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Biochemical Characterization of Human Prolyl Hydroxylase Domain Protein 2 Variants Associated with Erythrocytosis

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ABSTRACT: Prolyl hydroxylase domain proteins (PHD isozymes 1−3) regulate levels of the α-subunit of the hypoxia inducible factor (HIF) through proline hydroxylation, earmarking HIFα for proteosome-mediated degradation. Under hypoxic conditions, HIF stabilization leads to enhanced transcription and regulation of a multitude of processes, including erythropoiesis. Herein, we examine the biochemical characterization of PHD2 variants, Arg371His and Pro317Arg, identified from patients with familial erythrocytosis. The variants display differential effects on catalytic rate and substrate binding, implying that partial inhibition or selective inhibition with regard to HIFα isoforms of PHD2 could result in the phenotype displayed by patients with familial erythrocytosis.

Hypoxia inducible factor (HIF)[1] plays a central regulatory role in oxygen homeostasis. The HIF family of transcription factors is composed of α- and β-subunits. The α-subunit, present in three isoforms, is tightly regulated in response to molecular oxygen levels through hydroxylation on one of two proline residues present in the oxygen-dependent degradation domain (ODDD) (*2, 3*). This hydroxylation, catalyzed by prolyl hydroxylase domain proteins (PHD isozymes 1−3), earmarks HIFα for interaction with the von Hippel Lindau (VHL) protein, resulting in ubiquitination and subsequent proteasomal degradation (*4*−*7*). Under hypoxic conditions, PHD proteins fail to hydroxylate HIFα, leading to HIFα stabilization and dimerization with constitutively present HIFβ. The resulting heterodimeric transcription factor binds to hypoxia response elements, inducing the transcription of numerous genes that are involved in functions such as erythropoiesis, iron metabolism, angiogenesis, and energy metabolism (*8*−*10*). PHD proteins are members of the α-ketoglutarate (α-KG)-dependent and iron-dependent dioxygenase family of enzymes, and their activity is dependent upon oxygen, α-KG, and HIFα along with iron and ascorbate as obligate cofactors (*11, 12*). Recently, two heterozygous PHD2 point mutations were identified from patients with erythrocytosis, a disorder characterized by an increase in the number of circulating red blood cells (*13, 14*). The point mutation C950G resulted in the PHD2 variant Pro317Arg (P317R), while the G1112A mutation resulted in the Arg371His (R371H) variant. These findings identified a new cause of erythrocytosis, now denoted as familial erythrocy-

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[1] Abbreviations: α-KG, α-ketoglutarate; CODDD, C-terminal ODDD; ECYT, familial erythrocytosis; EPO, erythropoietin; ESI-MS, electrospray ionization mass spectrometry; HIF, hypoxia inducible factor; HTRF, homogeneous time-resolved fluorescence; ODDDD, oxygen-dependent degradation domain; PHD, prolyl hydroxylase domain; VHL, von Hippel Lindau; WT, wild-type.

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Table 1: Steady-State Kinetic Parameters Determined for WT, P317R, and R371H Forms of Human PHD2 Protein Using HIF-1α $CODDD_{498-603}$ and HIF-2α $CODDD_{467-572}$ as Substrates

	HIF-1α $CODDD_{498-603}$			HIF-2α $CODDD_{467-572}$		
	WT	P317R	R371H	WT	P317R	R371H
α-KG K_m[a] (nM)	217 ± 54	479 ± 73	365 ± 37	318 ± 317	830 ± 249	287 ± 28
HIF $K_{m,app}$[b,c] (nM)	544 ± 247	731 ± 205	181 ± 52	571 ± 81	3477 ± 547	230 ± 59
k_{cat}[b] (min^{-1})	0.32 ± 0.04	0.32 ± 0.03	0.073 ± 0.005	0.20 ± 0.06	0.22 ± 0.04	0.06 ± 0.01
$k_{cat}/K_{m,HIF\alpha}$ ($\times 10^{-3}$ nM^{-1} min^{-1})	0.59	0.44	0.40	0.35	0.062	0.24
decrease in catalytic efficiency[d] (%)	0	25	32	0	82	31

[a] Values were determined using the HTRF assay. [b] Values were generated using the $^{14}CO_2$ release assay. [c] Similar values were observed using the HTRF assay. [d] The catalytic rate under physiological conditions was defined as $k_{cat}/K_{m,HIF\alpha}$. The estimation is based on the knowledge that the cellular α-KG concentration (100−250 μM) is well above the α-KG K_m values determined for all three enzymes.

tosis 3 (ECYT3), and shed new light on the physiological role of PHD2 in erythropoiesis.

To improve our understanding of the biochemical basis of this disease, here we examine the characteristics of human wild-type (WT) PHD2 and the PHD2 varints R371H and P317R using HIF-1α C-terminal ODDD ($CODDD_{498-603}$) and HIF-2α $CODDD_{467-572}$ as substrates. As described in the Supporting Information, the WT, R371H, and P317R full-length $PHD2_{1-426}$ proteins were expressed in sf9 cells from modified Gateway baculovirus vectors and purified as recombinant proteins. The kinetic parameters were determined using either a radioactive $^{14}CO_2$ release assay or a homogeneous time-resolved fluorescence (HTRF) assay. The $^{14}CO_2$ release assay monitors the radioactivity of the $^{14}CO_2$ product formed during the hydroxylation-coupled decarboxylation of α-[1-^{14}C]ketoglutaric acid, while the HTRF assay detects the energy transfer between VHL protein and the hydroxylated HIFα products upon binding.

When the hydroxylation substrate used was the HIF-1α CODDD protein, which contains the primary hydroxylation site Pro564, the P317R variant exhibited no change in k_{cat} and a very slight increase in HIF-1α CODDD $K_{m,app}$ in comparison to those of WT PHD2 (Table 1). There was an approximately 2-fold increase in K_m for α-KG, suggesting that this substitution weakens α-KG binding. The K_m values were used as a relative measure of substrate binding affinity. In contrast to P317R, the R371H variant exhibited a 4.4-fold decrease in k_{cat} over WT using the HIF-1α CODDD substrate. Additionally, a decrease in the HIF-1α $K_{m,app}$ was observed, rendering the HIF-1α CODDD a better substrate for the R371H variant than for WT or P317R. The α-KG K_m for R371H increased slightly (365 ± 37 nM) compared to that of WT PHD2 (217 ± 54 nM). It was noticed that the α-KG K_m values we determined for PHD2 were in the range of 200−300 nM, much lower than the range of 50−60 μM reported previously[2] (*15*). These differential effects on the catalytic rate and substrate binding were also observed in HIF-2α CODDD hydroxylation at Pro531 (Table 1). For the P317R variant, no change in k_{cat} was observed in HIF-2α CODDD hydroxylation as was likewise observed in the HIF-1α CODDD hydroxylation reaction. A similar 2-fold increase in α-KG K_m was observed in P317R-catalyzed HIF-2α CODDD hydroxylation. The HIF-2α CODDD $K_{m,app}$ for P317R had a noticeable increase of more than 6-fold, suggesting that the Pro317 to Arg substitution disrupted the interaction between the enzyme and HIF-2α CODDD. For the R371H variant, a 3.3-fold decrease in k_{cat} was observed in comparison to that of the WT-catalyzed reaction. The α-KG K_m for R371H was very comparable to that of WT PHD2, while the HIF-2α CODDD $K_{m,app}$ decreased 2.5-fold, similar to the effect observed when HIF-1α CODDD was used as the substrate.

In summary, the two variants displayed differential effects on catalytic activity and substrate K_m. The Arg371 to His substitution showed a noticeable decrease in the rate of catalysis, but no negative effect on substrate K_m. In contrast, the P317R mutation displayed catalytic rates comparable to that of the WT but resulted in decreased HIF substrate K_m values. In particular, the P317R variant produced a much greater decrease in the HIF-2α CODDD K_m (6-fold) than in the HIF-1α CODDD K_m (1.3-fold).

In an attempt to assess the catalytic efficiency of the WT and variant PHD2 enzymes, we calculated the $k_{cat}/K_{m,HIF\alpha}$ values. Since the cellular α-KG concentration (100−250 μM) was well above the α-KG K_m values determined for all three enzymes, it is expected that the difference in α-KG K_m values between WT and variant enzymes should have little or no impact on the enzyme catalytic efficiency under physiological conditions. As shown in Table 1, the P317R variant showed a 25 and 82% decrease in catalytic efficiency in HIF-1α CODDD and HIF-2α CODDD hydroxylation, respectively. The R371H variant experienced an approximately 30% decrease in catalytic efficiency under physiological conditions regardless of the HIFα substrate. Since both variants are the result of heterozygous point mutations, the effect of the amino acid substitutions on the PHD2 catalytic activity in human cells may be estimated to be a 15% decrease (*13, 14*).

In the X-ray crystallographic structure of PHD2, both the Pro317 and Arg371 residues are pointing away from the α-KG binding site and are located in a putative HIFα binding site (Figure 1) (*16*). It is not surprising that the substitution of Pro317 with an Arg residue, which contains a larger side chain, would disrupt the binding of the HIFα substrate. It would be of particular interest to have a cocrystal structure of PHD2 with its HIFα protein substrate to improve our understanding of the differential effect of HIF-1α and HIF-2α binding to the P317R variant. Contrary to a previous report, here we show that the R371H variant does not affect HIF substrate binding (*14*); in addition, we found that it also does not affect α-KG binding. As mentioned previously, the K_m values reported herein are used as a relative measure of

[2] The low α-KG K_m values obtained were confirmed through two independent assay formats: the $^{14}CO_2$ release assay and the HTRF assay. Indeed, our results agree with a recent report on the tight binding of PHD2 to iron and α-KG. In that study, the author suggested that the α-KG K_m should be lower than 2 μM from ESI-MS studies and the unusually high α-KG K_m reported previously could be complicated by endogenous sources of α-KG (*1*).

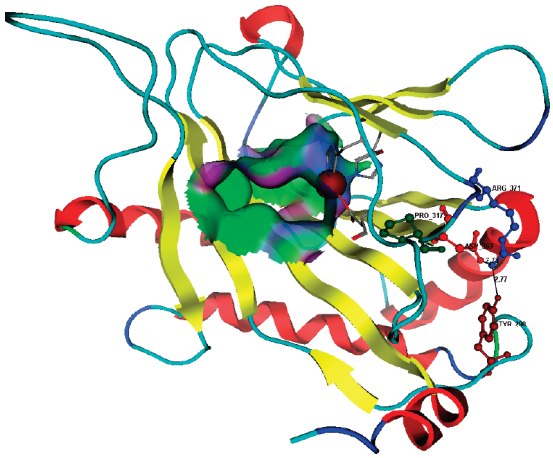

FIGURE 1: Crystal structure of PHD2, highlighting the locations of the two mutated residues, Arg371 (blue) and Pro317 (green). The two residues that form intramolecular hydrogen bonding interactions with Arg371 are Tyr290 [2.77 Å (dark red)] and Asp369 [2.73 Å (light red)]. Active site residues that interact with the active site Fe(II) are also shown (His374, His313, and Asp315) along with a surface representation of the catalytic site. The structure was generated using coordinates from the Protein Data Bank (entry 2G1M).

substrate binding affinity. The binding of R371H to HIF-1α CODDD and HIF-2α CODDD is actually tighter than with WT PHD2, most likely due to the introduction of a smaller histidine side chain. Arg371 is located three residues from the Fe(II) binding active site histidine (His374). The crystal structure reveals that Arg371 forms two H-bonds with nearby residues Tyr290 and Asp369 (Figure 1). The missing Arg side chain in the R371H variant and the loss of two H-bonds might lead to the misalignment of the active site His374 for catalysis, resulting in the observed decrease in the level of proline hydroxylation (k_{cat}) with this variant.

There is great interest in the development of prolyl hydroxylase inhibitors as oral anemia therapies. Some have hypothesized that inactivation of prolyl hydroxylases could activate HIF target genes, including erythropoietin (EPO), leading to increases in the level of red blood cell production (*17*−*19*). Indeed, several prolyl hydroxylase inhibitors have already been developed and are currently in clinical trials (*20, 21*). The results described here for the two variants associated with ECYT3 suggest that limited loss of the PHD2 catalytic efficiency is sufficient to induce erythrocytosis. More interestingly, the differential effect of P317R on HIF-1α and HIF-2α CODDD hydroxylation indicates that the discovery of small molecule agents that have a differential effect on HIF-1α and HIF-2α hydroxylation may be possible. Such agents could realize a therapeutic advantage by specifically stabilizing HIF-2α, which appears to be the main regulator of erythropoiesis (*17*−*19*). Interestingly, HIF-2α mutations, but not HIF-1α mutations, have also been reported to be associated with erythrocytosis (*22*−*24*).

Functional characterization of the P317R and R371H variants has previously been reported (*13, 14*). However, evaluations of substrate binding and activity change were not quantitative, and their studies were based on GST-tagged peptide substrates. In this study, we prepared HIFα CODDD protein substrates that contain a larger segment from the ODDD portion of the HIFα protein and substituted the bulky GST tag with a much smaller six-histidine tag. The availability of purified, recombinant enzyme and substrate afforded us an opportunity to assess the steady-state kinetic parameters for the PHD2 variants associated with ECTY3 for the first time. These results provided a quantitative understanding of the biochemical bases for this human genetic disease and importantly demonstrate that the two PHD2 variants display notably dissimilar kinetic parameters.

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SUPPORTING INFORMATION AVAILABLE

Detailed methodology. This material is available free of charge via the Internet at http://pubs.acs.org.

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