).

The importance of Pro564 of $CODD_{HIF-1\alpha}$ in binding to tPHD2 is consistent with the observation that all the $CODD_{HIF-1\alpha}$ variants which were observed to bind by NMR, induced similar chemical shift changes relative to the $PHD2_{181-402}$-Zn^{II}-2OG complex spectrum without $CODD_{HIF-1\alpha}$. Previous studies have shown that when P564 $CODD_{HIF-1\alpha}$ is substituted with some close proline-analogues, inhibition is observed (e.g. *L*-3,4-

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dehydroproline, IC_{50} = 8.5 μM) .[14] Similarly to P564A $CODD_{HIF-1\alpha}$, binding of P564G $CODD_{HIF-1\alpha}$ to $PHD2_{181-402}$-Zn(II) was weak by ^{1}H-^{15}N HSQC NMR. As the glycine- and alanine-substituted peptides are likely more flexible than the proline-residue wildtype, these results suggest that the relatively rigid conformation of the (hydroxylated) Pro-564 may play an important role in the induced fit mediated binding of the ODDs to the PHDs (Fig. S3A); the P564A/G substitutions may disrupt the 3_{10}-helix adopted by both the NODD and CODD LxxLAP motifs at the tPHD2 active site (Fig. S3A).[1a] Note, hydroxylated-prolyl HIFα can bind to tPHD2 in the absence of 2OG.[15]

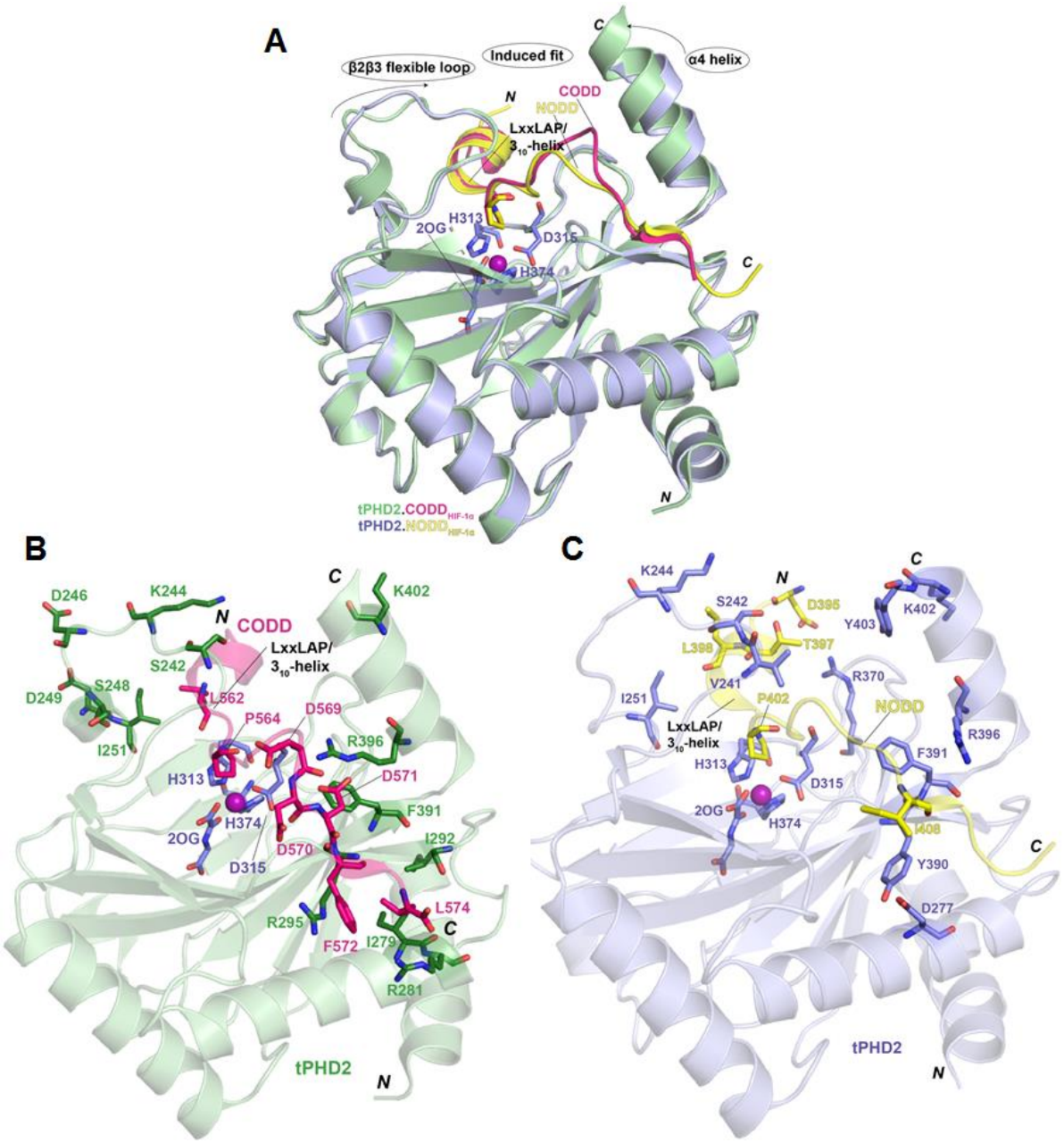

Figure 3. Proposed key determinants in binding of CODD and NODD to tPHD2. **A**. Overlaid views of tPHD2.2OG.Mn.$CODD_{HIF-1\alpha}$ (PDB ID: 5L9B) and tPHD2.2OG.Mn.$NODD_{HIF-1\alpha}$ (PDB ID: 5L9V) complexes, highlighting differences in interactions between CODD and NODD with the C-terminal α-helix (α4) of tPHD2.[1a] (Mn^{II} (purple sphere) substitutes for Fe^{II} in crystallisation).[1a] Metal binding residues (H313, H374 and D315) are in purple. **B.** View from a structure of tPHD2.2OG.Mn.$CODD_{HIF-1\alpha}$ (PDB ID: 5L9B)[1a] highlighting elements in CODD (fuchsia) binding to tPHD2 (green). S242, K244, D246, S248, D249 and I251 are part of the β2β3 tPHD2 loop, which is involved in induced fit binding. K402, Y403 and R396 are part of the tPHD2 C-terminal α4 helix. Note the salt-bridge between CODD D571 and the tPHD2 C-terminal region. **C.** View from a structure of tPHD2.2OG.Mn.$NODD_{HIF-1\alpha}$ (PDB ID: 5L9V)[1a] highlighting elements in NODD (yellow) binding to tPHD2 (light purple). Color coding as Fig. 3A. Note β2β3 loop residues interact with the xx residues of the $LxxLAP_{NODD}$ motif ($T398_{NODD}$ and $L399_{NODD}$). $R370_{tPHD2}$ interacts electrostatically with $D395_{NODD}$ and is important for NODD hydroxylation.[1a]

Overall, only small localised changes between the ^{1}H-^{15}N HSQC spectra of the $PHD2_{181-402}$-Zn^{II}-2OG-$CODD_{HIF-1\alpha}$ variants and $PHD2_{181-402}$-Zn^{II}-2OG-$CODD_{HIF-1\alpha}$ were observed (Table 1; Fig. 3A). Most of these were observed when an Ala was

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substituted at the C-terminal side of HIF-1α CODD (Fig. 1B). To investigate the relative importance of the regions to the N- and C-terminal sides of the hydroxylated proline in tPHD2 catalysis/binding, we made hybrid (HIF-1α) ODD peptides, i.e. hybrid CODD-NODD: LDLEMLAPAAGDTIISLDF-NH_2 and hybrid NODD-CODD: PDALTLLAPYIPMDDDFQL-NH_2 (Fig. 1B).

$CODD_{HIF\text{-}1\alpha}$ was preferentially hydroxylated by tPHD2 over both of the hybrid peptides (Fig. 2B). The hybrid CODD-NODD appeared to be slightly more hydroxylated than hybrid NODD-CODD. Both hybrid peptides were more efficiently hydroxylated than $NODD_{HIF\text{-}1\alpha}$ under standard assay conditions (Fig. 2B). The increased hydroxylation of hybrid NODD-CODD over NODD is likely, in part, due to the presence of a stabilising $D571_{CODD}$-$R396_{PHD2}$ salt-bridge as observed in the tPHD2.$CODD_{HIF\text{-}1\alpha}$ structure (Fig. S3B).[1a] An analogous acidic residue to HIF-1α CODD $D571_{CODD}$ (equivalent to HIF-2α CODD D536) is not present in either HIF-1α or HIF-2α NODD (Fig. 1B).[1a]

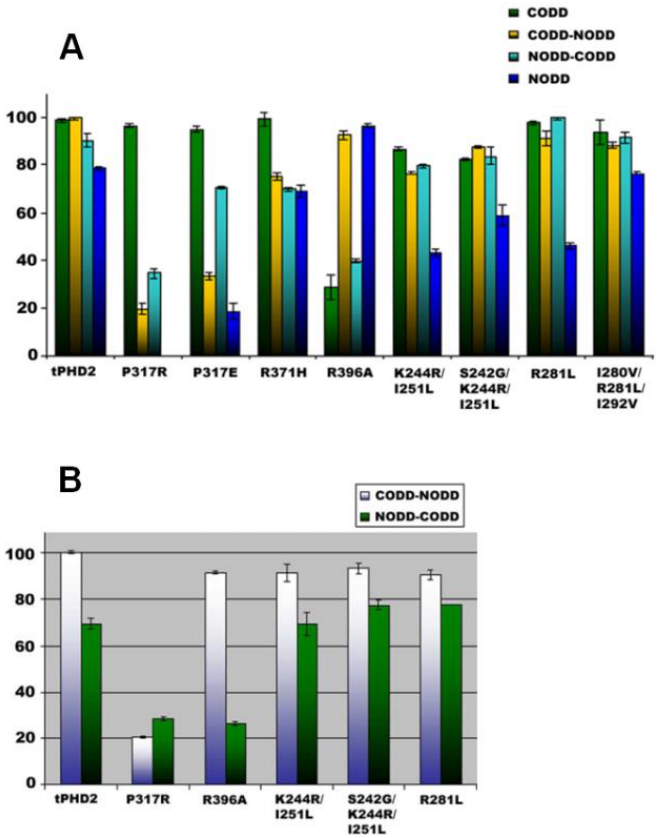

Figure 4. Hydroxylation of $CODD/NODD_{HIF\text{-}1\alpha}$ and ODD hybrid peptide substrates catalysed by wildtype tPHD2 and tPHD2 variants assayed using MS. **A.** 'Individual substrate assays', showing the % hydroxylation of ODD hybrid peptides. **B.** 'Competition experiments' with either hybrid CODD-NODD or hybrid NODD-CODD. $PHD2_{181\text{-}426}$ (2 μM) was incubated with $(NH_4)_2Fe(II)(SO_4)_2$ (50 μM), 2OG disodium salt (60 μM), sodium L-ascorbate (300 μM) and HIF-1α CODD variant peptides (50 μM), in Tris (50 mM), pH 7.5 at 25 °C. Samples were quenched with 1% ($^{v}/_{v}$) aqueous formic acid (15 min). Errors: standard deviations (n = 3). 100% hydroxylation corresponds to full substrate hydroxylation.

Hydroxylation and competition assays were then conducted with wildtype tPHD2 and tPHD2 variants.[1, 16] The latter were selected because they had been observed to show variations in $CODD_{HIF\text{-}1\alpha}$ and $NODD_{HIF\text{-}1\alpha}$ hydroxylation activities in comparison with wildtype tPHD2 (Fig. 4). The results showed that, as with wildtype tPHD2, all the tested variants, except for P317R/E and R396A tPHD2, demonstrated similarly high levels of hydroxylation of hybrid CODD-NODD and NODD-CODD. The P317R tPHD2 variant,[17] which is reported to be a CODD-selective variant[1] (and the presence of which is linked to erythrocytosis in patients),[17] manifested preferential hydroxylation of hybrid NODD-CODD over hybrid CODD-NODD. P317 of tPHD2 constitutes a hydrophobic region binding the ODD LxxLAP-3_{10}-helix and is likely relatively more important in binding the motif of NODD than CODD, and hence, the P317R (Fig. S3C) substitution is selective for blocking NODD over CODD.[1a]

R396T tPHD2 is a clinically observed variant of PHD2 that is NODD selective[1] and which is associated with breast carcinoma.[18] The R396A tPHD2 variant manifested ~90% and ~25% hydroxylation of the CODD-NODD and NODD-CODD hybrid peptides, respectively, in competition assays (Fig. 4B). This result compares with 100% and ~70% with the same hybrid peptides respectively using wildtype tPHD2 in the same competition assays (Fig. 4B). This observation further supports the importance of a salt-bridge interaction ($D571_{CODD}$-$R396_{PHD2}$) between the CODD C-terminal region and α4 of tPHD2 (Fig. S3B).

CONCLUSIONS

The overall results inform on key elements involved in ODD binding to the catalytic domain of PHD2, and by implication the other PHDs. Combined with previous biophysical and kinetic analyses,[4, 10] they support the proposal that NODD/CODD selectivity arises from multiple interactions including: (i) between the non-conserved 'xx' elements of the ODD LxxLAP motif and the PHD β2β3 loop; (ii) between the N-terminal part, and the conserved LxxLAP motif, of the ODD substrates and the PHD β2β3 loop region; and (iii) between C-terminal part of the ODDs and α4 of tPHD2 (see below). The evidence implies that although all these elements are important in binding both NODD and CODD, their relative importance varies in the binding of the two types of ODD (the relative importance may also change with specific PHD variants). The observation that the hybrid NODD-CODD and CODD-NODD peptides are substrates to differing extents compared to NODD and CODD is consistent with the proposal that the extent of relative NODD/CODD hydroxylation is regulated by discrete elements. The different effects of key elements in binding NODD and CODD are clearly manifested in terms of the roles of $R396_{tPHD2}$ and $P317_{tPHD2}$.

The results with singly substituted CODD peptides and the hybrid peptides (with both wildtype tPHD2 and the R396A tPHD2 variant) support the proposal that the interactions between the C-terminal part of the ODDs and the C-terminal region of tPHD2, including α4, are important in enhancing CODD *relative* to NODD hydroxylation; as observed here and previously,[5a] CODD is a better substrate than NODD. The reduced reactivity of R396A tPHD2 with CODD, but not NODD, is consistent with the observed salt-bridge between $R396_{tPHD2}$ (part of the α4 helix) and $D571_{CODD}$ (Fig. S3B). Note, given the evidence for induced fit during ODD binding,[1] it is possible that the $D571_{CODD}$:$R396_{tPHD2}$ salt bridge interaction is more dynamic

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in solution than indicated by the crystal structures and that $R396_{tPHD2}$ may also interact with D569 and D570 of HIF-1α CODD during binding.

The results also support the proposal that the interactions between the LxxLAP motif and the β2β3 loop are *relatively* more important in NODD than CODD binding/hydroxylation. $P317_{PHD2}$ is part of a hydrophobic patch binding the LxxLAP-3_{10}-helix and P317R tPHD2 does not hydroxylate NODD, but does hydroxylate CODD (Fig. 4A). Interestingly, both hybrid peptides were accepted by P317R tPHD2, though at reduced levels compared to CODD. With P317E tPHD2, the same trend was observed, but with some NODD hydroxylation being observed. Thus, whilst these results support the relatively more important role of $P317_{tPHD2}$ (β2β3 loop) in binding the LxxLAP residues in NODD rather than CODD, they suggest the substitutions can have subtle effects on ODD binding (note, in the P317R crystal structure, the R317 side chain was refined in more than one conformation – Fig. S3C).[1a]

The combined substrate selectivity studies and NMR binding results imply that the proline of the LxxLAP motif is important, not only for positioning the Pro-564 C^4 C-H bond appropriately for hydroxylation in the immediate vicinity of the Fe-binding centre, but also for productively organising the overall conformation of the PHD:ODD complex, part of which is observed as a 3_{10}-helix in the region of the active site Fe (Fig. 3A). Such a role is likely relevant to binding of both unhydroxylated and hydroxylated-prolyl ODDs to the PHDs, though the latter only binds in the absence of 2OG.[15] The importance of the LxxLAP proline-residue (P564 in HIF-1α CODD) is supported by results showing that the P564A and P564G $CODD_{HIF\text{-}1\alpha}$ variants only bind weakly to tPHD2, likely due to the lack of conformational constraints imposed by the proline residue; CODD is substantially disordered in solution.[19] By contrast with the Pro-564 substitutions, the other tested alanine substituted $CODD_{HIF\text{-}1\alpha}$ peptides were hydroxylated and manifested similar affinity for tPHD2 as $CODD_{HIF\text{-}1\alpha}$, except for the triple substituted aspartyl D569A/D570A/D571A CODD variant (see above) which disrupts the salt bridge interaction $D571_{CODD}$:$R396_{tPHD2}$[1a] (Fig. S3B).

To date, near all PHD inhibitors (including those in clinical trials) bind at the active site of the PHDs, ligating to the Fe^{II} and competing with 2OG.[8] Although their development may be challenging, our results imply that pursuing substrate selective inhibitors (either for HIFα isoforms and/or ODDs) may be viable. Recent work has revealed high affinity peptides not binding to the PHD active site.[20] The development of these and other allosteric compounds could be the starting point for the development of substrate selective inhibitors that work by modulating the induced fit processes involved in ODD binding.[1a] Such substrate selective inhibitors are of potential interest from a biomedicinal perspective and would be useful in assigning ODD function.

Experimental Section

Recombinant forms of tPHD2, the tPHD2 variants used, and ^{15}N-$PHD2_{181\text{-}402}$ were produced as reported.[1a, 4a, 9b, 10, 15, 17] Alanine variants of $CODD_{HIF\text{-}1\alpha}$ peptides were from Peptide Synthetics, U.K. All other peptides were produced by solid-phase synthesis as C-terminal amides as reported.[4a, 9a] Activity and competition assays were conducted using a MALDI-ToF mass spectrometry machine as reported.[4a, 9a] Binding experiments by ^{1}H-^{15}N HSQC NMR were carried out as reported.[1a] Assay conditions are given in the figure legends.

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Keywords: Prolyl hydroxylase domain enzymes • hypoxia inducible factor (HIF) hydroxylases • 2-oxoglutarate/α-ketoglutarate oxygenases • oxygen sensing • transcriptional regulation.

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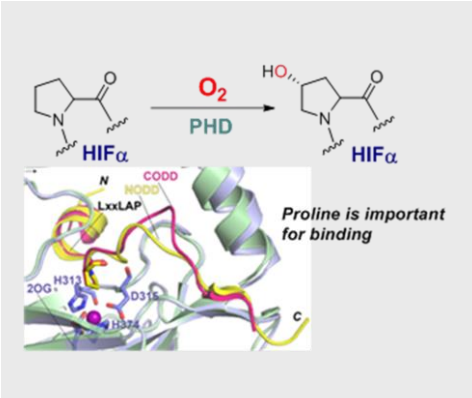

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