Different Modes of Inhibitor Binding to Prolyl Hydroxylase by Combined Use of X-ray Crystallography and NMR Spectroscopy of Paramagnetic Complexes

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Supporting Information

Experimental Methods

Expression and Purification of [^{15}N-Trp] Hif-PHD2$_{173-403}$ 4E6 *Trichoplusia ni* cells grown in Excel 405 media were collected by centrifugation. The cell pellet was resuspended in 2L of custom made BioExpress® 2000 (Cambridge Isotope Laboratories, CIL) ^{15}N-Tryptophan labeled insect media. 0.8 L of the culture was placed into two 2.80 L Fernbach shake flasks. The cultures were incubated at 28 °C for 2 hours shaking at 110 rpm. After two hours, 8 mL of Baculovirus stock expressing His6-DEVD-HIF-PHD2 (173-403) was added to each flask. The flasks were incubated at 28 °C for 48 hours shaking at 110 rpm. The cells were harvested by centrifugation and stored at -20 °C. Cells were resuspended in 20 mM Tris (pH 7.2), 0.5M NaCl, 5mM imidazole, 0.1% CHAPS and 10mM βME and lysed by a microfluidizer. Cleared lysate was applied to Ni-NTA resin and incubated for 2h. Protein was eluted with 250 mm imidazole and incubated overnight with caspase-3 to remove the six histidine tag. Cleaved HIF-PHD2 was concentrated to 15 mg/mL and applied to a size exclusion column (Superdex 200, Pharmacia) equilibrated with 50 mM Ammonium Acetate (pH 6.3), 2mM TCEP. Purified protein was typically analyzed by LC-MS and aliquots were snap frozen in liquid nitrogen. The yield was 17 mg of the protein (>90% pure by gel densitometry) from 20 g of cell paste.

Removal of divalent metal ions from the purified protein. 10 mL of protein (~1 mg/mL) from purification was twice dialized against 4L of 50 mM ammonium acetate, pH=5.5,1 mM TCEP, 5mM EDTA for 24 h and finally dialized into 20 mM HEPES buffer, 100 mM

NaCl, 2 mM TCEP, pH = 6.5 and concentrated to ~ 4 mg/mL. The final molar concentrations of divalent metal ions were below 1 % of the protein concentration.

NMR Spectroscopy. Each NMR sample contained [^{15}N-Trp]-PHD2$_{173-403}$ at 150 μM concentration with a stoichiometric amount of divalent metal ion and a stoichiometric amount of ^{13}C labeled or ^{13}C unlabeled **1**. The spectra were acquired at 293 K on AVANCE^{TM}-500 spectrometer equipped with (^{1}H,^{13}C,^{15}N) – QNP cryoprobe for ^{15}N-^{1}H HSQC and direct ^{13}C 1D experiments and RT SEF probe for ^{19}F experiments. The HSQC datasets were acquired with 80 scans and 128 experiments in the indirect dimension. The ^{13}C spectra for the diamagnetic and paramagnetic complexes were acquired with 8K and 32K scans respectively. The ^{13}C spectra for paramagnetic complexes were acquired for labeled and unlabeled **1** and the corresponding FIDs were subtracted before FT. The ^{19}F spectra were recorded with 2K scans.

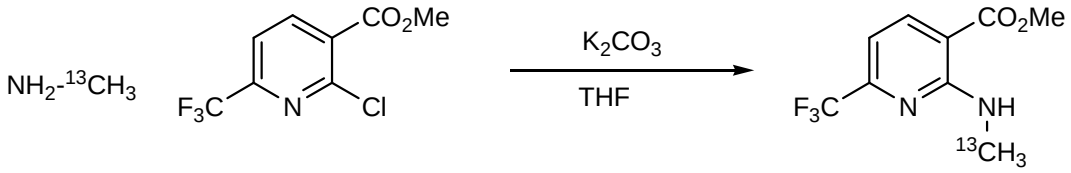
Structural Computations. Extensive simulations with the synthetical data, obtained from various tensor parameters and random variation of PCSs with 0.05 ppm standard deviation, have shown that accurate tensor and correct spectral assignments can be obtained from PCSs for the 7 N-H pairs of the four tryptophan residues in the catalytic domain of PHD2 (W$^{\varepsilon}$389 is in the blind zone of the paramagnetic metal center). Thus, tensor parameters and ^{15}N-H assignments were obtained through initial grid search followed by nonlinear least-squares optimization, using χ^2 statistics for the differences between the observed and calculated PCS values, performed for all permutations (4!3!) of the four amide and three indole groups in ^{15}N labeled tryptophan residues. These calculations were performed for 8 combinations of the experimental shifts resulting from the splittings in the spectrum with the paramagnetic ion (only three signals in ^{15}N-^{1}H HSQC spectra were split in either Co^{2+} and Fe^{2+} complexes). All 8 combinations yielded the same spectral assignment. The PCS combination with the smallest χ^2 value was consistent with two sets of signals of different amplitudes corresponding to the “X” (major) and “F” (minor) orientations of the ligand. Repeating the calculations with randomly biased experimental PCSs (within 0.05 ppm standard deviation) gave virtually the same results. The CH_3 and CF_3 signals in the diamagnetic complex were assigned on the basis of NOE patterns (not shown). The signal assignements in paramagnetic complexes were based on the paramagnetic line broadenings and signal integrations.

For optimizations of the ligand positions, based on Eq.1, we used the following target function:

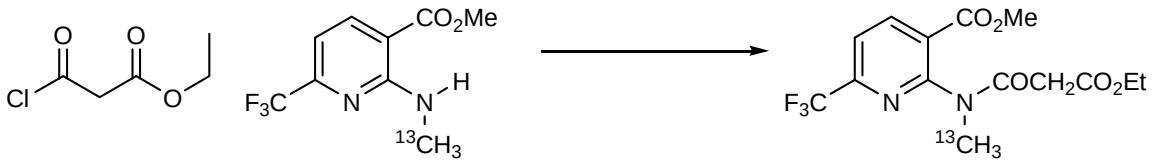
$$T = \sum_i \left[\left(\frac{\delta^p_{X,Fe} - \delta^{PCS}_{X,Fe}}{\delta^p_{F,Fe} - \delta^{PCS}_{F,Fe}} \right) - \left(\frac{\delta^p_{X,Co} - \delta^{PCS}_{X,Co}}{\delta^p_{F,Co} - \delta^{PCS}_{F,Co}} \right) \right]^2_i$$

The summation index is over the observed nuclei from the ligand. During optimizations, the ligand was allowed to undergo translations and rotations in 3D space plus torsional motions except for the amide bond which was in a trans-planar conformation. The distances between two oxygen atoms and the metal center were constrained to 2.1 ± 0.3Å and the hydrogen bonds between the carboxylic group of the ligand and arginine R383 were retained during the optimizations. The parameters for the magnetic susceptibility tensors were allowed to vary within their standard deviation limits (Table S2). The pseudocontact shift for the CF_3 group was calculated as an average value for all three fluorine atoms. The optimizations started from 1000 randomly biased "X" and "F" positions and only solutions with T<0.001 and fulfilling coordination plus hydrogen bond constraints were used for calculations of means and standard deviations in Table 1. All computations were done in MATLAB® (MathWorks, Inc.) computer software.

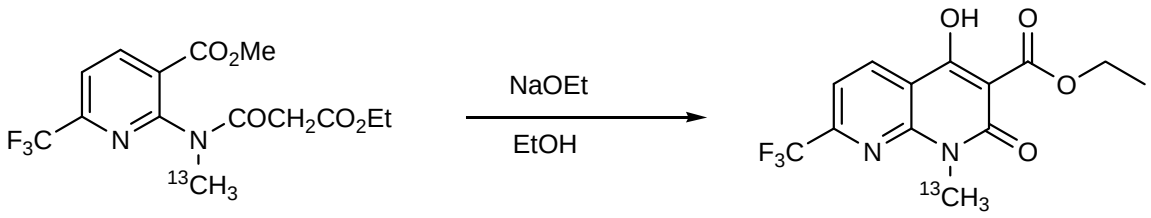
Synthesis of **1**



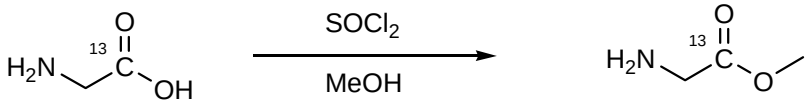
To a solution of methyl 2-chloro-6-(trifluoromethyl)nicotinate (1 g, 4 mmol) and THF (20 ml) was added K_2CO_3 (2 g, 13 mmol) and methylamine-^{13}C hydrochloride (0.3 g, 5 mmol) . The reaction mixture was stirred at rt overnight. After 17 hrs, the reaction was filtered and concentrated *in vacuo*. The product was used crude in the next step.



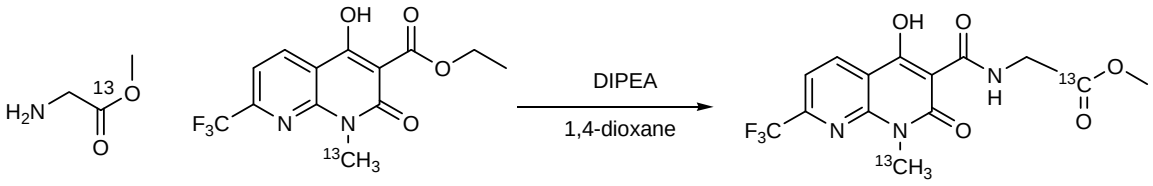
A solution of the amino-pyridine (1.0 g, 4 mmol), ethyl 3-chloro-3-oxopropanoate (0.6 g, 4 mmol) and 1,2-dichloroethane (10 ml) was stirred at 80 °C for 4.5 hrs. The reaction mixture was cooled to rt and concentrated *in vacuo*. EtOH was added to the oil and concentrated again. The orange oil residue was purified by silica flash chromatography (0-75% EtOAc:hex) to give the desired product as a light-yellow oil (0.9 g).



To a solution of the amino-pyridine (0.9 g, 3 mmol) and EtOH (10 ml, 171 mmol) was added 20 wt% NaOEt (2.5 ml, 8 mmol) at rt. A white precipitate formed. After 5 min. the reaction was complete. AcOH was added, and the mixture was filtered providing a white solid. The solid was washed with water and then dried under vacuum to give the desired as a white solid (0.6 g).

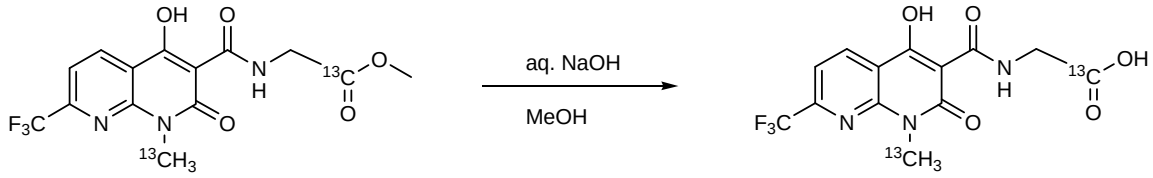


To a solution of 2-aminoacetic acid (200 mg, 2.7 mmol) and MeOH (10 ml), was carefully added $SOCl_2$ (0.19 ml, 2.7 mmol) dropwise. The solid gradually dissolved after several minutes. The reaction mixture was stirred at rt for 1 hr and was then concentrated *in vacuo* to give the desired product as a crystalline white solid (0.24 g). The product thus obtained was used in the next step without further purification.



To a solution of the azaquinolone (100 mg, 0.32 mmol) and 1,4-dioxane (10 ml), was added methyl 2-aminoacetate (0.14 g, 1.6 mmol) and DIPEA (0.28 ml, 1.6 mmol) at rt. The reaction mixture was heated to 100 °C for 2 hrs. The reaction mixture was

removed from heat and stirred for 15 hrs at rt. The reaction mixture was concentrated *in vacuo* to give a light yellow solid. The solid was treated with DCM and a white solid was obtained by filtration. The filtrate was purified by silica flash chromatography (0-50% EtOAc:Hex) to give the product as a crystalline white solid (0.07 g).



A mixture of the quinolone (0.070 g, 0.2 mmol), 5N NaOH (3 ml, 15 mmol) and MeOH (6 ml) was stirred at rt for 3 hrs. The reaction mixture was acidified with 5N HCl and chilled at 4 °C overnight. A white crystalline solid formed. The solid was filtered, washed with water and then dried under vacuum. The filtrate was concentrated until a white solid formed. This solid was filtered, washed with water and then dried under vacuum to give the desired product (0.045 g).

Table S1 Calorimetric K_d (μM) values for different metal ions binding to the catalytic domain of $PHD2_{181-417}$

Fe^{2+}	Zn^{2+}	Co^{2+}	Ni^{2+}
1.1±0.2[a]	0.27±0.05[a]	0.58±0.3[a]	<0.1[b]

[a] Obtained from ITC titration at 293 K in the protein buffer. [b]There was no measurable heat associated with nickel binding. The affinity was estimated from the DSC experiments where T_m for the nickel complex was at least 12 deg higher then T_m for the other complexes.

Table S2 Experimental and simulated PCS values in ppm for amide and indole groups in tryptophan residues in $PHD2_{173-403}$ complexed with **1** and different metals. The tensor parameters and their standard deviations were obtained from least-squares optimization of the PCS values. The same confidence limits were also obtained from the Monte Carlo simulation assuming 0.05 ppm standard error for each PCS value.

Metal		PCS for amides: N/H				PCS for indoles:N/H		
	Groups:	**W258**	**W334**	**W367**	**W389**	**W258**	**W334**	**W367**
Co^{2+}	exp	1.20 1.10	-0.61 -0.61	2.10 2.10	.56 .63	1.63 1.57	-1.46 -1.43	0.85 0.80
	Sim	1.16 1.09	-0.60 -0.62	2.02 2.15	0.51 0.69	1.65 1.54	-1.55 -1.38	0.91 0.87
	Tensor-X[a]	$\alpha = 297 \pm 1.5^{\circ}; \beta = 86 \pm 1^{\circ}; \gamma = 224 \pm 2^{\circ}; \Delta\chi_{ax} = -12 \pm 0.25 \times 10^{-32}\,m^3; \Delta\chi_{rh} = 6.9 \pm 0.25 \times 10^{-32}\,m^3$						
	Exp	1.55 1.40	-0.61 -0.61	2.10 2.10	0.23 0.21	2.20 2.20	-1.46 -1.43	0.85 0.80
	Sim	1.55 1.4	-0.59 -0.59	2.11 2.08	0.27 0.27	2.20 2.20	-1.52 -1.37	0.86 0.81
	Tensor-F	$\alpha = 296 \pm 2^{\circ}; \beta = 84 \pm 1.5^{\circ}; \gamma = 223 \pm 3^{\circ}; \Delta\chi_{ax} = -11.9 \pm 0.5 \times 10^{-32}\,m^3; \Delta\chi_{rh} = 3.8 \pm 0.5 \times 10^{-32}\,m^3$						
Fe^{2+}	Exp	-0.11 -0.12	0.12 0.06	2.13 1.73	-2.15 -2.2	-0.72 -0.72	0.07 -0.05	0.83 0.72
	Sim	-0.10 -0.12	0.17 0.07	2.11 1.77	-2.1 -2.25	-0.69 -0.74	0.10 -0.06	0.81 0.73
	Tensor-X	$\alpha = 160 \pm 1^{\circ}; \beta = 70 \pm 1.5^{\circ}; \gamma = 232 \pm 1^{\circ}; \Delta\chi_{ax} = 7.9 \pm 0.4 \times 10^{-32}\,m^3; \Delta\chi_{rh} = 4.05 \pm 0.4 \times 10^{-32}\,m^3$						
	Exp	-0.11 -0.12	0.12 0.06	1.68 1.44	-2.53 -2.34	-0.72 -0.72	0.07 -0.05	0.71 0.62
	Sim	-0.12 -0.13	0.13 0.025	1.63 1.49	-2.57 -2.3	-0.7 -0.75	0.08 -0.07	0.71 0.65
	Tensor-F	$\alpha = 154 \pm 1^{\circ}; \beta = 64 \pm 1^{\circ}; \gamma = 239 \pm 1.5^{\circ}; \Delta\chi_{ax} = 7.4 \pm 0.5 \times 10^{-32}\,m^3; \Delta\chi_{rh} = 4.9 \pm 0.35 \times 10^{-32}\,m^3$						

[a]The anisotropy of the magnetic susceptibility tensor is similar, although significantly larger in magnitude as compared to other Co(II) substituted proteins.[7]

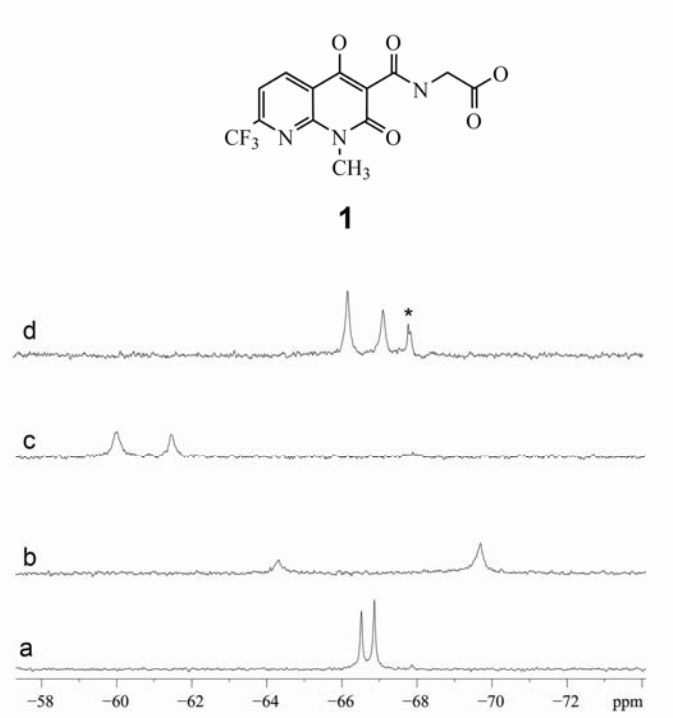


Figure S1. ^{19}F NMR spectra of $PHD2_{173-403}$ complexed with inhibitor **1** and different metal ions. **a**: Zn^{2+} (S=0), **b**: Fe^{2+} (S=2), **c**: Co^{2+} (S=3/2), **d**: Ni^{2+} (S=1), where S is the d-electron spin state. The asterisk corresponds to the CF_3 signal from the free ligand.

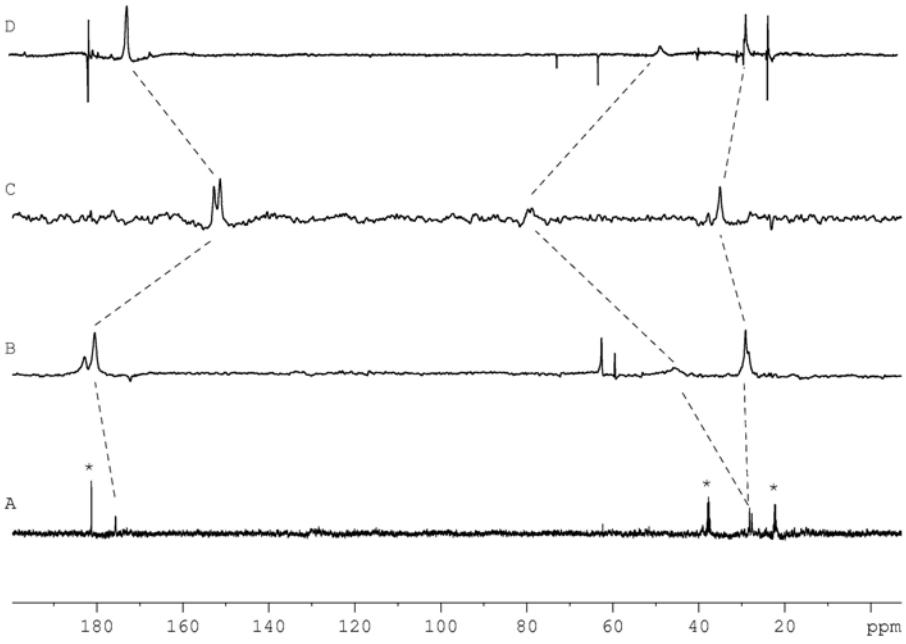


Figure S2. ^{13}C NMR spectra for $PHD2_{173-403}$ complexed with ^{13}C labeled **1** and different metals: A: Zn^{2+} ,B: Fe^{2+}, C: Co^{2+}, D: Ni^{2+} The dotted lines follow the paramagnetic shifts. The asterisks correspond to the buffer.

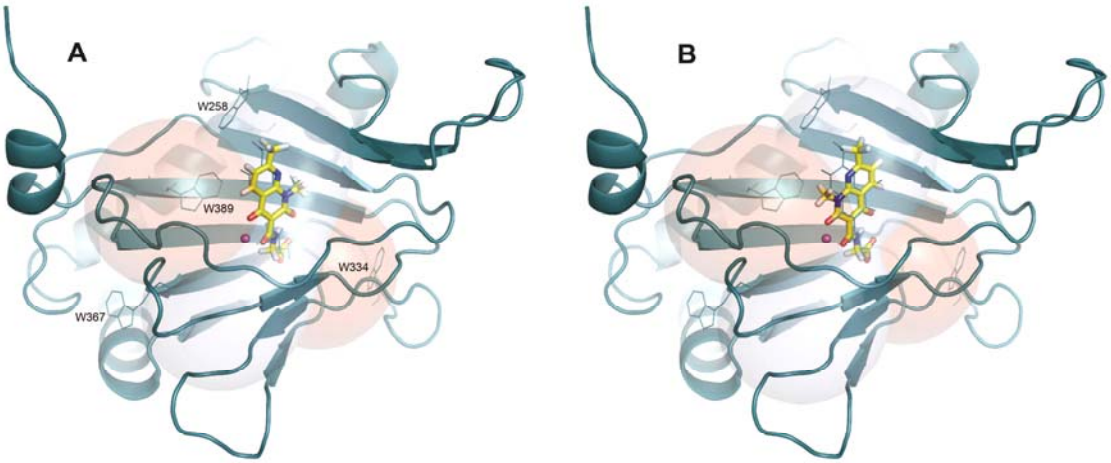


Figure S3. Different orientations of **1** in complex with PHD2 obtained from pseudocontact shifts (PCS) and X-ray structure of $PHD2_{180-417}$ (cartoon). **A**: the "X" orientation of the ligand, **B**: the "F" orientation of the ligand. The magnetic susceptibility tensors are depicted as the transparent isosurfaces calculated at cutoff values of -2 pmm (red) and 2 ppm (blue). The tryptophan residues used for calculating these tensors are labeled in **A**.

Complete Ref. (3)

(3) McDonough, M. A.; Li, V.; Flashman, E.; Chowdhury, R.; Mohr, Ch.; Liénard, B. M. R.; Zondlo, J.; Oldham, N. J.; Clifton, I. J.; Lewis, J.; McNeill, L. A.; Kurzeja, R. J. M.; Hewitson, K. S.; Yang, E.; Jordan, S.; Syed, R. S.; Schofield, Ch. J. *Proceedings of the National Academy of Science,* **2006**, 103(26), 9814-9819.