

Water-Based Ligand Screening for Paramagnetic Metalloproteins**

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Dedicated to Professor Jan Reedijk on the occasion of his retirement

NMR-based strategies for ligand screening and drug design are widely applied in pharmaceutical research.^[1–11] Given the versatility of the NMR technique, many different methods have been developed to answer questions such as 1) whether the ligand binds, 2) where it binds, and 3) what is the structure of the resulting complex. Some or all of these questions can be answered using two broad categories of experiments: so-called ligand-based (Figure 1 a) or protein-based (Figure 1 b) experiments.^[12–19]

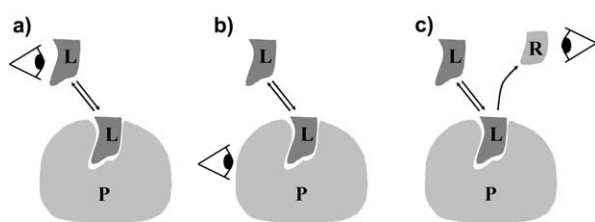


Figure 1. NMR-based strategies for ligand screening are either based on the observation of the bulk ligand, L, in exchange with the protein-bound ligand (a) or on the observation of the protein, P, in the presence of excess ligand (b). A subclass of ligand-based experiments is based on the observation of a reporter molecule, R, competing with the ligand for the same protein binding site (c).

Ligand-based experiments address question (1), while protein-based experiments typically address questions (2) and (3). Furthermore, ligand-based experiments require much smaller amounts of protein as their sensitivity depends on the ligand concentration (which can be in the high millimolar range) rather than on the protein concentration (usually in

the low micromolar range), which means that they are therefore faster and easily adaptable to high-throughput screening protocols. A particular subclass of ligand-based experiments, called competition experiments (Figure 1 c), is based on the observation of one reporter ligand which may or may not be displaced from its binding site by the screened molecules.^[20] In this case, question (2) can also be answered if the binding site of the ligand is known. The sensitivity of competition experiments is similar to that of ligand-based experiments as it is related to the concentration of the reporter ligand and not to that of the protein.

It occurred to us that any ligand-protein binding experiment often implies competition with a ubiquitous probe-ligand, usually water. Indeed, water molecules are almost always displaced upon binding of the protein ligand. Observation of the water signals instead of the signals of a reporter ligand should therefore provide a tremendous increase in sensitivity due to the very high concentration of water protons (110M compared to the submolar concentration of the reporter ligand). Unfortunately, this very same feature prevents the direct use of water in competition experiments. In fact, the very large molar ratio between water and protein, and the fact that only very few of the many protein-bound water molecules are displaced by the ligand, makes the differential effect negligibly small.^[4]

However there is one case where the NMR parameters of a protein-bound water molecule are so strongly altered that the effect is easily seen even when it is propagated from bound to bulk water molecules. This is the case of a water molecule coordinated to a paramagnetic metal ion (provided that it exchanges with bulk water molecules).^[4+] The presence of unpaired electrons, whose magnetic moment is 658-times larger than that of the protons, and the close proximity of the bound water protons to the paramagnetic center, increase the longitudinal (and transverse) relaxation rate of the latter by many orders of magnitude.^[21] We propose herein that water bound to a paramagnetic metal in the active site of a metalloenzyme, which is a pharmaceutically relevant target, can be used as an efficient reporter ligand in competition experiments, thereby answering questions (1) and (2). We also show that the efficiency of this method compares very favorably with the others commonly used. Given the fact that metalloenzymes are quite widespread, and that the metal can

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Supporting information for this article is available on the WWW under <http://www.angewandte.org> or from the author.

+ Protein-bound water is indeed used in ligand screening, but only as a means of transferring magnetization to ligand molecules bound nearby (so-called WaterLogsy experiments).

++ The maximal effect is observed when the residence time of the metal-bound water molecule is shorter than, or of the same order as, the longitudinal relaxation time.

either be natively paramagnetic or be substituted by a paramagnetic one, this method should be of fairly general applicability. We have used manganese(II)-activated protein phosphatase 5 (PP5) as an example. Manganese(II)-activated enzymes alone constitute around 15% of all metalloenzymes in the Protein Data Bank (PDB),^[*] and several members of the two most studied protein targets in drug-discovery (kinases and phosphatases) are manganese-activated.^[22–33] Manganese(II) is an ideal probe for demonstrating the feasibility of this approach due to its strong paramagnetism and its favorable electronic relaxation properties.^[21]

PP5 contains two domains: a phosphatase domain and a regulatory domain.^[34,35] The former is responsible for the enzymatic activity. X-ray structures have revealed that the phosphatase active site is formed by two metal ions placed at the bottom of a shallow pocket. The nature and stoichiometry of the metal ions natively present in the catalytic domain of PP5 are still controversial given that Fe^{2+} , Zn^{2+} , and Mn^{2+} compete for the two metal-binding sites and are all found in varying amounts in enzyme preparations.^[35] The active site structure of PP5 is shown in Figure 2. The two metal ions (reported as Mn^{2+} in the original publication) are bridged by a phosphate ion (P^i) and by a solvent-donated ligand (W^3 , presumably a hydroxide). One of the two metals is coordinated by a water molecule (W^2), and other crystallographic molecules are present in the active site.^[36]

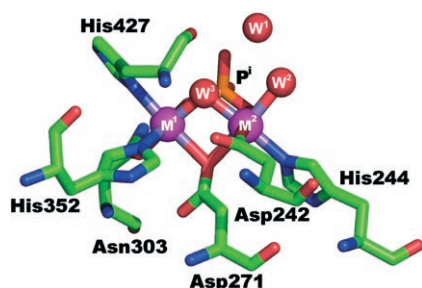


Figure 2. Active site structure of the catalytic domain of PP5. One metal ion is coordinated by Asp271, Asn303, His352, and His427 and the other by Asp242, His244, Asp271, and a water molecule (W^2). A phosphate ion (P^i) and a further solvent-donated ligand (W^3 , presumably a hydroxide ion), bridge the two metal ions.

Four compounds known to be generic phosphatases inhibitors, with molecular weights in the range 166–373 Da, were selected to test the method. These compounds can be considered as typical members of a compound library to be used in a high-throughput screening program to identify active site-directed PP5 ligands. Binding of these inhibitors at the catalytic site is believed to prevent the access of the substrate to the catalytic pocket and subsequent interaction with the metal ions.^[26] In doing so, the ligands may either displace the active site water or block its exchange with bulk water molecules: in either case, the propagation of relaxation enhancement to the bulk water molecules should be abol-

ished. PP5 may therefore be a good test case for competition experiments with water as the reporter molecule.

The selected ligands were: 1) cantharidin (inset of Figure 3a and Figure S1 in the Supporting Information), a biologically active natural compound known to inhibit several phosphatases at nanomolar concentrations;^[37,38] 2) endothall, a nanomolar inhibitor of protein phosphatase 2A (PP2A);^[39] 3) *exo*-3,6-epoxy-1,2,3,6-tetrahydrophthalic anhydride, a compound structurally related to norcantharidin, a micromolar inhibitor of PP2A;^[40] and 4) (–)-*p*-bromotetramisole, which was selected due to its ability to mimic the action of orthovanadate, a micromolar inhibitor of calcineurin (PP2B).^[41] The structures of the latter three compounds are available as Supporting Information (Figure S1).

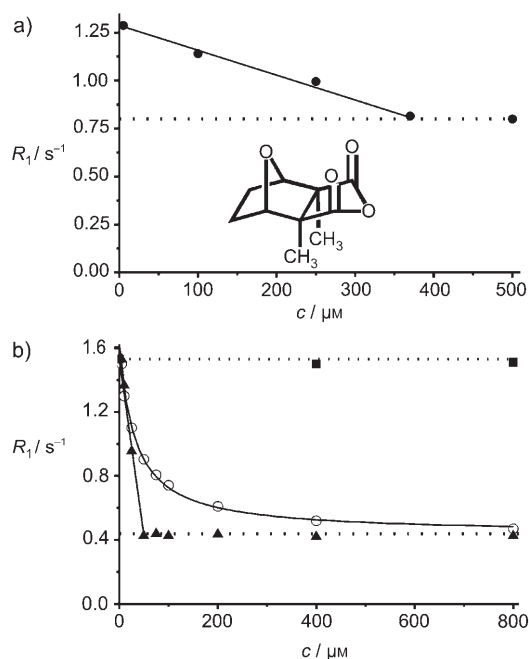


Figure 3. a) Decrease of the water proton longitudinal relaxation rate R_1 at 400 MHz and 295 K in a 370 μM solution of Fe,Mn-PP5 upon addition of cantharidin (inset). b) Titration curves of solutions of Fe,Mn-PP5 (50 μM) with endothall (○), (–)-*p*-bromotetramisole oxalate (■), and *exo*-3,6-epoxy-1,2,3,6-tetrahydrophthalic anhydride (▲) at 0.02 MHz and 295 K.

Water proton longitudinal relaxation rates (R_1) were measured at 400 MHz for 2 μL samples of Fe,Mn-PP5 (370 μM) in the presence of increasing amounts of cantharidin. A linear decrease in R_1 was observed up to addition of one equivalent of cantharidin, after which the effect leveled off (Figure 3a). The residual relaxation rate is mostly due to the sum of the relaxation rate of pure water (approx. 0.35 s⁻¹) and the diamagnetic contribution from other water molecules bound to PP5 far from the active site, which can be measured for solutions of Fe,Zn-PP5 (not shown). The effect of cantharidin is therefore consistent with binding of the ligand to the catalytic pocket preventing the access of the solvent to the paramagnetic center.

[*] Unpublished results from our laboratory.

It is apparent from Figure 3a that an effect is clearly detectable although it is not strong enough to allow the protein concentration to be reduced much below the high micromolar range. On the other hand, given the strong intensity of the water signal, the sample volume can be much smaller than the 2 μL volume of the present experiment (see below), which means that the quantity of enzyme needed is modest (much less than one nanomol).

The concentration of the enzyme could be further decreased if the paramagnetic effect were stronger. This is predicted to happen at low magnetic field, where the dipole-dipole coupling between the unpaired electrons and water protons is maximal. Indeed, it is well known that solutions of paramagnetic metalloproteins exhibit large proton longitudinal relaxation rates at magnetic fields much lower than 400 MHz. To explore the field-dependence of the longitudinal relaxation rate of water protons in solutions of Fe,Mn-PP5 with and without cantharidin, relaxation rate profiles at magnetic fields ranging between 0.01 and 40 MHz were acquired for 50 μM Fe,Mn-PP5 using a Fast-Field Cycling relaxometer.^[42] The relaxometric profile of Fe,Mn-PP5 (Figure 4) is indeed much higher over the whole low-field

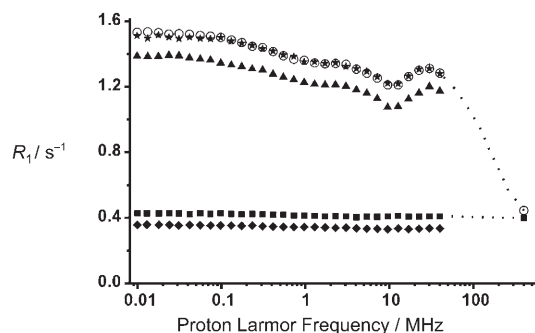


Figure 4. ^1H NMRD profile of a 50 μM Fe,Mn-PP5 solution at 288 (*), 295 ($\circ$), and 303 K ($\blacktriangle$); a 50 μM Fe,Zn-PP5 solution at 295 K ($\blacklozenge$); and a 50 μM Fe,Mn-PP5 solution at 295 K in the presence of an equimolar concentration of cantharidin ($\blacksquare$). Dotted lines have been added to aid visualization of the field dependence of the water relaxation rate.

range with respect to that recorded at 400 MHz and is typical of an Mn^{II} profile.^[21] The addition of an equimolar concentration of cantharidin to a solution of the paramagnetic protein lowers the relaxivity to very low values, that is, the values of Fe,Zn-PP5 (Figure 4). This decrease is much more striking than at 400 MHz even though the enzyme concentration is about eight times lower.

The acquired relaxometric profiles clearly indicate that a large gain in sensitivity can be achieved by performing the relaxation rate measurements at low magnetic fields, where the difference in relaxivity between the free and inhibited forms of the protein is more than one order of magnitude larger than at 400 MHz.

Such a strong effect also permits a safe estimate of the ligand dissociation constants, K_{D} , provided they are close to, or higher than, the concentration of the enzyme. Only an upper limit of about 1 μM can be estimated for cantharidin by this method, while K_{D} for cantharidin is in the nanomolar

range.^[38] The relaxation rate for endothall decreases linearly with the concentration, similarly to cantharidin, while for *exo*-3,6-epoxy-1,2,3,6-tetrahydrophthalic anhydride the dependence on the concentration is non-linear, thereby indicating a weaker binding constant (Figure 3b). The fit of the experimental relaxation rates, measured at the same magnetic field, as a function of the concentration of the latter inhibitor provided a K_{D} value of $19 \pm 2.5 \mu\text{M}$. This value compares well with the value of $13 \pm 5 \mu\text{M}$ determined by an enzymatic assay (see Supporting Information). Finally, no effect was detected upon the addition of (–)-*p*-bromotetramisole (Figure 3b), thus showing that this molecule does not bind PP5 at the metal binding site.

Although the R_1 values were acquired in a wide range of magnetic fields using a fast field-cycling relaxometer in the present work, the same information regarding the protein-ligand interaction could be achieved by performing the measurements at a specific value of the proton Larmor frequency. In particular, the large difference in relaxivity present in the region 15–30 MHz in this case can be exploited by using commercial fixed-field relaxometers operating, for example, at 20 MHz. These instruments are relatively inexpensive and widely used in food analysis, biology, and materials science, and their sensitivity is not much smaller than that of a field-cycling relaxometer.

The amount of protein needed for the analysis is, together with speed, the main determinant of the success of a high-throughput screening methodology. The abundance of water protons makes the application of the present approach possible with very small sample volumes. It can be concluded from the results presented herein that samples with enzyme concentrations as low as 15 μM can be analyzed at low field. With a conservative estimate of the minimal solution volume of 15 μL , the amount of protein needed would therefore be as low as 60 picomols, much lower than any other NMR-based method. At high field the minimal enzyme concentration would be much higher (around 400 μM) as the paramagnetic effect is much less pronounced at high fields. On the other hand, the sensitivity is so high that the sample volume could, in principle, be decreased by several orders of magnitude (down to a few nanoliters).^[*]

In a recent review,^[14] Meyer et al. published a comparative table (Table 2) of the merits and drawbacks of the most popular NMR-based methods in drug discovery. Table 1 is based on this table with an additional column for the present water-based method. In summary, the water-based strategy can be applied to both large and small proteins, does not require labeling, provides the binding epitope on the protein,^[**] and does not have lower or upper limits for K_{D} (ligand solubility being the upper limit), although it does

[*] As an additional bonus, such a small volume ensures that the radiation damping phenomenon that occurs for strong NMR signals and potentially makes relaxation measurements inaccurate cannot be operative

[**] A reviewer has pointed out that the ligand could bind away from the metal center and water accessibility still be altered by allosteric effects. This is relatively uncommon, and in any case detecting such active-site-relevant allosteric effects could actually be considered an advantage.

Table 1: Comparison of the present water-based approach with the most common ligand-screening strategies (from Meyer et al.,^[14] Table 2).^[a]

	SAR by NMR ^[1]	STD NMR ^[2]	Spin labeling ^[4]	Diffusion editing ^[43]	Inverse NOE pumping ^[44]	Water-Logsy ^[45]	Water-based
Large protein (>30 kDa)	limited	yes	yes	no	yes	yes	yes
Small protein (<10 kDa)	yes	no	yes	yes	no	no	yes
Isotope-labeled protein required	yes	no	no	no	no	no	no
Binding epitope on protein	yes	no	no	no	no	no	yes
Binding epitope on ligand	no	yes	no	no	yes	yes	no
Amount of protein [nmol] at 500 MHz	25	0.1	≈ 1	≈ 100	≈ 25	≈ 25	Low field: 5 $\mu\text{L} \times 15 \mu\text{M} = \mathbf{0.06}$ High field: 100 nL ^[b] $\times 400 \mu\text{M} = \mathbf{0.04}$
K_D tight binding	no limit	100 μM	100 μM	≈ 100 μM	1 nM	100 μM	no limit
K_D weak binding	≈ 1 mM	≈ 10 mM	≈ 10 mM	≈ 1 mM	≈ 1 mM	≈ 10 mM	no limit
Identification of ligand	no	yes	yes	yes	yes	yes	no

[a] The most favorable features for each strategy are highlighted in bold and italics. [b] The amount of protein could be further reduced by appropriate design of a nanodelivery system.

not provide the binding epitope on the ligand and does not allow the ligand to be identified in a mixture. Above all, water-based screening permits the use of unprecedentedly small amounts of protein per single assay.

The data presented here show that this water-based approach can be successfully integrated into a drug discovery strategy aimed at designing ligands for metalloenzymes where a paramagnetic metal ion is already present or can be inserted in place of the diamagnetic native metal. This analysis can be applied to large libraries of compounds or to a portfolio of ligands already identified by different high-throughput screening strategies, thereby allowing the molecules that interact at the metal binding site to be selected and providing, at the same time, information regarding the affinity constant and the binding mode.

Experimental Section

The catalytic domain of PP5 corresponding to the construct 169–499 was expressed in soluble form in *E. coli* and prepared as either the Fe,Zn or Fe,Mn derivative (see Supporting Information). The Fe,Mn enzyme was found to be fully active ($3.9 \pm 0.8 \text{ U } \mu\text{g}^{-1}$), while the Fe,Zn derivative was virtually inactive ($0.1 \pm 0.03 \text{ U } \mu\text{g}^{-1}$). This finding suggests a physiological role for the manganese ion and further supports the use of the Fe,Mn derivative in PP5-directed ligand screening, selection, and optimization experiments.

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