residue is labeled with ^{13}C while the *i*th+1 amino acid is labeled with ^{15}N. Acquisition of a two-dimensional HNCO spectrum (which correlates the H and N resonances of the *i*th residue with the CO resonance of the *i*th-1 residue)[60,61] will consist of a single peak, which acts as a "spy" on the state of the binding site of interest. Because only one peak is observed, the demands on the sensitivity and resolution of the data can be relaxed, increasing both the molecular mass and solubility range of the method relative to that of the uniformly labeled approach. Another approach, described later in this chapter, that bypasses the need for full resonance assignments of the target is to use a well-characterized test ligand, for example, a known inhibitor, to induce perturbations in the ^{15}N-^{1}H correlation spectrum and then screen for new ligands that perturb the same resonances. This approach was used successfully for screening and fragment-based design of potent inhibitors for prostaglandin D synthase.[62]

Ligand-directed methods

Because of the limitations of target-directed methods described in the previous section, ligand-directed methods have become the most widely applied detection scheme for fragment screening by NMR. Although they contain less information about the ligand binding site than target-directed methods, they are more generally applicable and more rapidly implemented, making them highly valuable in the early drug discovery process. Ligand-directed methods enjoy a significant advantage, in that there are no limitations on the molecular mass of the target (and in fact are usually better suited to large targets). In addition, the protein required for ligand-directed methods need not be isotopically labeled, which provides more flexibility in the choice of expression systems that can be used to generate the sample. Finally, because it is the ligand that is observed, and the experimental mixtures contain a large excess of ligand relative to the protein, experiments can be performed with much less protein than in target-directed experiments, thus reducing the amount of protein that must be expressed and purified and also extending the solubility range of the targets that can be screened.

Despite these advantages, ligand-directed methods have two disadvantages relative to target-directed methods that should be considered before studies are undertaken. The first is that these methods require the ligand to be in rapid exchange between the bound and free state and are consequently limited to weakly binding fragments ($K_d > \sim 10^{-7}$ M). This is usually not a serious limitation as low MW fragments are expected to bind weakly and have been prescreened for good solubility. The second disadvantage is that these methods do not provide information about the nature of the binding site on the target receptor and as a result cannot distinguish nonspecific and/or biologically irrelevant association from binding at the desired site of the target.

Saturation transfer difference methods

Ligand-directed methods may be divided into to two distinct classes of experiments. One type of experiment relies on detecting a difference in an NMR observable parameter that is dependent on a change in the rotational correlation time (τ_c) of the small molecule ligand, resulting from interaction with the target receptor. Another class of experiments is based on transfer of magnetization between the target receptor and ligand that occurs during the time that the two molecules are bound to one another. In either method, the off rate of the ligand/protein complex must be fast on the NMR time scale ($K_{off} > \sim 100\ s^{-1}$), such that the signal observed represents the population weighted average of the free and bound states of the ligand.

An example of the latter type of experiment is saturation transfer difference (STD) spectroscopy,[63] which is one of the most robust, and frequently employed, methods for detecting fragment binding by NMR. In this method, a train of frequency selective ^{1}H radio frequency (RF) pulses is applied for a number of seconds at a frequency that excites some resonances of the protein target but none of the ligand resonances of the fragment (or mixture of fragments) (Figure 4.4). During this saturation time, magnetization is transferred by spin diffusion from the selectively irradiated nuclei to all protons of the protein. In addition, this magnetization is transferred from the target protein to any small molecules that bind to the target. Provided that the ligand is in fast exchange on the NMR time scale, many target/ligand binding events will take place during the saturation time, resulting in a partial saturation of the fragments that have bound. A 90° pulse is then applied and a proton spectrum is recorded. A second data set is then acquired, this time applying the same selective ^{1}H RF saturation, except at a frequency that does not disturb the magnetization state of the nuclei of either the target protein or ligands. Ligands that do not interact with the protein do not have their proton magnetization perturbed in either spectrum, and subtraction of the data sets results in a null difference spectrum. For ligands that do interact with the target protein, a difference spectrum is observed and is used to determine the identity of the binding compound in the mixture of compounds (Figure 4.5). STD experiments (and virtually all other NMR-detected fragment screening methods) are most often applied to systems containing a soluble, purified protein but can be extended to more challenging and heterogeneous systems. For example, Claasen et al.[64] have reported using a double difference STD experiment to observe peptide binding to the membrane protein integrin expressed on the surface of intact platelets. Ligand binding can also be observed to integrin reconstituted into liposomes.[65]

WaterLOGSY

An alternative method to transfer magnetization from the target protein to the binding ligand has been described by Dalvit and coworkers.[66,67] In the WaterLOGSY (Water/Ligand Observed via Gradient SpectroscopY) experiment,