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Using NMR Solvent Water Relaxation to Investigate Metalloenzyme−Ligand Binding Interactions

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This report demonstrates that solvent water relaxation measurements can be used for quantitative screening of ligand binding and for mechanistic investigations of enzymes containing paramagnetic metal centers by using conventional NMR instrumentation at high field. The method was exemplified using prolyl hydroxylase domain containing enzyme 2 (PHD2), a human enzyme involved in hypoxic sensing, with Mn(II) substituting for Fe(II) at the active site. K_D values were determined for inhibitors that hinder access of water to the paramagnetic center. This technique is also useful for investigating the mechanism of suitable metalloenzymes, including order of ligand binding and modes of inhibition.

Introduction

NMR spectroscopy is widely used for the study of small molecule−protein interactions; saturation transfer difference (STD[a]),[1] transferred NOE,[2] and chemical shift perturbation techniques have emerged over recent years to play useful roles in ligand screening.[3−6] Although widely employed, these techniques may suffer from low intrinsic sensitivity and can require careful optimization of experimental conditions. For instance, STD enhancements can be rather weak and these analyses typically require a large excess of ligand, which may result in nonspecific binding or cause problems if the ligands are of limited solubility. The transferred NOE method requires a delicate ligand−protein ratio, and the results can be difficult to interpret in cases of relatively large ligands such as for peptide−protein or polysaccharide−protein interactions. Chemical shift perturbation using $^{1}H-^{15}N$ HSQC requires access to ^{15}N labeled protein and can be difficult to apply in systems larger than 25 kDa.[7] These factors may limit the applicability of these methods as primary screening tools.

Relaxation enhancement has been used as a tool to study paramagnetic metalloprotein−ligand interactions since the early 1960s;[8] the technique is based on the fact that the magnetic moment of an electron is 658 times greater than that of a proton nuclear spin.[9] Therefore, in the presence of a paramagnetic metalloprotein, the longitudinal and transverse relaxation rates of nuclei within a binding ligand may be enhanced, while for nonbinders the relaxation rates will remain unchanged. The changes in relaxation behavior depend on factors including the applied magnetic field (B_0), molecular correlation times, accessibility of the active site, and the properties of the paramagnetic metal itself.[8,10−12] The same paramagnetic relaxation enhancement (PRE) effect can be observed through solvent water proton relaxation rates. Various groups have made use of this effect to study paramagnetic metal binding interactions with proteins and other macromolecules by monitoring changes in the bulk water relaxation rate.[13−17]

Recently, a novel competition method has been proposed for screening small molecules binding to paramagnetic metalloproteins by employing water as the reporter molecule and monitoring changes in the bulk water proton longitudinal relaxation rate (R_1).[18] In the absence of any ligand, providing that access is possible, water molecules may chelate to the paramagnetic metal and undergo exchange with water molecules in the bulk solvent matrix. This leads to a net increase in the bulk water relaxation rate. The binding of ligands to the active site will normally hinder or prevent access of water molecules to the paramagnetic center and so decrease the observed bulk water relaxation rate.[19] Because the exchange of water will not normally be directly reliant on the exchange kinetics of the reversibly forming protein−ligand complex (as in the case for STD-NMR), this technique does not suffer from the limits imposed by other ligand-based NMR screening methods.[18] For example, while the association of very strongly binding ligands is often difficult to detect directly with ligand-observed methods, alterations in water accessibility may still arise under these conditions and remain readily detectable through water relaxation measurements. Thus, provided the enzyme under investigation contains a paramagnetic center at the binding site, this present approach can overcome the difficulties and limitations often associated with other ligand based NMR screening techniques.

The effect of paramagnetic relaxation is maximized at low field, generally between 0.01 to 100 MHz;[18] hence the original application of this solvent-based method for ligand screening was focused toward the use of specialist low field NMR instruments or fast field-cycling relaxometers.[18,20] While it was demonstrated that it is possible to conduct screening experiments at high field (400 MHz) on very small sample volumes (~2 μL) using an inhibitor with a K_D in the

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[a] Abbreviations: 2OG, 2-oxoglutarate; NOG, *N*-oxalylglycine; PHD, prolyl hydroxylase; STD, saturation transfer difference; PRE, paramagnetic relaxation enhancement; HIF, hypoxia inducible factor; ESI-MS, electrospray ionization mass spectrometry; ILOEs, interligand NOEs; CODD, *C*-terminal oxygen-dependent degradation domain; NODD, *N*-terminal oxygen-dependent degradation domain; BNS, bicyclic naphthalenylsulfonyl compound; BIQ, bicyclic isoquinolinyl compound.

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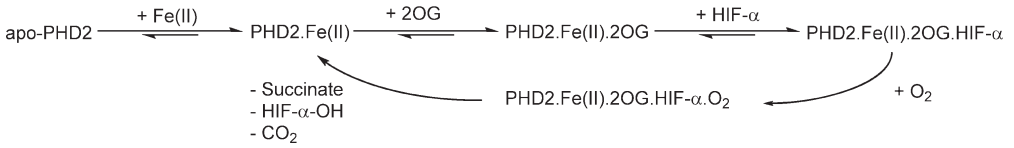
Scheme 1. Outline Reaction Scheme for PHD2 Catalysis; 2OG = 2-Oxoglutarate



nanomolar region, the application to screen weaker inhibitors has not been demonstrated at high field and may suggest limited applicability in biochemical research laboratories employing conventional high-field NMR spectrometers.

By using the catalytic domain of human prolyl hydroxylase domain 2 ($PHD2_{181-426}$, or PHD2 hereafter) as a model system, we have optimized the experimental conditions of this water relaxation technique at high field and demonstrate its wider application as a NMR screening technique using conventional instrumentation. Furthermore, we have also demonstrated its potential as a method to investigate metalloenzyme reaction and inhibition mechanisms by monitoring the formation of complexes between protein, cofactor and substrates.

Results

Methodology. PHD2 is a Fe(II) and 2-oxoglutarate (2OG) dependent oxygenase that is involved in a key step in human oxygen sensing as part of the response to limiting oxygen levels (hypoxia) (for reviews, see refs 21 and 22). Under normal oxygen levels (normoxia), PHD2 initiates the oxygen dependent degradation of the α-subunit of hypoxia inducible factor (HIF-1α) by catalyzing the hydroxylation of two prolyl residues (positions 402 and 564 in human HIF-1α).[23,24] The PHD2−Fe(II) complex forms a ternary complex with the cofactor 2OG and its protein substrate HIF-α (or the peptide fragments $HIF\text{-}1\alpha_{556-574}$ (*C*-terminal oxygen-dependent degradation domain, CODD) and $HIF\text{-}1\alpha_{395-413}$ (*N*-terminal oxygen-dependent degradation domain, NODD)) (Scheme 1). In the case of the peptide substrates being described here, the hydroxyproline (Hyp) peptides $CODD_{Hyp564}$ or $NODD_{Hyp402}$ are produced, with carbon dioxide and succinate as coproducts.[25] PHD2 is a current pharmaceutical target for new treatments for ischemic disease and anemia.[26,27] Following the finding that PHD activity is inhibited by *N*-oxalylglycine (NOG),[28,29] a 2OG analogue, several groups have described the identification of inhibitors of PHD2.[30−36] Herein we report the application of bulk water relaxation measurements to investigate mechanistic features and inhibition of PHD2.

An absolute requirement for the relaxation-based screening experiment is the presence of a paramagnetic center inside the protein that is accessible to bulk water. PHD2 is unusual among other 2OG oxygenases in that it forms a stable complex with diamagnetic high spin Fe(II).[37] Although there may be small amounts of paramagnetic Fe(III) species inside the PHD2 active site, quantitative information is difficult to extract. Previous studies have indicated that the Fe(II) metal ion in the PHD2 active site can be substituted by different transition metals.[38] Mn(II) forms a 1:1 complex with apo-PHD2[38,39] and was selected as the metal of choice in this study because of its favorable paramagnetic properties.[8,40] By measuring the bulk water relaxation rates at a fixed Mn(II) concentration and varying PHD2 concentrations, we confirmed that the binding of Mn(II) to apo-PHD2 is strong (see Supporting Information, Figure S1). In the screening experiments described below, a 2-fold molar excess of Mn(II) was used to saturate the active site. However, in cases where the small molecule is a nonbinder or a very weak binder, the presence of free paramagnetic metal ions in solution can potentially be a source of artifacts through direct chelation of the free metal and should be avoided. Therefore, in this situation, a 1:1 molar ratio of protein to Mn(II) was used so that any contribution to relaxation rate changes from small molecules chelating to free metal ions was minimized.

Longitudinal relaxation time constants (T_1) were measured at 500 MHz using the standard inversion recovery scheme.[41] Solvent water T_1 constants were measured directly in a H_2O-D_2O mixture as opposed to the originally proposed method in which samples are measured in 100% H_2O.[18] A H_2O-D_2O ratio of 12.5%:87.5% was chosen so that the range of T_1 values could be kept roughly between 1.8 to 3.5 s in order to keep the length of inversion recovery experiments reasonably short. Conventional 3 mm tubes were used to strike a balance between the ease of titration and avoiding radiation damping that may arise from the intense H_2O resonance. The small proportion of H_2O to D_2O would also assist in the avoidance of radiation damping. The reduction of filling factor when using a 5 mm probe was not of concern because of the strong HDO signal being measured.

Ligand Screening. Figure 1a presents solvent water relaxation data for three different 2OG titrations from separate sample preparations by plotting the rate of relaxation (R_1) against the ligand concentration. Analysis in the presence of succinate, a product of PHD2 catalysis and a relatively weak inhibitor,[42] is plotted for comparison purposes. In this presentation format, variability between titration curves is apparent for 2OG and it is not immediately apparent whether 2OG or succinate is the stronger binder. The absolute R_1 values, such as the initial relaxation rate when no ligand is added ($R_{1(0)}$) and the minimum relaxation rate when a 1:1 complex is formed, are strongly influenced by variations in the concentrations of paramagnetic ion and protein. Small variations in Mn(II) and protein concentrations from separate sample preparation stages will therefore influence the ability to identify differences in binding affinities when comparing across samples.

However, the actual parameter of interest is the relative change in observed relaxation rate on titration of the ligand. We therefore propose that in order to normalize these variations, it is advantageous to plot and compare the relative relaxation rates $R_1/R_{1(0)}$ against the titrated ligand concentration, as shown in Figure 1b. With this method of analysis, it is possible to readily distinguish between strong and weak binders. The curve for a strong binder (2OG[37]) is characterized by a steep slope and rapid plateauing at, or close to, a 1:1 protein−ligand ratio, whereas the curve for a weak binder (succinate[42]) is characterized by a gentler slope which does not plateau until a large ligand excess is reached. The R_1 ratio at which this saturating equilibrium condition is reached is likely governed by factors including kinetics of the

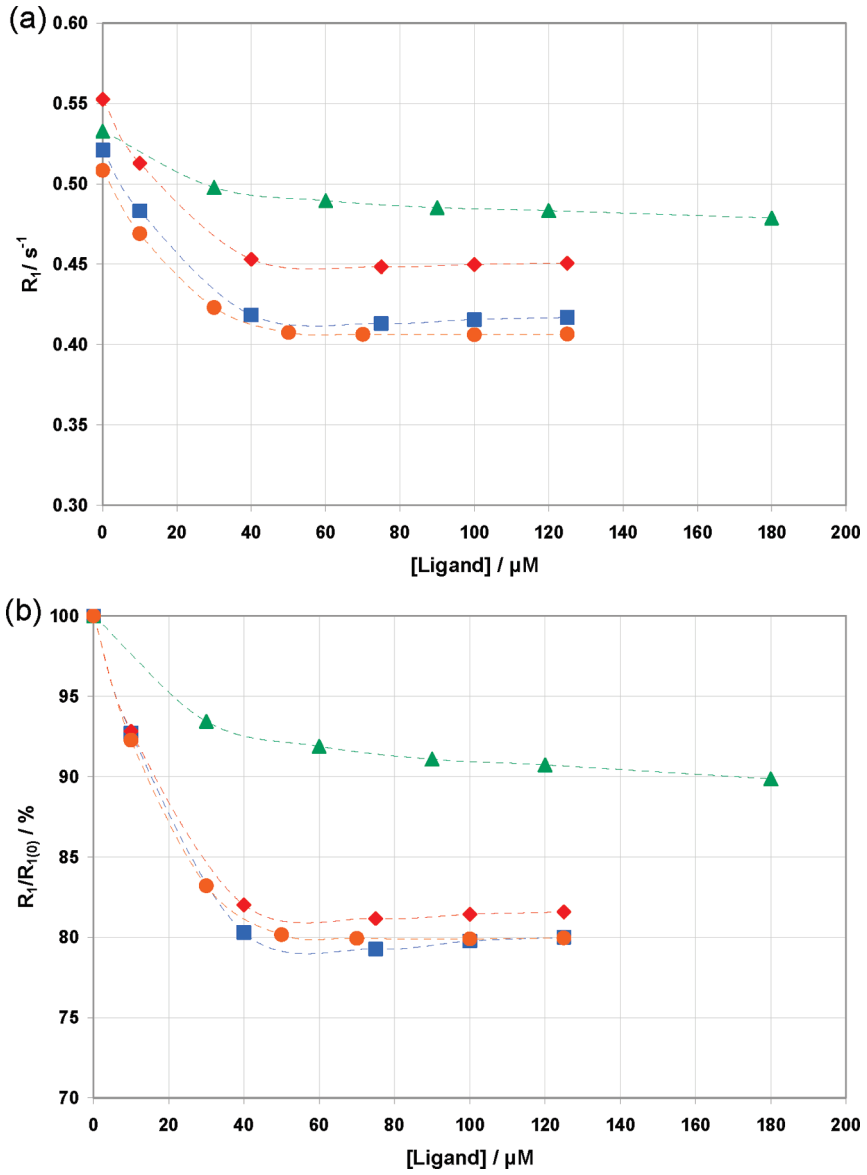


Figure 1. Titrations of 2OG in three separate sample preparations (blue squares, red circles, and red diamonds), and the titration of succinate (green triangles) into 50 μM PHD2. Plotted against ligand concentration is (a) the directly measured solvent relaxation rate (R_1) and (b) the relative relaxation rate $R_1/R_{1(0)}$, in which $R_{1(0)}$ is the initial relaxation rate when no ligand is added. Slight differences in Mn(II) and protein concentrations from different sample preparations complicate comparisons across samples, but by normalizing the rates as $R_1/R_{1(0)}$, binding differences are made apparent. In this and all subsequent figures, dashed lines are included to aid visualization.

reversibly forming protein−ligand complex, the accessibly of water molecules, their mode of binding (e.g., directly to metal or otherwise), and the number of water molecules that are being displaced. The position of the plateau is also somewhat sensitive to the intrinsic variability that occurs between different sample preparations, arising from difficulties in determining protein concentrations accurately and the slight variation in metal ion concentration between different stock solutions. Such variability is apparent for the 2OG titration data in Figure 1b, and we observe this to be typical behavior between discrete sample preparations. For clarity therefore, the normalized rates, $R_1/R_{1(0)}$, versus ligand concentration presentation format is used throughout the remainder of this report.

The amounts of protein and ligand molecules required for this relaxation method are comparable to, or less than, other NMR based screening techniques because it is the intense HDO signal that is being measured. Nevertheless, consideration should be given when choosing the relative amounts of protein and ligand for the experiment because there is trade-off between protein concentration, total experiment time, and the sensitivity of the method in differentiating ligands of similar binding affinity (Figure 2). When using ~25 μM protein and 100 μM Mn(II), the experimental time is shortened because there is a higher concentration of free Mn(II) in solution that is readily accessible to water molecules, thus leading to faster relaxation rates ($R_{1(0)} = 0.60$ s^{-1}). However, in this case, the extent of reduction in relaxation rates on ligand addition is diminished with the titration curve plateauing at around 90%, reducing the separation between the curves of similar affinity ligands. In contrast, by using ~75 μM protein with 100 μM Mn(II), the variation in rates is enhanced, now plateauing at around 65%, and the method becomes more sensitive to small differences in binding affinities. One consequence of this, however, is that the time required for the experiment is greater due to the higher concentration of protein bound Mn(II)

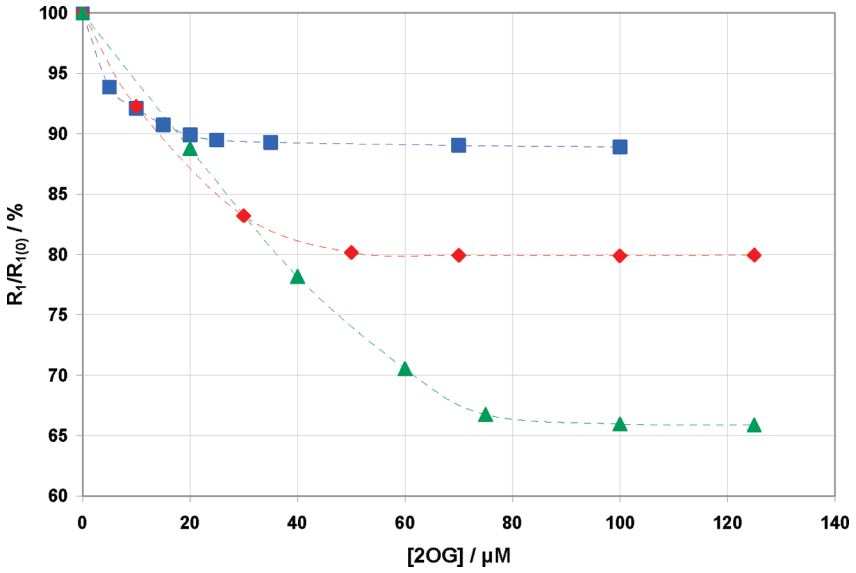


Figure 2. Titration of 2OG into 25 μM PHD2 (blue squares), 50 μM PHD2 (red diamonds), and 75 μM PHD2 (green triangles) with 100 μM Mn(II). The minimum R_1 value depends on the residual relaxation rate of the H_2O-D_2O mixture, which in turn is dependent upon the amount of free Mn(II) present in solution.

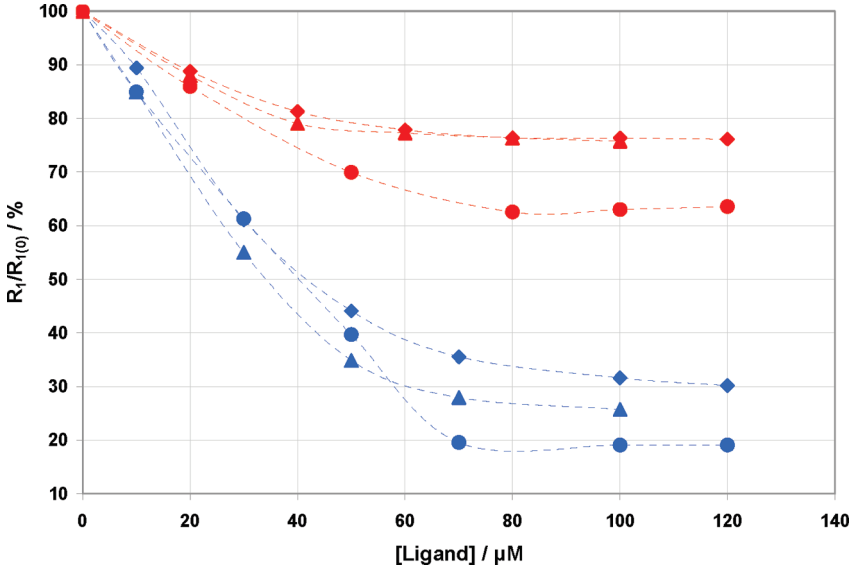


Figure 3. The titration of 2OG (solid triangles), NOG (solid diamonds), and BNS (solid circles) into 50 μM PHD2 with 100 μM Mn(II). The data plotted in red and blue are conducted at 500 and 23 MHz, respectively.

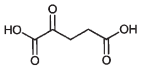
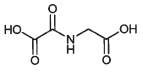
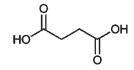
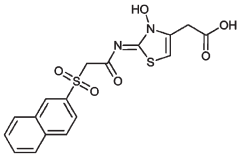
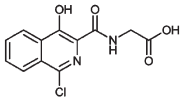
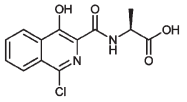
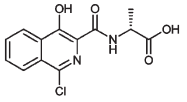
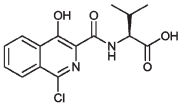
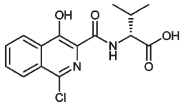
species with correspondingly fewer free ions in solution ($R_{1(0)}$ = 0.41 s^{-1}). As comparison, the water relaxation rate observed for the apo-PHD2 solution (50 μM, 12.5% H_2O) in the absence of Mn(II) was 0.11 s^{-1}.

To investigate the validity and sensitivity of the method at high field, we then conducted experiments at low field, as in the work of Bertini et al.,[18] and compared the results with our high field data. Three PHD2−ligand systems were analyzed for comparison. 2OG is a natural cofactor of PHD2 and has a K_D value of about 1 μM as determined by nondenaturing ESI-MS,[37] while *N*-oxalylglycine (NOG) is a 2OG mimic that binds slightly weaker than 2OG.[23] A potent bicyclic naphthalenylsulfonyl inhibitor (BNS) was also tested (Figure 3).[34,36]

The low field experiments were conducted in 100% H_2O at 23 MHz, and both high and low field experiments were measured at 313 K so that the rate of ligand exchange was increased, enabling a more accurate determination of K_D, as suggested by Bertini et al.[18] Such K_D measurements become feasible because only near stoichiometric amounts of ligand are required.

As anticipated, the minimum relaxation rate is smaller at low field, indicating greater sensitivity under these conditions; however, the relative change of R_1 between the three ligands, and hence the calculated K_D values, are essentially the same at both low and high field. Qualitatively, results obtained at high and low fields confirm that the bicyclic inhibitor BNS is a much stronger binder than 2OG and NOG. Quantitatively, the K_D values obtained for 2OG at both low and high field are ~2 μM, while for NOG they are ~3 μM (see Supporting Information, Figures S2−S5). Note that these values are slightly higher than the reported values

Table 1. Dissociation Constants (K_D) Determined by the Solvent Relaxation Technique at 298 K and IC_{50} Values[34,46] for 2OG, Succinate and a Range of Inhibitors

Ligand	Structure	K_D / μM	IC_{50} / μM
2OG		0.9 ± 0.1	N/A
NOG		3.1 ± 0.3	18.5
Succinate		> 100	85.3
BNS		<1	0.021
BIQ		<1	0.073
L-Ala-BIQ		<1	0.512
D-Ala-BIQ		~22	230
L-Val-BIQ		~35	> 400
D-Val-BIQ		~33	> 400

for 2OG and NOG,[23,37] possibly because in this work we are analyzing binding to a PHD2−Mn(II) rather than a PHD2−Fe(II) complex. Thus, both qualitative screening and the quantitative determination of binding kinetics appear feasible at high field and with conventional, widely used NMR instrumentation.

Determination of Inhibitor Binding Affinities. The bicyclic isoquinolinyl inhibitor (BIQ) is a potent inhibitor of PHD2, with an IC_{50} value of 0.07 μM.[43−45] Most PHD2/HIF hydroxylase inhibitors have amino acid side chains that bind in the 2OG binding pocket, and previous work has shown that the selectivity of these inhibitors can be modified by varying the amino acid moiety.[33] A series of D/L-amino acid substituted BIQ analogues have been synthesized; the binding of some of these to PHD2 is limited by steric constraints.[46] These were screened for their binding affinities using the solvent relaxation technique (Table 1). We found that the introduction of a methyl group (alanine derivative) altered the selectivity of BIQ binding significantly, with L-Ala-BIQ binding to PHD2 in a similar fashion to BIQ, whereas D-Ala-BIQ was a much weaker binder. The relative weaker binding of D-Ala-BIQ is likely due, at least in part, to steric hindrance with the side chain of valine-376 in the 2OG binding pocket.[43,46] Solvent relaxation experiments gave K_D values of < 1 μM and ~22 μM for L-Ala-BIQ and D-Ala-BIQ respectively (see Supporting Information, Figure S8), consistent with the binding data obtained from nondenaturing ESI-MS (see Supporting Information, Figure S15).

The substitution of the glycine moiety of BIQ by bulky amino acids such as valine also sterically weakens the binding of BIQ inhibitors to PHD2. In fact, D- and L-Val BIQ show very similar levels of weak binding as indicated by MS analyses (Supporting Information, Figure S15). Solvent relaxation experiments gave K_D values of ~33 μM and ~35 μM for D- and L-Val BIQ, respectively (see Supporting Information, Figures S9−S10). Our results therefore demonstrate and further support the accuracy and sensitivity of this technique while using more conventional NMR instrumentation.

Investigation of PHD2−Mn(II)−2OG−HIF-α Complex Formation. CODD and NODD are 19 amino-acid peptide substrates of PHD2 which are fragments of the natural human HIF-1α; both are hydroxylated by PHD2 at the *trans*-C4 position of a prolyl residue.[23] In studies on the binding of larger peptides such as these, it is often difficult to apply ligand-based NMR techniques; for example, in the transferred NOE method, it becomes difficult to distinguish between NOE signals arising from free and bound peptides because they have the same sign, while in saturation transfer difference (STD) NMR, peak overlap can make it difficult to irradiate selectively the protein resonances. Despite the relatively large size of the NODD and CODD peptide substrates, the application of the solvent relaxation technique at high field is straightforward because only the HDO signal is observed.

Previous biophysical and kinetic studies show that PHD2 has a preference for hydroxylating CODD over NODD,[47−49] and K_m values for CODD and NODD were reported as 37 and 44 μM, respectively.[48] Our results suggest CODD is a significantly stronger binder than NODD, with K_D values of ~14 and ~85 μM, respectively, derived from solvent relaxation data (see Supporting Information, Figures S11−S12). Because neither CODD or NODD directly chelate the metal center,[39] these data demonstrate that it is possible to screen for compounds that bind in the vicinity of the active site metal yet need not interact with this directly, provided that their association displaces water molecules from the active site metal (or indeed enhances their access) or alters their exchange with bulk water. These data are also consistent with the proposed PHD2 mechanism of hydroxylation which, by analogy with work on other 2OG oxygenases, involves displacement of water complexed to the active site iron upon CODD or NODD binding, such that a coordination position becomes free for an oxygen molecule to coordinate to the metal prior to the oxidative decarboxylation step.[50−52]

We have also investigated whether this technique can be used for mechanistic studies. CODD and NODD are both known to form complexes with the PHD2−metal complex and 2OG. CODD binds to PHD2 in a well-defined cleft, adopting a loose and extended conformation; 2OG chelates directly to the metal center in a bidentate fashion.[39] A clear additive effect is observed by the water relaxation technique when CODD is added to PHD2−2OG or when 2OG is added to PHD2−CODD (Figure 4). When CODD is first titrated into PHD2 (red squares, Figure 4a), the curve slowly plateaus to around 90% at a substrate to protein ratio of 2:1, indicating formation of a PHD2−CODD complex. Subsequent addition of 2OG (red triangles, Figure 4a) further decreases the $R_1/R_{1(0)}$ ratio to around 70%, indicating the formation of a PHD2−CODD−2OG complex. An equivalent effect is observed when 2OG is titrated into PHD2

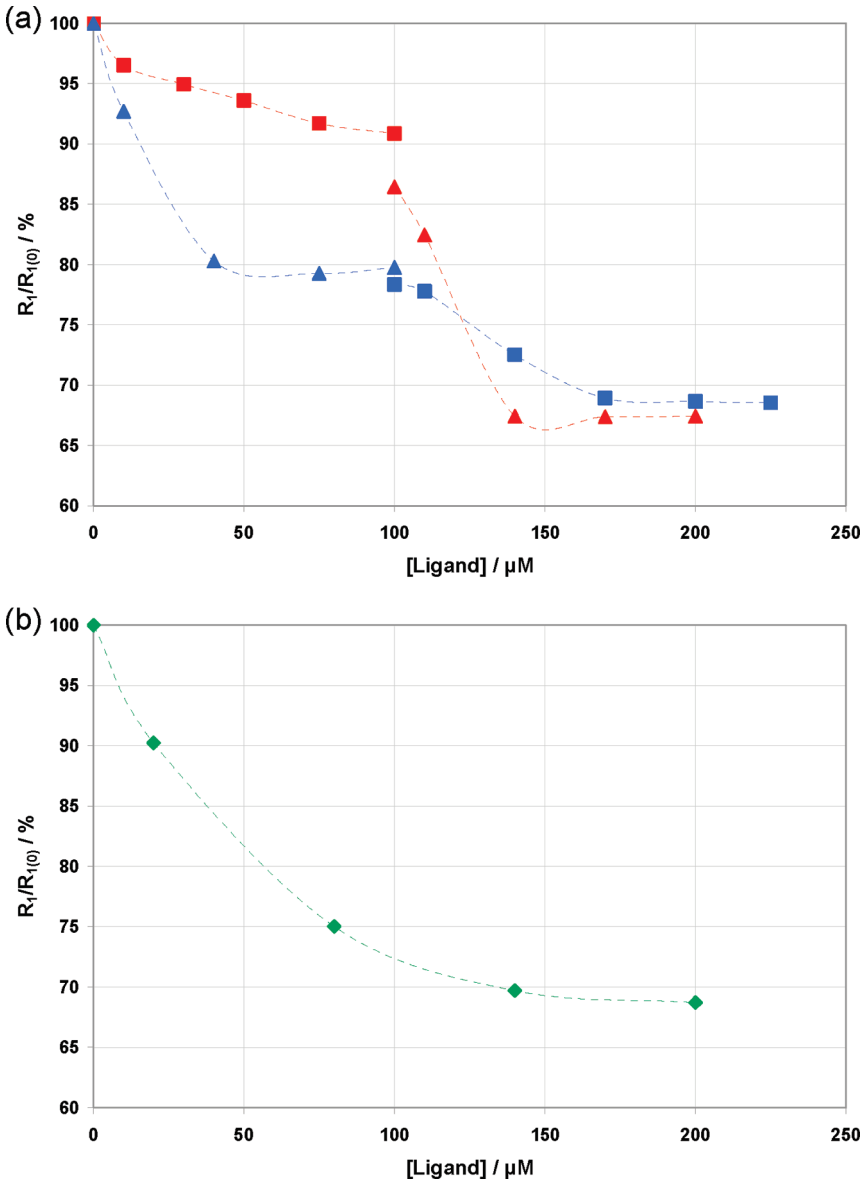


Figure 4. (a) The sequential titration of CODD (solid squares) and 2OG (solid triangles), and (b) simultaneous titration of equal molar CODD and 2OG (solid diamonds) into 50 μM PHD2 as a function of total ligand concentrations. For (a), different colors are used to represent different sequences of addition (red: CODD then 2OG; blue: 2OG then CODD).

first (blue triangles, Figure 4a) in which the $R_1/R_{1(0)}$ ratio plateaus at around 80%, and further titration of CODD (blue squares, Figure 4a) decreases this to around 70%. The titration of an equimolar mixture of 2OG−CODD into PHD2 yields a similar net effect although the profile displays a steeper slope than 2OG or CODD alone due to the binding of both substrate and cofactor throughout. The slight differences in the titration end points observed for the three systems likely arises from the intrinsic variability that occurs between different sample preparations, as described for Figure 1b. The observation of these sequential binding profiles is consistent with previously reported mechanistic and structural studies of PHD2, in which PHD2 forms a ternary complex with 2OG and CODD,[37] and in which 2OG and CODD are observed to bind to neighboring sites in the protein active site.[39,43] It also provides insights into the extent of water accessibility upon the binding of individual substrates. The K_Ds for 2OG in the absence and presence of CODD are measured as 0.9 ± 0.1 and 1.0 ± 0.2 μM, respectively. Similar titration behavior was also observed in ternary systems involving PHD2−2OG−NODD (see Supporting Information, Figure S16).

This additive effect also allows the study of different modes of inhibition. NOG is an unreactive analogue of 2OG, which competes with 2OG for binding to the active site metal.[29,53] As for 2OG, additive effects were observed when CODD was added to PHD2−NOG, indicating the formation of a ternary complex (Figure 5a). In contrast, when CODD (or 2OG) is added to PHD2−BIQ or PHD2−BNS inhibitor complexes, no additive effects were observed (Figure 5b). BIQ and BNS chelate directly to the metal center and therefore compete with 2OG binding, but in addition they also appear able to block CODD from approaching the active site metal, consistent with crystallography studies[39,43] (or alternatively that they serve to completely displace water from around the metal center such that subsequent binding effects cannot be detected, although this

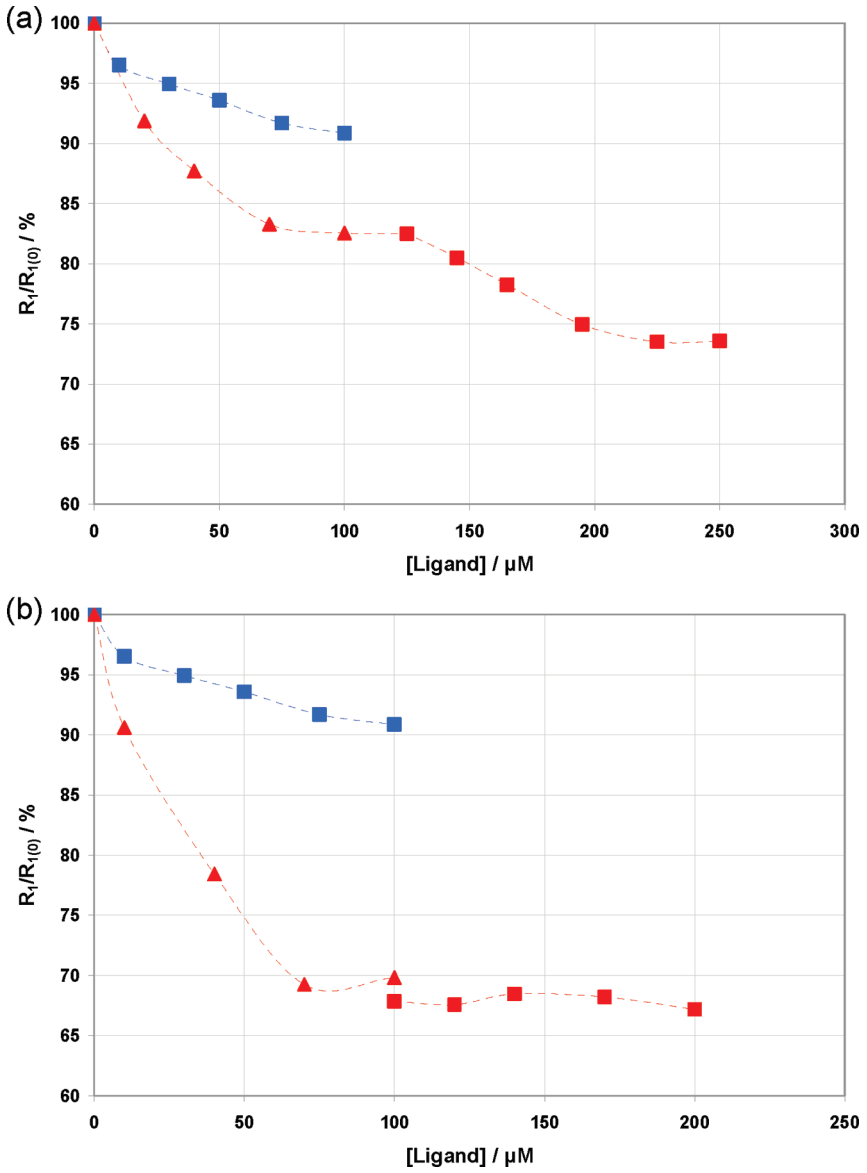


Figure 5. Binding titration curves showing (a) the titration of CODD (red squares) into a solution of PHD2 + NOG (red triangles) and (b) the titration of CODD (red squares) into a solution of PHD2 + BNS (red triangles). In both, the titration of CODD into PHD2 is also plotted as reference (blue squares). Additive effects can be observed in (a) indicating the formation of a PHD2−NOG−CODD complex, while in (b) the BNS inhibitor prevents CODD from approaching the PHD2 active site metal.

seems less likely as they are bidentate metal ligands). Thus, the additional bulk associated with these ligands also offers a second mode of inhibition through blocking substrate binding. Information such as this could prove valuable in inhibitor optimization.

Discussion and Conclusions

We have herein demonstrated the general applicability of ligand screening with paramagnetic metalloproteins through the study of bulk water relaxation, notably using high field NMR spectrometers. It is important to emphasize that the technique is relatively simple to carry out both from experimental and data analysis perspectives. The method is highly sensitive to ligand binding events directly at, and in the vicinity of, the paramagnetic metal center. It enables the differentiation of binding affinities between ligands and, in favorable cases, allows the value of dissociation constants to be determined. Furthermore, it is less restricted than many other screening methods by the upper and lower affinity limits imposed by ligand exchange kinetics.

We have further demonstrated the wider applicability of this technique to detect multiple binding events such as in the formation of ternary protein−substrate complexes, which may have potential applicability in mechanistic studies of enzyme processes and in the design of enzyme inhibitors. Other NMR techniques that are able to identify ternary complex formation, such as interligand NOEs (ILOEs),[54,55] lack the relative simplicity and sensitivity of the solvent relaxation method presented here, requiring very long experiment times and careful optimization of ligand−protein ratios for their success. The ability to screen the formation of ternary complexes in a high throughput fashion is therefore potentially powerful in mechanistic studies on enzymes and in drug design. For instance, Becattini et al. have reported a methodology for drug discovery through the application of ILOEs in which a library of small molecules with simple structures was screened for ternary complex formation.[56] Covalent linkage

of molecules that bind simultaneously may then lead to larger and potentially more potent inhibitors. Provided the target enzyme is a paramagnetic metalloprotein, this solvent screening technique may provide an attractive alternative to ILOEs, in that it may enable the straightforward identification of ternary complex formation.

We also suggest this technique can be applied in a high-throughput fashion for the ligand screening of paramagnetic metalloproteins; by conducting a 2-point assay with and without potential ligands, a significant change in solvent relaxation rate may be correlated with ligand binding. The screening of each potential ligand can be as short as 10 min and may be performed using robotic sample handling.

Experimental Section

Materials. All chemicals were from Sigma-Aldrich, with the exceptions of the CODD (sequence DLDLEMLAPYIPMDDDFQL) and NODD (sequence DALTLLAPAAGDTIISLDF) peptides (Peptide Protein Research Limited), Tris-D11 (Cambridge Isotope Laboratories), and D_2O (Apollo Scientific). BNS was a kind gift from Amgen. BIQ and analogues of BIQ were synthesized by reported methods.[46]

Expression and Purification of PHD2. Recombinant PHD2 was expressed in *Escherichia coli* BL21 (DE3) and purified by cation exchange and size exclusion chromatography as described previously.[37] Apo-proteins were obtained by incubation with EDTA after cation exchange chromatography. Purified PHD2 was buffer exchanged into 50 mM Tris-D11 (100% D_2O, pD 7.1) using Amicon Ultra-4 or Amicon Ultra-15 filter units (MW cutoff: 10 kDa) (Millipore). At least three rounds of dilution and concentration were performed at 277 K. PHD2 aliquots were stored at 193 K.

NMR Experiments. High-field experiments were conducted at 500 MHz using a Bruker Avance II spectrometer equipped with a standard 5 mm *z*-gradient TXI probe. Unless otherwise stated, all experiments were conducted at 298 K. Conventional 3 mm diameter NMR tubes (Norell or Sigma-Aldrich) containing 50 μM PHD2 were used in all experiments unless otherwise indicated. Solutions were buffered using Tris or Tris-D11 (pH 7.5) dissolved in 12.5% H_2O and 87.5% D_2O. A stock solution of 800 μM Mn(II) was used throughout and diluted to final concentrations of 50 or 100 μM. Stock solutions of ligand molecules were made up freshly to concentrations between 1.6 and 2.5 mM. Samples of 1 μL or 2 μL were titrated into NMR tubes. Inversion recovery experiments were performed with two scans with a relaxation delay of at least 5 times T_1 between transients. Pulse tip-angle calibration using the single-pulse nutation method was undertaken for each sample ensuring accurate 90° and 180° pulses.[57] Curve fitting to allow for the possibility of radiation damping[58,59] was used as a check during development work and proved that under the conditions employed there was no contribution from this to the recovery profiles recorded.

Low-field experiments were conducted using an Oxford Instruments MQC spectrometer operating at 23 MHz. Conventional 5 mm diameter tubes (Norell) were used throughout. All experimental details are as for the high field work, except the solution was buffered in 100% H_2O Tris (pH 7.5), and experiments were conducted at 313 K.

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Supporting Information Available: Solvent relaxation measurement for the binding of Mn(II) to PHD2; fitted K_D curves for the binding of small molecule inhibitors to PHD2; ESI-MS data for the binding of BIQ analogues to PHD2; titration data for NODD and 2OG; details for the synthesis of BIQ analogues. This material is available free of charge via the Internet at http://pubs.acs.org.

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