Ligand Screening

Paramagnetic Metal Ions in Ligand Screening: The Co^{II} Matrix Metalloproteinase 12**

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An increasing number of validated drug targets are being revealed as the components of the human genome become known. At the same time, there is an increasing need to speed up the drug discovery process, especially in the early stages of ligand screening to design candidate drugs. With respect to other techniques, NMR spectroscopy is less prone to generate false positives or false negatives, and can be used to couple qualitative high-throughput screening with quantitative information on the binding affinity and structure of the target–ligand complex. Furthermore, NMR spectroscopy is probably the best technique to detect weak binders and to identify secondary binding sites. A wide portfolio of weak binders is a precious asset, as weak binders are the building blocks for experimentally based strategies for rational and semirational drug design.

A popular ligand screening protocol is based on recording the ^{1}H NMR signals of a mixture of compounds from a library of chemicals in a spin-echo Carr–Purcell–Meiboom–Gill (CPMG) NMR pulse sequence experiment.[1] The intensity of the signals under these conditions is inversely related to the transverse relaxation rate R_2 of the corresponding protons. Upon addition of micromolar quantities of the target protein, and if any of the compounds in the mixture interacts with the protein, the R_2 of the protons in the molecule bound to the protein increases owing to the long rotational correlation time τ_r of the macromolecular adduct. If the bound ligand is in fast exchange with the bulk ligand, a selective decrease in the signal intensity for that compound is observed. The sensitivity of the experiment depends on the size of the macromolecule (the larger the size, the larger the value of τ_r, the larger the effect) and on the affinity of the ligand—the larger the affinity (provided fast exchange conditions are maintained), the larger the signal reduction for a given amount of added

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protein. The method is progressively less sensitive for binders with dissociation constants larger than the typical ligand concentration (50–500 μM). Increasing the protein concentration is feasible but of course costly.

Stronger increases in ligand R_2 values are expected if the target protein contains a paramagnetic center. Unpaired electrons can undergo dipole–dipole coupling with the ligand nuclei if they are sufficiently close to each other; the strength of the coupling depends on the reciprocal sixth power of the electron–nucleus distance.[2] It has been shown that addition of spin labels in the vicinity of the ligand binding site in a protein can significantly enhance the increase in R_2 of the bound ligand, thereby allowing detection of the interaction of weaker ligands.[3] Spin labels have also been exploited for second-site screening by coupling with a ligand for a first binding site.[4] The use of paramagnetic metal ion complexes instead of spin labels has also been proposed.[5] Surprisingly, there has been little interest in exploiting paramagnetic metal ions that are either native constituents of metalloenzymes (such as iron, copper, or manganese) or that can be substituted in the place of diamagnetic native metals (such as lanthanides for calcium or cobalt for zinc ions). Many hydrolytic zinc enzymes are now recognized drug targets, matrix metalloproteinases (MMPs) being a well-known example. The cobalt(II) ion is known to be an excellent substitute for zinc in many metalloproteins,[6] and it often maintains a substantial fraction of the catalytic activity. In particular, cobalt(II) has been shown to substitute zinc in the active site of MMP-1, and the resulting derivative is still catalytically active.[7]

We therefore felt that it was worthwhile to investigate the possibility of substituting cobalt in MMPs to increase the sensitivity of the drug screening experiment. The enzyme of choice was the catalytic domain of human MMP-12 (from (Met 105)Gly 106 to Gly 263, with a Phe-171-Asp mutation).[8] The X-ray crystal structure of this protein has been solved to atomic resolution,[9] and the structures of several adducts with druglike molecules are also known.[8–10] We have prepared the cobalt derivative of MMP-12, proven its binding to the catalytic zinc site through HSQC and electron spectroscopy, and checked on the basis of equilibrium dialysis that its dissociation constant was low enough for the planned experiments (see the Supporting Information). Spin-echo experiments were then conducted on several compounds and mixtures of compounds, using either the cobalt derivative or the native zinc-containing protein.

Test solutions were prepared with several medium to weak binders of MMPs, both alone or in a mixture with two noninteracting molecules (2-ethoxyanisole and *N*,*N*-dimethylaniline). The chosen ligands were 4-hydroxybiphenyl, 4-aminobiphenyl, 4-phenoxyaniline, 3-(2-hydroxyethyl)indole, and anthraquinone-2-sulfonate. Ligand concentrations were kept constant at 50 μM. In all cases, addition of native ZnMMP caused a decrease in the signals of those ligands that bind the enzyme. However, the amount of ZnMMP required to observe an effect varied from about 5–8 μM for 4-hydroxybiphenyl, 4-aminobiphenyl, and 4-phenoxyaniline (whose dissociation constants are estimated at about 10–100 μM) and 10–15 μM for 3-(2-hydroxyethyl)indole up to 50 μM for anthraquinone-2-sulfonate. Conversely, the amount of CoMMP required to obtain a similar effect was around 1.5, 3, and 5 μM, respectively. At the end of the experiments, the strong MMP inhibitor *N*-isobutyl-*N*-(4-methoxyphenylsulfonyl)glycyl hydroxamic acid (NNGH, Biomol Inc., USA) was added to the ZnMMP–ligand and CoMMP–ligand mixtures. In all cases the signal intensities reverted almost exactly to those seen in the absence of the enzyme, confirming that we are specifically monitoring binding of the ligands to the enzyme active site. The results for 4-aminobiphenyl and anthraquinone-2-sulfonate are shown in Figure 1. It appears

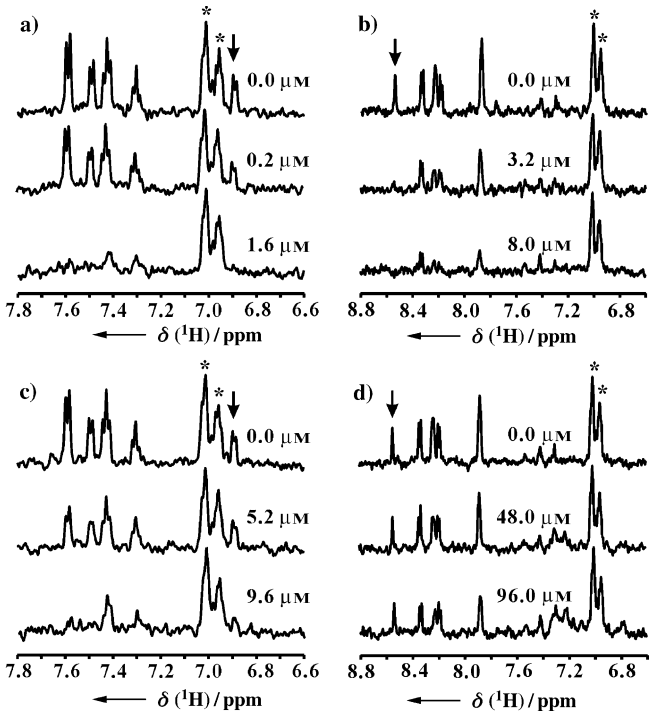

Figure 1. Sections of the ^{1}H NMR spectra (500 MHz, 298 K) of 4-aminobiphenyl (a, c) and anthraquinone-2-sulfonate (b, d) in the presence of increasing amounts of CoMMP-12 (a, b) and ZnMMP-12 (c, d). The signals marked with an asterisk correspond to reference compounds; the arrows indicate 4-aminobiphenyl protons next to the amino group (a, c) and anthraquinone protons next to the sulfonate group (b, d).

that the sensitivity is significantly enhanced (by a factor of about 4 to greater than 10). Thus, these data constitute the proof of principle that substitution with cobalt (and possibly other paramagnetic metals) can be a general strategy to enhance the sensitivity of ligand screening by NMR spectroscopy.

Careful analysis of the spectra in Figure 1 reveals that more information is gained in the experiments with the paramagnetic metal than those with the native diamagnetic metal. Whereas in the latter a similar decrease in the intensity of all the signals of the ligands is observed (consistent with the R_2 enhancement being due to τ_r in all cases, that is, to a global property of the molecule), in the experiments with the paramagnetic metal the decrease in the intensity of the signals is strongly inhomogeneous. This is because of the different distance from the cobalt(II) ion to each proton in the bound

molecule. Therefore, even in the fast, high-throughput mode, a screening based on paramagnetic effects permits a qualitative estimate of the orientation of the ligand within the catalytic site. This means that from the present experiments meaningful information can be obtained on all the ligands investigated. In the case of 4-aminobiphenyl, for example, the differential effect is the least marked (Figure 1 a, c). This is consistent with the ligand occupying the hydrophobic S_1' pocket, which extends on one side of the catalytic metal in such a way that the far end of the pocket is not much farther away from the metal than the entrance. Yet, the protons next to the amino group (marked by arrows in Figure 1) are slightly more affected than those belonging to the distal phenyl ring, indicating that the amino end of the molecule sticks out of the cavity and is slightly closer to the metal than the other end. The differential effect is dramatic for anthraquinone-2-sulfonate (Figure 1 b, d). The signals of the ring protons next to the sulfonate group (marked by arrows) undergo a dramatic decrease in intensity upon addition of even a small amount of CoMMP. Conversely, a very large amount of ZnMMP is required to alter the signal intensities of anthraquinone β-sulfonate, and the effect is essentially the same for all signals. Clearly, anthraquinone is the weakest binder (dissociation constant >1 mM) of the present series of ligands, but some of its signals undergo a dramatic paramagnetic effect. These results are inconsistent with the anthraquinone moiety entering deeply into the S_1' pocket, and point instead to a situation in which the sulfonate group interacts with the metal, allowing the nearby protons to be very close (about 4 Å) to the metal itself.

In summary, the method we propose has the potential of combining fast, high-throughput screening with structural information of the same quality as that obtained, for example, from HSQC experiments, which are somewhat more time-consuming and require higher enzyme concentrations. Moreover, thanks to recent progress in protein engineering and chemistry, this approach can also be extended to proteins without natural metal-binding sites.[11]

We conclude by giving some rough estimates of the expected distance-dependent effects: For a protein of the size of the present one (ca. 17 kDa, $\tau_r \approx 7$ ns), the R_2 values of bound ligand protons should be around 60 s^{-1}. The R_2 enhancement due to the paramagnetic cobalt(II) ion is dominated by Curie relaxation[2] and can be calculated easily. The results are given in Table 1 for experiments performed at 500 MHz. Paramagnetic effects are sensed as far as 8–9 Å, are sizable at 6–7 Å, and become dramatic below 6 Å. The data in Table 1 should be fairly independent of the size of the molecule (both diamagnetic relaxation and Curie relaxation increase linearly with τ_r). On the other hand, the advantage of using a paramagnetic metal should be even greater at higher field, because Curie relaxation increases quadratically with field, while only the chemical-shift anisotropy (CSA) contribution to diamagnetic proton relaxation increases quadratically with field. Finally, the values given in Table 1 were calculated for a cobalt(II) ion having three unpaired electrons ($S = 3/2$). For metal ions such as manganese(II) ($S = 5/2$) or gadolinium(III) ($S = 7/2$), the paramagnetic effects should be much stronger, because Curie relaxation depends on the square of the $S(S+1)$ product.

Table 1: The R_2 enhancements at 298 K calculated for ligand protons at various distances from a high-spin cobalt(II) ion in a metalloenzyme.

Metal–proton distance [Å]	$(R_{2para} + R_{2dia})/R_{2dia}$
∞	1
9	1.25
8	1.5
7	2
6	4
5	9
4	33
3	180

Experimental Section

CoMMP-12 was prepared by exhaustive dialysis of ZnMMP-12 against a buffer containing 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES; 10 mM), $CaCl_2$ (5 mM), $CoCl_2$ (0.3 mM), NaCl (300 mM), and acetohydroxamic acid (200 mM) at pH 7 (see Supporting Information).[8] The samples for NMR spectroscopy were prepared by adding concentrated solutions of the ligand in [D_6]DMSO to D_2O containing tris(hydroxymethyl)aminomethane (Tris) buffer (10 mM), NaCl (300 mM), and $CaCl_2$ (5 mM). These samples were then titrated with concentrated solutions of ZnMMP-12 or CoMMP-12. Relaxation-edited NMR experiments were performed on a DRX Bruker 500-MHz spectrometer equipped with a TXI cryoprobe.

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