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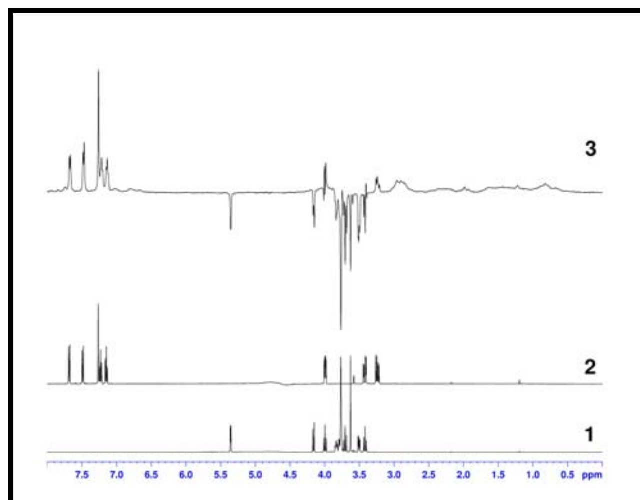
Using Bulk Water to Detect Ligand Binding

Introducing the WaterLOGSY Experiment

Characterizing the binding between ligands and macromolecules is of paramount importance to a number of different branches of chemical and biological research. One of many examples is the field of medicinal chemistry and drug discovery, an active area of research at Dalhousie. Given the sensitivity of most NMR parameters to intermolecular influence, it is not surprising that NMR spectroscopy has and continues to play a pivotal role in this research. Numerous binding NMR experiments have been devised and are broadly categorized according to which of the two molecules is monitored; the macromolecule or ligand. Experiments in the former group typically involve monitoring chemical shift changes in the presence of ligand. Unfortunately, the ability to conduct these experiments is commonly hindered by factors such as the size of the target, lack of a suitable sample, and lack of access to sufficient magnetic field strengths. With the requirements being less stringent for the ligand-based group of experiments, this latter category has received broader application. All experiments in this category are founded on the fact that NMR observables such as NOE's, linewidths and diffusion rates vary dramatically for small molecules compared to heavy molecules. When a small ligand molecule (typically < 750 Da) binds to a heavy target molecule (typically > 30 kDa), rotational correlation times and diffusion rates are lengthened (compared to the free-state) and consequently an atypical heavy molecule NMR result is expected. In the absence of any affinity, a typical small molecule result is obtained; this distinction provides the basis for NMR screening experiments.

A significant percentage of the ligand-based binding work has employed NOE's (the transferred-NOE effect). Pioneered by Moriz Mayer and Bernd Meyer, the popular Saturation Transfer Difference (STD) experiment has been in use in the NMR-3 facility for many years (and a topic of an earlier bulletin). The experiment is essentially a steady-state NOE difference experiment involving selective saturation of target protons and relies on spin diffusion to propagate saturation to bound ligands. Another popular NOE-based ligand binding experiment is Water-LOGSY (**Water**-**L**igand **O**bserved via **G**radient **S**pectroscop**Y**) from Claudio Dalvit's lab (Dalvit *et al*, J. Biomol. NMR, 18, 2000, pp 65-68). It is a variant of STD where the source magnetization originates from bulk solvent (H_2O) protons instead of target protons. The typical implementation is via transient-NOE with bulk water protons selectively inverted. During the mixing time, the inverted magnetization cross-relaxes (CR) with bound ligand protons via a number of possible (and simultaneous) mechanisms, all supported by a long water T_1: (1) direct CR from H_2O molecules trapped at the target-ligand interface (2) direct CR from labile (exchanged with H_2O) target protons at the binding site such as NH and OH protons and (3) indirect CR via labile target protons remote from the binding site but propagated there by spin diffusion. Each of the three mechanisms acts in concert to yield a negative CR rate due to the slow tumbling of the target-ligand complex. During the long mixing time, inverted ligand molecules dissociate and "fresh" ligand binds, CR and dissociates. This turnover leads to a growing pool of inverted ligand magnetization in free solution which is preserved by the long free-ligand T_1. For non-binders, the CR rate and intermolecular NOE with H_2O is positive (a typical small molecule result due to fast molecular tumbling). Thus, peak intensities have opposite signs for binding and nonbinding molecules. A Water-LOGSY spectrum obtained on our Bruker AV-500 spectrometer is shown in the figure. The sample was 0.2 mM human serum albumin (HSA) with 10 mM sucrose (non-binder) and L-tryptophan (binder) in 90% H_2O:10% D_2O. Spectra 1 and 2 are the ^1H spectra of sucrose and tryptophan, respectively whereas spectrum 3 is the Water-LOGSY result (128 transients, 1.8 s mixing time, DPFGSE water suppression, and 25 ms $T_{1\rho}$ filter to reduce protein background). Positive peak intensities for L-tryptophan and negative for sucrose identifies the former as binding to HSA and the latter with no measurable affinity.



The spectroscopist is cautioned that unlike STD, where there is significant freedom in saturation frequency, with WaterLOGSY the spectroscopist is restricted to the H_2O resonance. This introduces potential intramolecular NOE contamination due to the simultaneous inversion of resonances that may overlap with H_2O (the experiment is designed to be strictly intermolecular). For overlapping ligand protons, negative CR rates are only conceivable for ligands that bind and so false positives from this source are not concerning. Overlapping target protons are more problematic but can be controlled during the inversion step. Since inversion is achieved with ePHOGSY (a gradient echo), the echo delay can be used as a T_2 filter (made sufficiently long) to eliminate transverse target resonances. Obviously, the smaller the protein, the longer the required delay but as this delay is lengthened, the spectroscopist must be cognisant of potential losses due to translational diffusion. Note that this source of contamination can be assessed, if desired, by running a 2nd experiment with a purposely short echo delay or by performing the experiment in D_2O. Another concern arises from the so-called "free-state contributions"; direct water to ligand NOE's that bypass the complex. Since bound and free-state contributions have opposite signs, false negatives are possible if these exceed the bound-state contribution. For this reason (and unlike STD), large ligand to protein molar ratios should be avoided. Note that the bound-state contribution can always be isolated by performing WaterLOGSY experiments on two separate samples; one with target molecules and one without and taking the difference. Lastly, the spectroscopist is reminded that labile protons from non-binding compounds can lead to positive exchange peaks (as for small molecule NOESY) and to not treat these as "hits".

In summary, WaterLOGSY has been found to yield impressive sensitivity and to be remarkably devoid of artefacts. The experiment has arguably become the method of choice for screening compounds for binding. If your research program involves (or could be furthered) by an understanding of binding phenomenon involving small ligands and large receptors, I encourage you to give this technique consideration. I want to thank PhD candidate **Stephanie Forget** for preparing the NMR samples for me as well as **Robin Stein** and **Rainer Kerssebaum** from Bruker BioSpin for their assistance in getting this experiment implemented.