Supplementary Material (ESI) for Molecular BioSystems

Electronic Supplementary Information

Hypoxia-inducible factor prolyl hydroxylase has a high affinity for ferrous iron and 2-oxoglutarate

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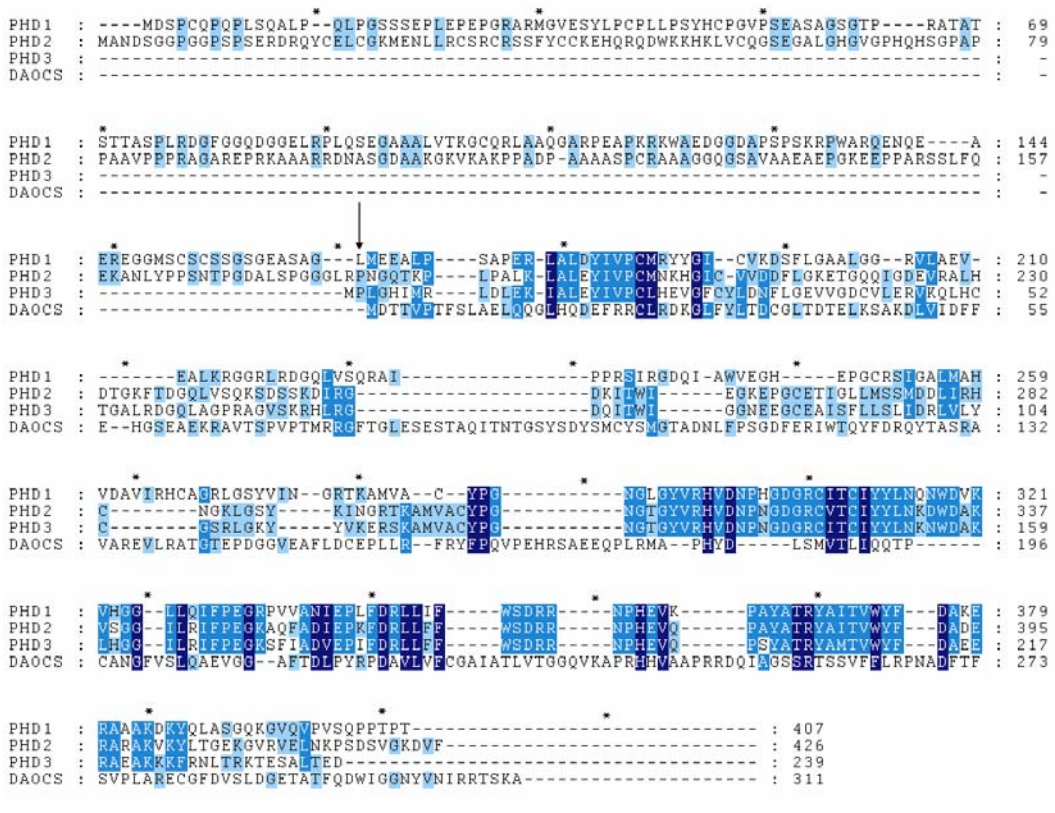
S1. Sequences of PHD1, 2 and 3, and DAOCS, aligned using ClustalW [1].



The N-terminus of the truncated PHD2 (residue 181) is indicated with an arrow, and was based upon homology with DAOCS and other 2OG enzymes. Stars mark every 20 residues. Shading represents sequence conservation: Dark blue highlights 100% conservation (or equivalent residues), light blue 80% conservation and aqua 60% conservation.

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S2. NMR Experimental Details.

Assignment of resonances in all peptides was achieved by HSQC and TOCSY experiments and is shown in Table 1. The expected large shifts in the resonances of both the proline γ-proton (3.55ppm) and the proline γ-carbon (45.1ppm) on hydroxylation is accompanied by a shift in the resonance of both the flanking β- and δ- carbons (7.5 and 7.2ppm respectively).

	Synthetic $HIF_{556-574}$		Synthetic $HIF_{556-574}$ (*trans*-4-hydroxylated at Pro-564)	
Position on proline ring	δ_H	δ_C	δ_H	δ_C
α	4.41	61.4	4.52	60.1
β	1.87, 2.26	30.3	1.82, 2.22	37.8
γ	1.99 (2H)	25.6	4.54	70.7
δ	3.64, 3.75	49.0	3.78 (2H)	56.2

Table 1. Assignment of resonances in synthetic HIF peptides.

For the synthetic $HIF_{556-574}$ fragment (Peptide Protein Research Ltd., Fareham, UK.) and the synthetic hydroxylated $HIF_{556-574}$ fragment (Biopeptide Co. LLC, San Diego, USA), 1.5mg of peptide was dissolved in 500µl D_2O + 0.1% 1,4-dioxane, and spectra were recorded on a Bruker DRX500 with a 5mm TBI probe ($^1H\{^{13}C,BB\}$) at 298K. For the synthetic $HIF_{556-574}$ fragment after incubation with hnt-PHD2, approximately 100µg of HPLC purified peptide was dissolved in 7µl 90% H_2O/ 10% D_2O and the spectrum was recorded on a Bruker AV600 with a 1mm TXI microprobe ($^1H\{^{13}C,^{15}N\}$) at 295K. Reference 1,4-dioxane can be seen in Figure 3(i) at δ_H 3.75ppm, δ_C 67.5ppm. The resonance in Figure 3(i) at δ_H 3.7ppm, δ_C 53.5ppm is an unidentified impurity present in both synthetic samples. Resonances in Figure 3(ii) at δ_H 3.7ppm and δ_C 60.0ppm and 71.0ppm are impurities from the enzyme incubation.

Incubation and purification conditions used to produce enzyme modified $HIF_{556-574}$ fragment

To produce $HIF_{556-574}$ that had been hydroxylated by hnt-PHD2, the following reaction was repeated five times. Incubation of 1m DTT (Melford Labs.), 0.6mg/ml catalase (Sigma), 5mM 2-oxoglutarate (Sigma), 4.4mM synthetic $HIF_{556-574}$ fragment, 400µM hnt-PHD2, 1mM iron(II) ammonium sulphate hexahydrate in a final volume of 100µl 50mM HEPES pH7.0 was carried out at 37°C for 30minutes. The reaction was stopped with an equal volume of methanol added at 4°C. Precipitated protein was removed by centrifugation and purification of the resulting peptide was carried out as described[2] on a Jupiter C4 column (250mm x 10mm) in a gradient of acetonitrile in 0.1% trifluoroacetic acid.

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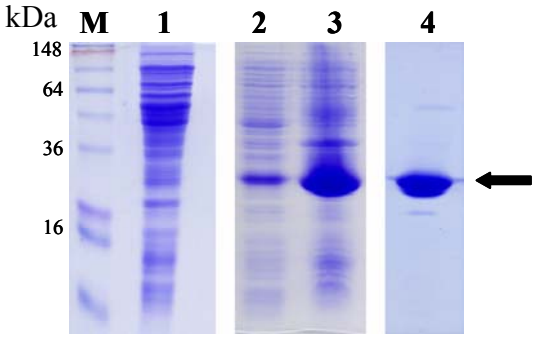
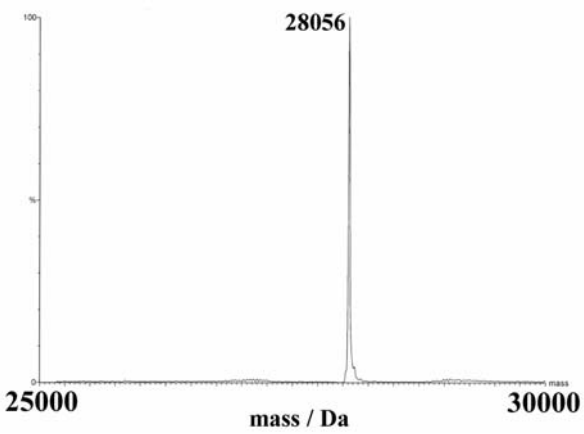
S3. Overexpression and Purification of PHD2.

A, SDS PAGE gels showing expressed and purified PHD2. Protein marker (M) sizes are indicated to the left of the gels. Lane 1 shows uninduced BL21(DE3) cells, lanes 2 and 3 show the expression of soluble and insoluble PHD2 respectively. Lane 4 shows highly purified soluble PHD2. The arrow indicates the position of PHD2 at approximately 28 kDa. **B**, ESI-MS of purified PHD2 showing the existence of a single species.

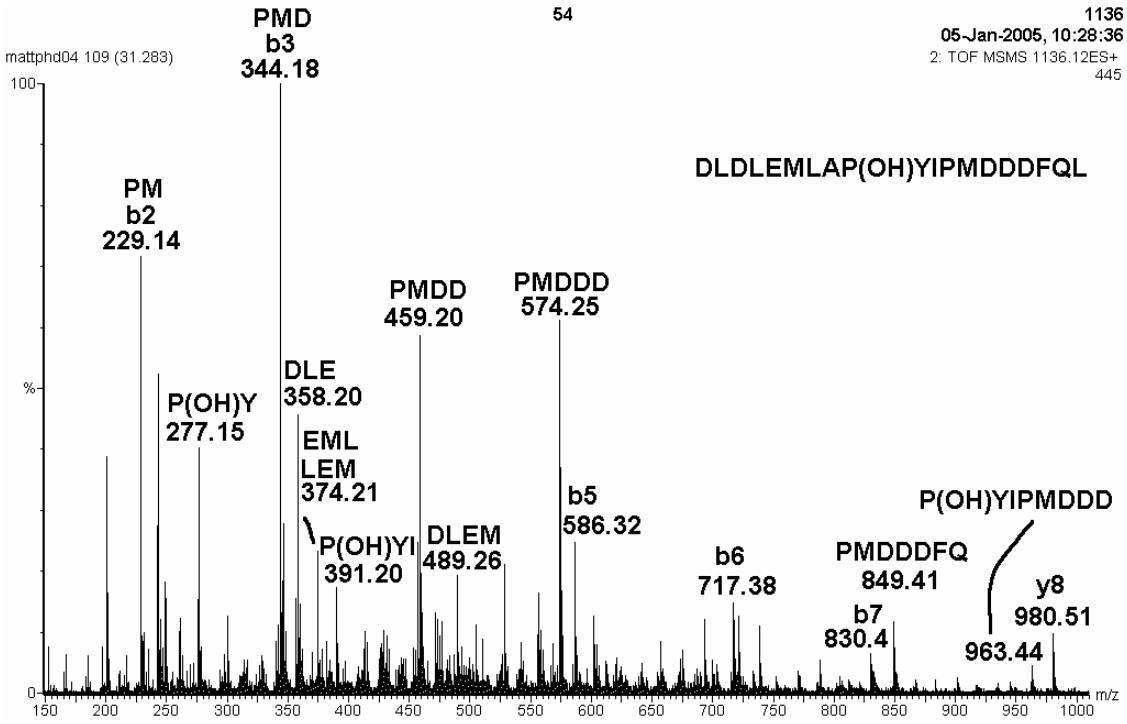
A



B



S4. MS/MS spectrum of $[M+2H]^{2+}$ = 1136 for hydroxyprolyl HIF peptide resulting from incubation with nt-PHD2 and 2OG.



S5. Atomic Absorption Spectroscopy: Experimental Details

Atomic absorption spectroscopy is a technique that determines the presence of metals in liquid samples by measuring their characteristic light absorption upon aspirating into a flame. Experiments were performed by Mr. A Hewson (Inorganic Chemistry Laboratory, Oxford University) using a Thermo Electron Corporation, Atomscan16 spectrometer, with nt-PHD2 samples at 7.7µM in 50mM Tris-HCl pH 7.5.

S6. Micro-PIXE: Experimental Details and Results

Micro-PIXE enables the simultaneous detection of many elements, and in the case of proteins their quantification by comparison with the number of sulphur atoms calculated from the protein sequence[3]. PHD2 contains 12 sulphur-containing residues out of 252 (about 5%). Protein samples (10 mg/ml) were dried onto the mylar film of standard microPIXE sample holders. Protons from the 2MV Tandetron accelerator at the University of Surrey Ion Beam Centre were used to make a 1mm × 1mm scan of the sample area. As indicated by the sulphur map from this scan, four point measurements around the protein drop, plus one background sample were then taken. Estimation of sample thickness and composition were first carried out by modelling the Rutherford back-scattering (RBS) proton spectra with a two-layer model comprising C and O (Mylar support component) and C, N, O, S, Cl (protein component). Fitting of the induced X-ray

spectra was based on these models but allowing for in addition K and P as contaminants in the Mylar film support and Fe, Ni, Cu and Zn as possible components of the proteinaceous layer.

Results (assuming 12 sulphur atoms per protein molecule) for four spots picked from the sulphur map as being regions of high protein concentration are given in Table 2 below.

Run (spot on sample)	Element	Composition ppm	Composition atoms/molecule
24	S	744	
	Fe	42	0.39
	Zn	51	0.40
25	S	811	
	Fe	43	0.36
	Zn	57	0.41
26	S	1402	
	Fe	59	0.29
	Zn	71	0.30
27	S	907	
	Fe	42	0.32
	Zn	56	0.36

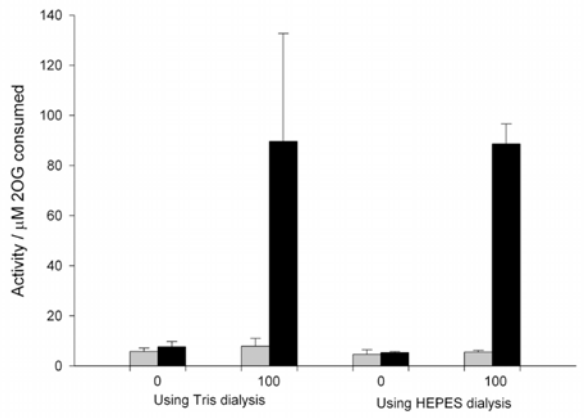
Iron and zinc were found to be the main metallic components present.

Table 2. Complete Micro-PIXE results for Fe and Zn composition in PHD2.

The results show reasonable agreement between the four point spectra, indicating an average of 0.34 Fe and 0.38 Zn atoms per protein molecule. A point spectrum collected on a drop of the buffer alone confirmed that it made no contribution to the sulphur or iron signals. However some, but not all (up to 20%) of the zinc signal could have arisen by contamination. Analysis of a sample of nt-PHD2 also revealed iron and zinc as the major metals (data not shown).

S7. Dialysis Experiments

PHD2 solution (0.5ml, 4.7mg/ml) was dialysed at 4°C into 5L of dialysis buffer for 5 days with one change of buffer. Specific activity was measured (incubation at 37°C for 12 minutes) and the results can be seen below. The dialysis buffer used and concentration of $HIF_{556-574}$ substrate added (μM) can be seen along the x-axis. Grey and black bars show the absence and presence of ascorbate, respectively.



S8. EPR Experimental Conditions

Continuous-wave EPR spectra of nt-PHD2 preparations were obtained at a temperature of 50 K and a frequency of 9.757 GHz with a modulation amplitude of 0.5 mT and a microwave power of 2 mW in a Bruker E580 spectrometer.

S9. ESI-Mass Spectrometry

Electrospray ionisation mass spectrometry (ESI-MS) was conducted on a Micromass (now Waters) Q-TOFmicro quadrupole-time of flight mass spectrometer. For LC/ESI-MS (and LC/ESI-MS/MS) the instrument was coupled to an Agilent 1100 capillary LC system equipped with a Phenomenex Jupiter C4 (15 cm x 500 □M) column using a gradient of water (0.1 % formic acid) and acetonitrile (0.1 % formic acid) as the mobile phase. For soft ionisation ESI-MS the standard Micromass source was replaced with an Advion BioSciences NanoMate^{TM} chip-based nano-ESI source. Protein samples were sprayed from 10 mM NH_4OAc (pH 7) using a chip nozzle voltage of 1.66 kV, and cone voltages of 20, 80 and 170 V (80 V being the standard value used). Collisional cooling of ions was achieved by partially closing a valve on the rotary vacuum pump, leading to an increased pressure in the intermediate vacuum region of the mass spectrometer. CsI was used for calibration.

References

1. R. Chenna, H. Sugawara, T. Koike, R. Lopez, T. J. Gibson, D. G. Higgins and J. D. Thompson, *Nucleic Acids Res,* 2003, **31**, 3497.
2. L. A. McNeill, K. S. Hewitson, T. D. W. Claridge, J. F. Seibel, L. E. Horsfall and C. J. Schofield, *Biochem. J.,* 2002, **367**, 571.
3. E. F. Garman and G. W. Grime, *Prog Biophys Mol Biol,* 2005, In press.