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ESI-MS Studies on Prolyl Hydroxylase Domain 2 Reveal a New Metal Binding Site

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Inhibitor screens with metalloenzymes, many of which are important targets for medicinal chemistry, are often complicated by the problem of defining the metals present and the stoichiometry of metal and inhibitor binding. Various techniques, such as X-ray crystallography, X-ray absorption spectroscopy,[1] circular dichroism, UV/Vis spectroscopy, and electron spin resonance spectroscopy[2] have been developed for metal binding studies. However, these techniques can be time consuming. Non-denaturing ionisation electrospray mass spectrometry (ESI-MS) has emerged as a technique for studying noncovalent protein–ligand interactions.[3–5] ESI-MS has also been used to study metal–ligand binding (for review, see Ref. [6]), ligand–metal binding to proteins (for review, see Ref. [7]), metal stoichiometry in enzyme–inhibitor interactions,[8] and, in combination with a dynamic chemistry approach, for the identification of metalloenzyme inhibitors.[9] Although there are some concerns as to whether the MS data always correlates well with that in solution, mild ionisation MS techniques offer a rapid method to investigate metal stoichiometry and inhibitor binding to proteins.

We report ESI-MS metal binding studies on the catalytic domain of prolyl hydroxylase domain 2 (PHD2), an iron(II) and 2-oxoglutarate (2OG) oxygenase that acts as an oxygen sensor in humans.[10–13] In combination with point mutagenesis analyses, the ESI-MS studies revealed an unanticipated second metal binding site. The work exemplifies the use of ESI-MS for the analyses of metalloenzymes of medicinal interest.

Initially, we prepared the apo form of N-terminally truncated PHD2 (residues 181–426),[14] which is suitable for mild ionisation ESI-MS analyses (Figure 1 a). We then investigated the binding of Fe^{II} and observed, consistent with previous results, that one Fe^{II} ion bound even in the presence of a twofold excess of Fe^{II} (Figure 1 b). Titration experiments using ESI-MS indicated that the K_D for Fe^{II} is <1 μM, which is consistent with data from other techniques.[14] Cobalt(II) ions inhibit PHD2 and other human PHDs, an effect that is proposed to rationalise the ability of Co^{II} to artificially induce the hypoxic response.[10] We therefore investigated the binding of a set of transition metals to apo-PHD2. Unexpectedly, the results revealed that certain metal ions, including Zn^{II}, Cd^{II}, Cu^{II}, and Co^{II} bound with a metal/PHD2 stoichiometry $>1:1$ (Table 1). In particular, the binding of a second metal was observed in the presence of Zn^{II}, Cd^{II}, and Cu^{II} (Figure 1 c,d,e). Second metal binding was partially observed in the case of Co^{II}, which formed complexes PHD2·Co/PHD2·Co_2 in a 1:1 ratio (Figure 1 f). Only one Ni^{II} and Mn^{II} were observed to bind, even in the presence of twofold excess metal ion (Figure 1 g,h). No metal ion binding was observed in the case of Ca^{II} and Mg^{II} (Figure 1 i,j). Further titration studies conducted on apo-PHD2 using ESI-MS revealed that the K_D for the first Zn^{II} is <1 μM and is higher for the binding of the second Zn^{II} ion at ~2 μM.

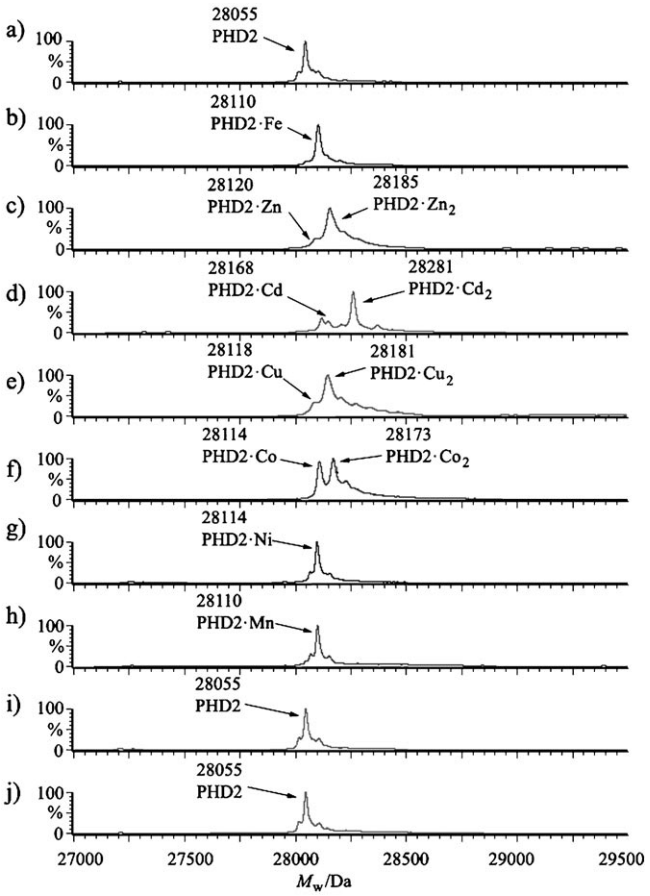

Figure 1. Deconvoluted ESI mass spectra from a) apo-PHD2 in the presence of 2 equiv of the following divalent metal ions: b) Fe^{II}, c) Zn^{II}, d) Cd^{II}, e) Cu^{II}, f) Co^{II}, g) Ni^{II}, h) Mn^{II}, i) Ca^{II}, and j) Mg^{II}.

To investigate whether the Zn^{II}, Cd^{II}, and Cu^{II} ions compete at the Fe^{II} PHD2 binding site, we then investigated the potential for second metal ion binding to the PHD2·Fe complex

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Table 1. Metal binding stoichiometry to apo-PHD2.

Exogenous metal	Detected complexes	
	1 equiv metal	2 equiv metal (ratio)[a]
None	PHD2	PHD2
Fe^{II}	PHD2·Fe	PHD2·Fe
Zn^{II}	PHD2·Zn	PHD2·Zn/PHD2·Zn_2 (1:4)
Cd^{II}	PHD2·Cd	PHD2·Cd/PHD2·Cd_2 (1:4)
Cu^{II}	PHD2·Cu	PHD2·Cu/PHD2·Cu_2 (1:3)
Co^{II}	PHD2·Co	PHD2·Co/PHD2·Co_2 (1:1)
Ni^{II}	PHD2·Ni	PHD2·Ni
Mn^{II}	PHD2·Mn	PHD2·Mn
Ca^{II}	PHD2	PHD2
Mg^{II}	PHD2	PHD2

[a] Figure 1; see Experimental Section for details.

(Supporting Information). As before, no clear binding of a second metal was observed, except for Zn^{II}, Cd^{II}, Cu^{II}, and Co^{II}. In these cases, at least predominantly, only two metal ions were observed to bind, with little or no binding of three metal ions. Evidence that Zn^{II} and Cd^{II} ions can compete with Fe^{II} for binding at the active site came from high-resolution ESI-FTICR-MS analyses (Supporting Information) that, unlike the previous ESI-MS analyses, were able to distinguish between the different PHD2·metal complexes. In contrast to the MS results reported herein, a recent crystal structure of PHD2 (residues 181–426, with 1 mM Fe^{II} and 0.71 mM PHD2 in the well solution) revealed only a single iron binding site, with ligands comprising an HXD/E, H motif (His 313, Asp 315, His 374) that is characteristic of 2OG oxygenases (Figure 2).[15]

Analytical ultracentrifugation experiments showed that apo-PHD2 and PHD2·Fe are present predominantly in monomeric form in solution. Activity assays were performed in cases of PHD2·Fe and apo-PHD2 in the presence of Fe^{II} and revealed that the two forms had equal activity under standard assay conditions.

Mutation studies were then carried out on PHD2 with the aim of identifying the residues involved in a second metal binding site. The mutants were prepared as for the PHD2·Fe form and screened for binding of Zn^{II}, Cd^{II}, and Cu^{II}, as before.

Because the C terminus of the 2OG oxygenase sub-family to which PHD2 belongs has been implicated in substrate binding in some cases (for review, see Ref. [16]), we made two C-terminal truncations $PHD2_{181–402}$ and $PHD2_{181–393}$, along with mutations to three active site residues that we thought might be involved in a second metal binding site (Y303F, Y310F, and D254A). In each of these cases we still observed binding of a second metal (Zn^{II}, Cd^{II}) to the mutant PHD2 (unpublished data).

As binding of a second metal was observed for Zn^{II} and Cd^{II}, and because cysteine residues are often involved in chelation of these metals in proteins, we then targeted three cysteine residues (C201, C208, C302) located at the surface of PHD2 for mutation analyses. C302A·Fe was found to bind an additional Zn^{II} or Cd^{II} ion, similarly to wild-type $PHD2_{181–426}$ (Figure 3 c,f). However, for both C201A·Fe and C208A·Fe a significant decrease in the binding of a second metal (Zn^{II}, Cd^{II}) was observed. Thus when Zn^{II} was added to C201A·Fe, the observed ratio of C201A·Fe/C201A·Fe·Zn was 5:1 (Figure 3 a), in contrast to that of wild-type enzyme, for which the ratio of PHD2·Fe/PHD2·Fe·Zn is 1:4. A similar observation was made when Cd^{II} was added to C201A·Fe, with a ratio for C201A·Fe/C201A·Fe·Cd of 5:1 (Figure 3 d).

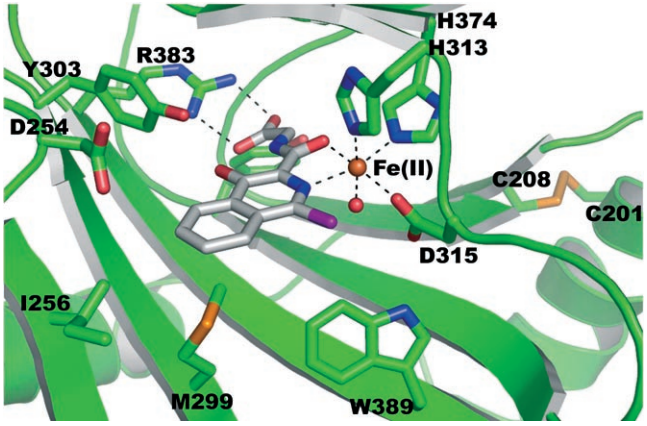

Figure 2. View from the PHD2 active site showing the octahedral coordination of Fe^{II} (orange) by His 313 (green), Asp 315 (green) and His 374 (green), a water molecule (red), and the side chains of the inhibitor (white); (PDB: 2HBT).[15] Secondary structure elements are shown.

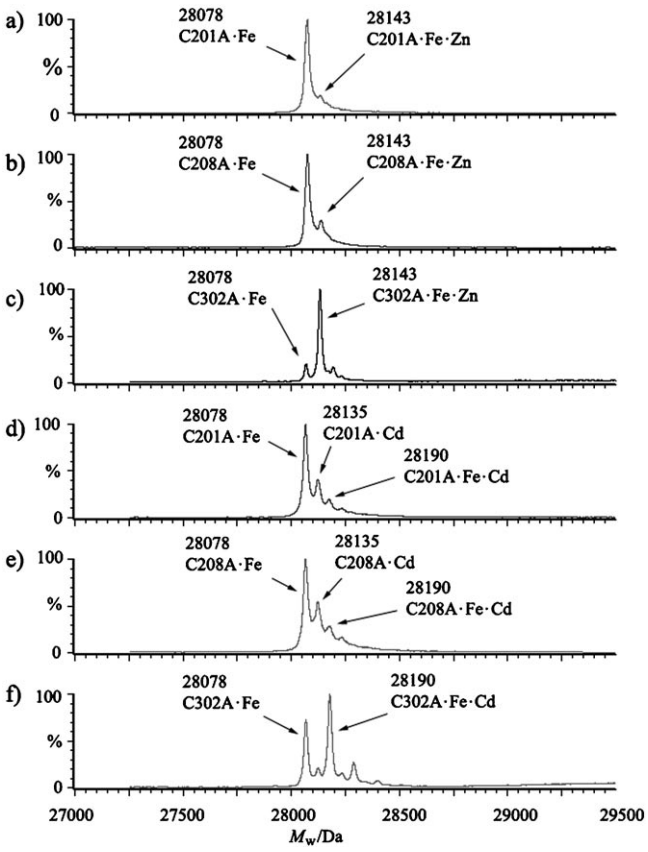

Figure 3. Deconvoluted ESI mass spectra from three cysteine mutants of PHD2 in the presence of the divalent metal ions indicated (1 equiv each): a) C201A + Fe^{II} + Zn^{II}, b) C208A + Fe^{II} + Zn^{II}, c) C302A + Fe^{II} + Zn^{II}, d) C201A + Fe^{II} + Cd^{II}, e) C208A + Fe^{II} + Cd^{II}, f) C302A + Fe^{II} + Cd^{II}.

For the analyses of C201A·Fe and Cd^{II}, the observation of an additional peak at 28135 Da provided evidence for the formation of a C201A·Cd complex. This result shows that Cd^{II} can partially displace Fe^{II} at the PHD2 active site (the resolution of the ESI-MS analyses was insufficient to observe displacement of Fe^{II} by Zn^{II}). The C208A mutant displayed similarly decreased affinity for binding of a second metal (Zn^{II} and Cd^{II}). Thus, the observed C208A·Fe/C208A·Fe·Zn (Figure 3b) and C208A·Fe/C208A·Fe·Cd (Figure 3e) ratios were 3:1 and 4:1, respectively. However, the Y197F·Fe complex was found to bind an additional Zn^{II} or Cd^{II} ion, similarly to the wild-type $PHD2_{181–426}$, showing that a phenolic hydroxy group is not required for the binding of these metals. In contrast, when Cu^{II} was added to C201A·Fe and C208A·Fe, C201A·Fe·Cu and C208A·Fe·Cu complexes were observed, respectively, implying that the binding of Cu^{II} as a second metal is different from that of Zn^{II}.

Overall the results reveal that the catalytic domain of PHD2 contains at least one second metal binding site. In the case of Zn^{II}, binding likely involves the side chains of C201 and C208 (Figure 4). At this stage, the biological relevance, if any, of this metal binding site is unknown. However, it is notable that the N-terminal domain of PHD2 (not part of the catalytic domain studied herein) contains a proposed zinc binding domain.[10,12] Crystallographic analyses have shown that Cys201 and Cys208 can form a disulfide bond (Figure 2, PDB: 2HBT). Although disulfide bonds in intracellular proteins are thought to be rare, this is notable given the oxygen-sensing role of PHD2; it is possible that metal binding to Cys201/208 may modulate the extent of disulfide formation. Finally, the results further exemplify the utility of ESI-MS for the efficient investigation of metal binding stoichiometry.

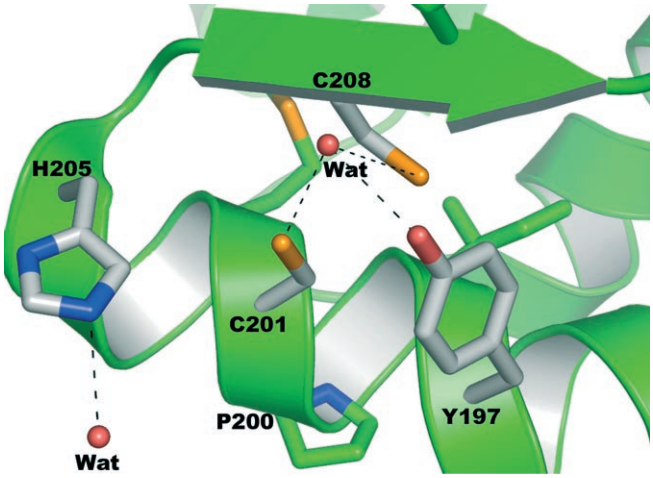

Figure 4. Putative second metal (Zn^{II}, Cd^{II}) binding site on the surface of PHD2 showing Cys201 and Cys208; (PDB: 2G19). Secondary structures elements are shown.

Experimental Section

The catalytic domain of human PHD2 (residues 181–426) and mutants were produced as N-terminally His_6-tagged proteins in *E. coli* and purified by metal-affinity chromatography.[14] The His_6 tag was cleaved with recombinant thrombin, and PHD2 was further purified by gel filtration chromatography (details of the preparation of the mutants will be published elsewhere). Human PHD2 and mutants were desalted using Microcon YM-10 (M_w cutoff: 10000 Da) centrifugal filters (Millipore, Bedford, MA, USA) in ammonium acetate (15 mM, pH 7.5). Seven rounds of dilution/concentration were performed at 4 °C and 13000 *g*. The stock solution was diluted with the same buffer to a final concentration of 100 μM. $FeSO_4·7H_2O$ was dissolved in 20 mM HCl at a concentration of 100 mM. This was then diluted with Milli-Q water to give final working concentrations of 100 and 200 μM. Other metal salts were dissolved in Milli-Q water at concentrations of 100 and 200 μM. The protein was mixed with metal salt (1 or 2 equiv) at a concentration of 15 μM prior to ESI-MS analysis. Data were acquired on a Q-TOF mass spectrometer (Q-TOF micro, Micromass, Altrincham, UK) linked to a Nanomate (Advion Biosciences, Ithaca, NY, USA) with a chip voltage of 1.70 kV and a delivery pressure of 1.70 kPa. The sample cone voltage was typically 200 V, with a source temperature of 40 °C and an acquisition/scan time of 10 s/1 s. Calibration and sample acquisition were performed in the positive ion mode in the range of 500–5000 *m/z*. The pressure at the interface between the atmospheric source and the high-vacuum region was fixed at 6.60 mbar. External instrument calibration was achieved by using sodium iodide. Data were processed with MASSLYNX 4.0 (Waters). ESI-FTICR-MS data were acquired on a Bruker Apex Qe 9.4-Tesla Fourier Transform Ion Cyclotron Resonance (FTICR) mass spectrometer (Bruker Daltonics, Billerica, MA, USA) fitted with an Apollo ESI nano source operated in positive ion mode and controlled by Hystar 3.2 software. Broadband excitation was used to analyse a mass range from 200–3000 Da, with FFTW 1Ma points acquired with a FID of 512k. The high-vacuum analyser region pressure was 1.0×10^{-10} mbar, with a scan accumulation time of 0.01 s. Calibration was carried out using Agilent ESI tuning mix (catalogue number G2421A, Palo Alto, CA, USA). Data were analysed using Data Analysis version 3.3 (Bruker Daltronics).

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Keywords: hypoxia · mass spectrometry · metalloenzymes · prolyl hydroxylase

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