Conformational Analysis

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Evidence for a Stereoelectronic Effect in Human Oxygen Sensing**

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Human cells respond to limiting oxygen by upregulation of the α,β-heterodimeric hypoxia inducible transcription factor (HIF; for review see Ref. [1]). Under limiting oxygen availability, HIF increases expression of genes that work to counteract hypoxic effects by enhancing blood-vessel growth and red-blood-cell production. In "normoxic" cells *trans*-4-prolyl-hydroxylation of HIF-α enables its recognition by a ubiquitin ligase, of which the von Hippel Lindau tumor suppressor protein (pVHL) acts as a targeting component. Thus, prolyl-hydroxylation marks HIF for proteasomal degradation, and down-regulates the expression of HIF target genes (Figure 1).[1] HIF-α hydroxylation is catalyzed by prolyl hydroxylase domain containing enzymes (PHDs), which are Fe^{II} and 2-oxoglutarate (2OG) dependent oxygenases.[2] The absolute requirement of the PHDs for dioxygen enables them to act as oxygen sensors.[3] In human cells there are two HIF hydroxylation sites (in HIF-1α, these are Pro402 and Pro564) and three PHD1-3 enzymes, of which the most important in normal tissues is PHD2.[4] The selectivity of the interactions between hydroxylated and non-hydroxylated HIF-α forms with pVHL and the PHDs is central to the oxygen-sensing mechanism. Herein we describe studies on the chemistry of these interactions, which provide evidence for a role for the stereoelectronic gauche effect in oxygen sensing.

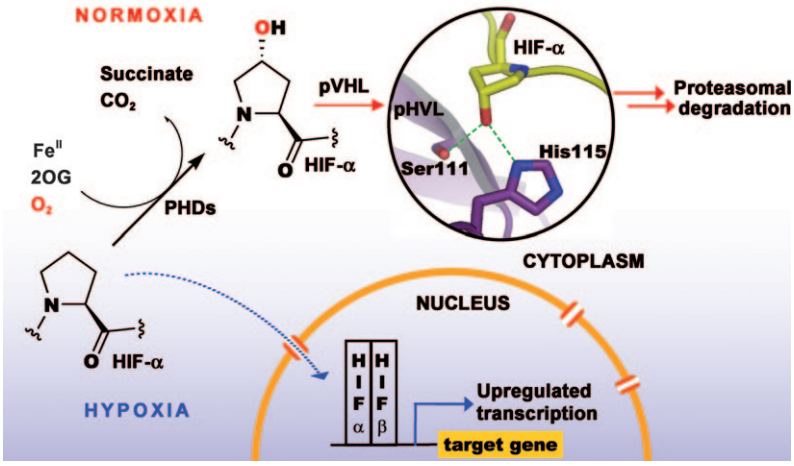

Figure 1. The human HIF oxygen-sensing mechanism highlighting the role of HIF-α prolyl hydroxylation.

The pyrrolidine ring of prolyl residues can adopt C^4-*exo* and C^4-*endo* conformations, with unsubstituted prolyl residues exhibiting a small preference for the C^4-*endo* conformation (Figure 2).[5] Substitution with electronegative groups at the C^3 or C^4 positions establishes a vicinal N-C-C-X arrangement between the prolyl amide group and the electronegative atom.[6] It is proposed that the "gauche effect" causes electron-withdrawing *trans*-4 and *cis*-4 prolyl substituents to effect a bias towards the C^4-*exo* and C^4-*endo* conformations, respectively.[5] Thus, it is predicted that prolyl *trans*-4-hydroxylation will bias the C^4-*endo*/*exo* equilibrium to the *exo* form. Indeed the C^4-*exo* prolyl conformation present in *trans*-4-hydroxyprolyl residues is crucial in maintaining the integrity of the collagen triple helix.[5] Recently, Gorres et al. have reported evidence that prolyl analogues with a C^4-*endo* preference are substrates of the collagen prolyl-4-hydroxy-

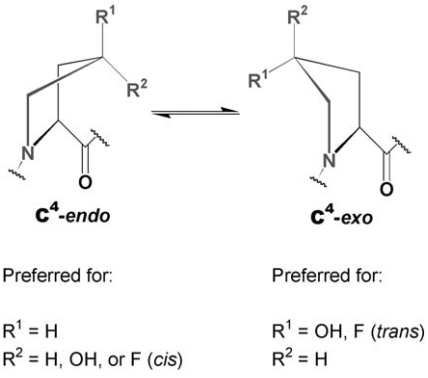

Figure 2. Ring conformations of 4-substituted prolyl residues.

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lase.[7] Herein we report our investigations on a role for the gauche effect in the HIF system.

We initially synthesized HIF-1$\alpha_{556-574}$ peptides incorporating the Pro564 hydroxylation site with prolyl analogues that are predicted to preferentially adopt C^4-*endo* or C^4-*exo* conformations and assayed them for binding to, and as substrates of human PHDs. Upon incubation with PHD2$_{181-426}$ (tPHD2, a readily prepared truncated form of PHD2), a HIF-1$\alpha_{556-574}$ peptide with *trans*-4-fluoroprolyl-564 residue was not hydroxylated, but stimulated 2OG turnover (Supporting Information, Figures S1, S2). In contrast, whilst both HIF *cis*-4-hydroxyprolyl and *cis*-4-fluoroprolyl-564 analogues also displayed high 2OG turnover, they underwent conversion into a 4-oxoprolyl product, presumably by hydroxylation followed by elimination of water or fluoride, respectively (Figure 3). The "indirect" cleavage of a C–F bond by PHDs is notable since such enzyme-mediated processes are rare.[8] Considering the stereoelectronic gauche effect, these results imply favored hydroxylation of prolyl analogues with a preference for the C^4-*endo*, compared to a C^4-*exo*, prolyl conformation.

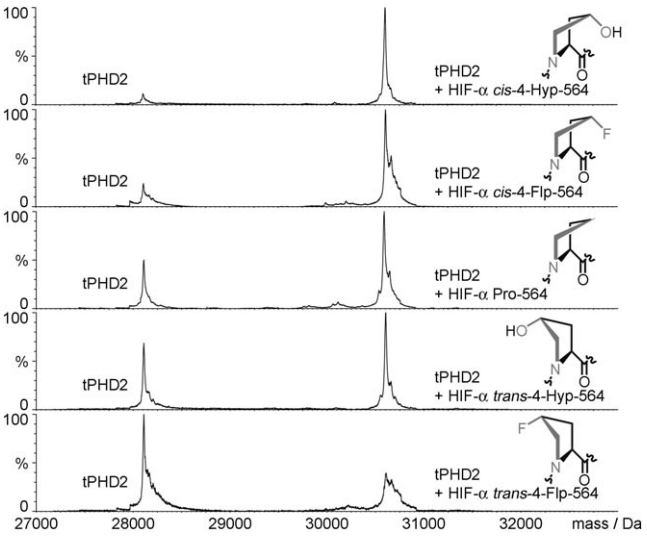


Figure 4. Non-denaturing ESI-MS analyses of tPHD2 in the presence of equimolar Fe^{II} and HIF-1α peptides with prolyl-564 analogues; peaks represent tPHD2·Fe (left) and tPHD2·Fe·HIF-α complexes (right).

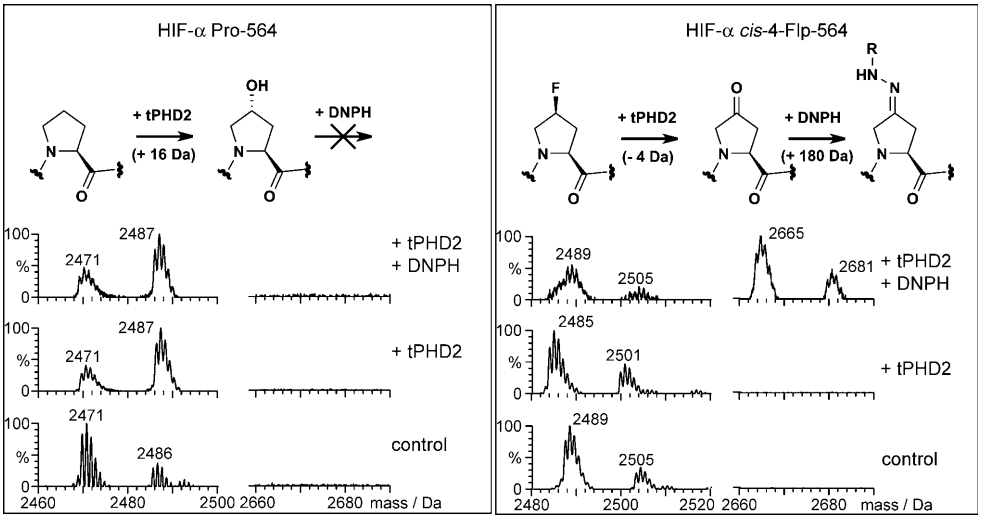
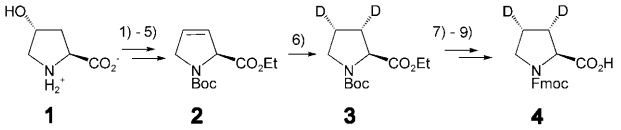


Figure 3. MALDI-TOF MS of HIF-1α peptides with Pro564 and *cis*-4-Flp564 analogues incubated with tPHD2. DNPH = 2,4-dinitrophenyl hydrazine; Flp = fluoroprolyl; R = 2,4-dinitrophenyl.

We investigated the affinity of HIF substrates containing the prolyl analogues for tPHD2. In support of the catalytic assays, the affinity of HIF-1$\alpha_{556-574}$ Pro564 analogues with a preference for the C^4-*endo* prolyl conformation to tPHD2 was found to be approximately five-fold stronger than for analogues stabilizing the C^4-*exo* conformation (by non-denaturing ESI-MS analyses; see Figure 4). We propose that the C^4-*endo* prolyl conformation is required for productive catalysis, that is, for reaction of the likely {Fe^{IV}=O} intermediate[9] with the C^4-*trans* prolyl hydrogen atom.

To investigate the stereochemical course of *trans*-4-prolyl hydroxylation of HIF-α, we prepared proline **4** appropriately labeled with deuterium at C^4, incorporated it into the Pro564 position of a HIF-1$\alpha_{556-574}$ peptide, and incubated the peptide with tPHD2. Synthesis of **3** proceeded essentially according to Oba et al. (Scheme 1).[10] However, we were unable to perform the saturation step with the reported diastereoselectivities. Several catalysts were investigated (Supporting Information, Table S1) and Crabtree's catalyst yielded approximately 96% of the desired diastereomer **3** (Supporting Information, Figure S3). Incubation of a HIF-1$\alpha_{556-574}$ peptide containing *trans*-4-D at the hydroxylation site Pro564 with tPHD2 gave hydroxylated HIF-α with more than 95% loss of the C^4 deuterium (mass increase of +15 Da), revealing that hydroxylation occurs with retention of stereochemistry (Figure 5; see the Supporting Information, Figure S4 for high-resolution analyses). These results imply that PHD catalysis proceeds by hydroxylation of HIF-1α Pro564 in its C^4-*endo* conformation and with retention of stereochemistry. Upon hydroxylation, a bias to the C^4-*exo* conformation may aid in product release.

To investigate the generality of our proposals we carried out incubations of HIF-1$\alpha_{395-413}$ peptides incorporating the N-terminal Pro402 hydroxylation site, or prolyl-402 analogues, with tPHD2 (Supporting Information, Figure S5). The results support a similar binding mode of the substrate prolyl residue at both the Pro402 and Pro564 HIF-1α hydroxylation sites. Together with the work of Gorres et al.,[7] our results indicate that a preference for the C^4-*endo* conformation may be a general property of prolyl-4-hydroxylases. Inspection of the



Scheme 1. Synthesis of labeled proline; 1) 1.1 equiv $SOCl_2$, EtOH, 16 h, reflux; 2) 3 equiv Et_3N, 1.2 equiv $(Boc)_2O$, CH_2Cl_2, 17 h; 3) 1 equiv PPh_3, 1 equiv di-*i*Pr azodicarboxylate, THF, 0 °C, then 1 equiv CH_3I, 21 h; 4) 1.1 equiv PhSeNa (1.1 equiv Ph_2Se_2, EtOH, 1.1 equiv $NaBH_4$, 16 h), 16 h, reflux; 5) 10 equiv H_2O_2, THF, 4 h, 0 °C; 6) 1 mol% catalyst, CD_3OD, 5 atm D_2, 5 days; 7) 1 M HCl, 3 h, reflux; 8) Dowex 50W-X8, lyophilization; 9) 1 equiv Et_3N, H_2O, 0.9 equiv Fmoc succinimide, MeCN, pH 9. Boc = *tert*-butyloxycarbonyl, Fmoc = (9H-fluoren-9-ylmethoxy)carbonyl.

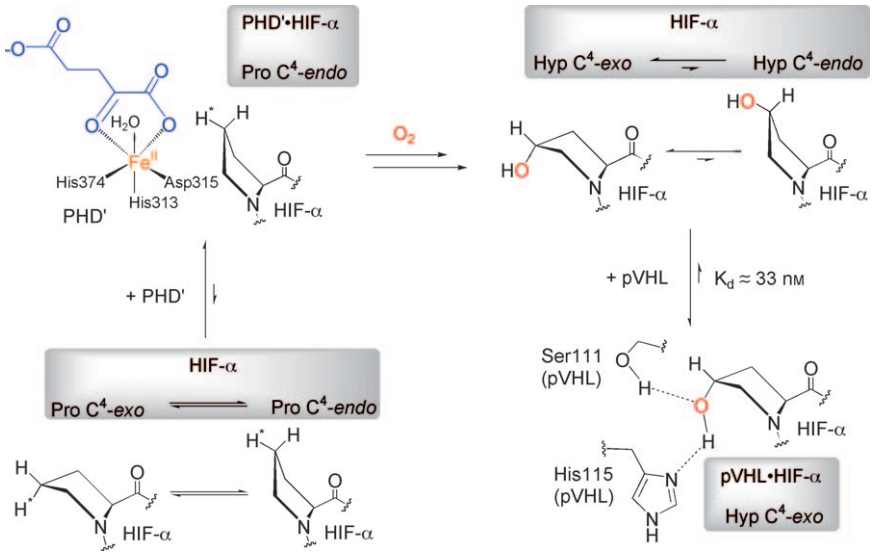


Figure 5. Proposed role of conformational changes at HIF-1α Pro564/402 in molecular recognition. Selected components shown only; when H* = D, loss of D was observed. PHD′ = PHD·Fe^{II}·2OG.

reactions catalyzed by human 2OG oxygenases reveals that hydroxylation frequently occurs vicinal to an electronegative atom. This result suggests possible involvement of stereoelectronic effects in other 2OG oxygenase reactions, including asparaginyl/aspartyl 3-hydroxylation, phytanoyl CoA 2-hydroxylation, lysyl 5-hydroxylation, and γ-butyrobetaine 3-hydroxylation.[2]

Given the apparently important role of the prolyl conformation in PHD catalysis, we then investigated its role in pVHL binding. The ability of a ternary pVHL/elongin C/B protein complex (VCB) to discriminate between hydroxylated and non-hydroxylated HIF-1$\alpha_{556–574}$ is remarkable (K_d = 33 nM and 34 μM, respectively).[11] In VCB·HIF crystal structures,[11,12] the HIF *trans*-4-hydroxyprolyl-564 residue adopts the C^4-*exo* conformation; the hydroxy group is positioned to make two hydrogen bonds (to pVHL Ser111 and His115, Figure 1). HIF-1α peptides with Pro564 and Pro402 analogues containing C^4 substituents known to bias the ring to C^4-*endo* or C^4-*exo* conformations, as well as the 4-oxoprolyl-564 derivative, were screened for pVHL binding. Excepting the HIF *trans*-4-hydroxyprolyl-402/564 peptides, none of the *cis*-4-fluoro-/hydroxyprolyl or 4-oxoprolyl analogues bound to pVHL within the detection limits of a pull-down assay (Supporting Information, Figure S6);[11] significantly neither did the *trans*-4-fluoroprolyl analogues. Non-denaturing ESI-MS analyses also indicated that none of the *cis*-4-fluoro-/hydroxyprolyl-564 analogues bound significantly to VCB (Supporting Information, Figure S7).

To quantify the relative binding of the HIF peptides to VCB, we carried out fluorescence-based assays.[13] Whilst unmodified HIF-1$\alpha_{556–574}$ did not bind, the *cis*-4-hydroxyprolyl analogue displayed a low affinity for pVHL (5.8 ± 0.1 % as compared to *trans*-4-hydroxyprolyl); none of the other analogues bound (Supporting Information, Figures S8, S9). These results reveal the remarkable selectivity of the pVHL binding pocket for HIF *trans*-4-hydroxyprolyl-402/564 and, since fluorine cannot substitute for the *trans*-4-hydroxy group, demonstrate that both hydrogen bonds from pVHL to the hydroxy group of HIF *trans*-4-hydroxyprolyl must contribute significantly to the binding of HIF to pVHL (Figure 5). Given the recent identification of the reversibility of histone methylation, including by 2OG oxygenases (for review see Ref. [2]), these results raise the possibility that the signaling effect of HIF hydroxylation may be functionally ablated by oxidation to the 4-oxoprolyl derivatives.

Overall our results reveal that catalysis of HIF prolyl hydroxylation by the oxygen-sensing human PHD1-3 enzymes induces a conformational bias from C^4-*endo* to C^4-*exo* (Figure 5) that is an integral part of the hypoxic response mechanism. Although the importance of stereoelectronic effects in biology is established in carbohydrate chemistry with the anomeric effect,[14] its role in post-translational modifications and catalysis by other types of enzymes has not been widely considered outside the context of collagen stabilization.[5] Coupled with recent reports suggesting that post-translational hydroxylation of intracellular proteins may be more widespread than previously perceived,[15] our results suggest that this effect may be involved in modulating other protein–protein interactions.

Experimental Section

Methods Summary; see the Supporting Information for details and abbreviations. Recombinant human PHD2$_{181–426}$ (tPHD2) and VCB complex were produced in *E. coli* and purified by affinity- and gel-filtration chromatography. VCB was labeled with Eu^{III}-cryptate complex for FRET assays as reported.[13] Enzymatic activity was analyzed by incubating tPHD2 with Fe^{II}, 2OG, and peptide and assayed by 1-[^{14}C]-2OG decarboxylation, DNPH derivatization, MALDI-TOF, FT-ICR MS, and MS/MS analyses. Investigation of reaction stereochemistry employed HIF-1α peptides prepared by solid-phase peptide synthesis with proline analogues including **4**. The

interaction of HIF-1α analogues with pVHL was assessed by IVTT [^{35}S]-pVHL capture assays, non-denaturing ESI-MS, and FRET.

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